Susceptibility To Basal Cell Carcinoma (BCC): The Influence Of Individual Characteristics And Polymorphism In Loci Encoding Detoxifying Enzymes.

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Susceptibility To Basal Cell Carcinoma (BCC): The Influence Of Individual Characteristics And Polymorphism In Loci Encoding Detoxifying Enzymes.

John T. Lear.

BCC is the commonest malignancy in white people and presents a great burden to health care provision. The genetic factors and individual characteristics associated with susceptibility to this cancer are still largely unclear. Since ultraviolet radiation constitutes an oxidative stress to skin cells, the ability to deal with the products of oxidative stress-induced damage may be important in determining BCC risk. The influence of detoxifying enzyme gene polymorphisms (glutathione S-transferases (GST) GSTM1, GSTT1, GSTM3 and cytochrome P450's (CYP) CYP1A1 and CYP2D6) on susceptibility to BCC was assessed. The influence of individual characteristics was also studied. Skin type 1, blue/green eyes, red/blonde hair and social class 1 or 2 were all significant risk factors for BCC. Truncal site of BCC was associated with multiple lesions. Patients with melanoma were younger, more likely to be female, have red/blonde hair and a truncal tumour when compared to those with a BCC. Patient's with skin cancers of different histological types were more likely to have truncal BCC and be of skin type 1. The GSTM1 A/B genotype was protective against multiple tumours. GSTM1 null in combination with some of these factors influenced BCC number and time to next tumour presentation. GSTT1 null influenced BCC accrual and tumour site. Interactions between GSTM3 AA, skin type 1 and other genotypes were associated with multiple BCC. CYP1A1 m1m1 was associated with increased numbers of lesions. The CYP1A1 Ile/Ile genotype was more common in those with a truncal tumour. The CYP2D6 EM genotype influenced total number of BCC, rate of accrual and time to next tumour presentation. These results indicate that individual variation in the ability to deal with products of ultraviolet and/or chemical-induced oxidative stress is important in BCC pathogenesis and indicate a potential use of individual characteristics and genetic markers in BCC follow-up strategies.

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CHAPTER 1

1.1. Abbreviations

Ah	Aryl hydrocarbon hydroxylase
ARMS	Amplification refractory mutation system
BCC	Basal cell carcinoma
CI	Confidence intervals
CYP	Cytochrome P450
GST	Glutathione S-transferase
HLA	Human leucocyte antigen
MED	Minimal erythema dose
MHC	Major histocompatibility complex
MM	Malignant melanoma
NAT	N-acetyl transferase
NMSC	Non-melanoma skin cancer
PAH	Polycyclic aromatic hydrocarbons
PCR	Polymerase chain reaction
PTC	Human homologue of drosophila *patched* gene
PUVA	8-methoxypsoralen plus ultraviolet A radiation
RFLP	Restriction fragment length polymorphism
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
SPF	Sun protection factor
SRST	Sun reactive skin type
TNF	Tumour necrosis factor
UV	Ultraviolet
XP	Xeroderma pigmentosum

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CHAPTER 2 - INTRODUCTION

2.1. Overview

Cutaneous basal cell carcinoma (BCC, rodent ulcer, rodent carcinoma, see figure 2.1.1.) was first reported in 1827 and is now the commonest malignancy in Caucasians. Its incidence is on the increase world-wide. The disease is responsible for considerable morbidity (Lee, 1973; Johnson et al., 1984). However, it is the least studied of the common cutaneous malignancies. There have been relatively few large case-control studies of risk factors in BCC (only 1 in the U.K., McHenry et al., 1995) and many studies have grouped BCC and cutaneous squamous cell carcinoma (SCC, see figure 2.1.2.) under the title non-melanoma skin cancer (NMSC), even though they demonstrate different pathological behaviour. There is also little data on the factors that influence number, rate and site of BCC and time to next tumour. Ultraviolet (UV) radiation constitutes an oxidative stress to skin. Detoxication reactions are important in BCC with allelism at the glutathione S-transferase GSTM1 locus influencing susceptibility (Heagerty et al., 1994). In this chapter I will review the current literature on the incidence, mortality, clinical features, risk factors, treatment, recurrence, genetics and aetiology of BCC. I will also discuss the biochemistry of detoxication with particular reference to the enzymes I have studied.

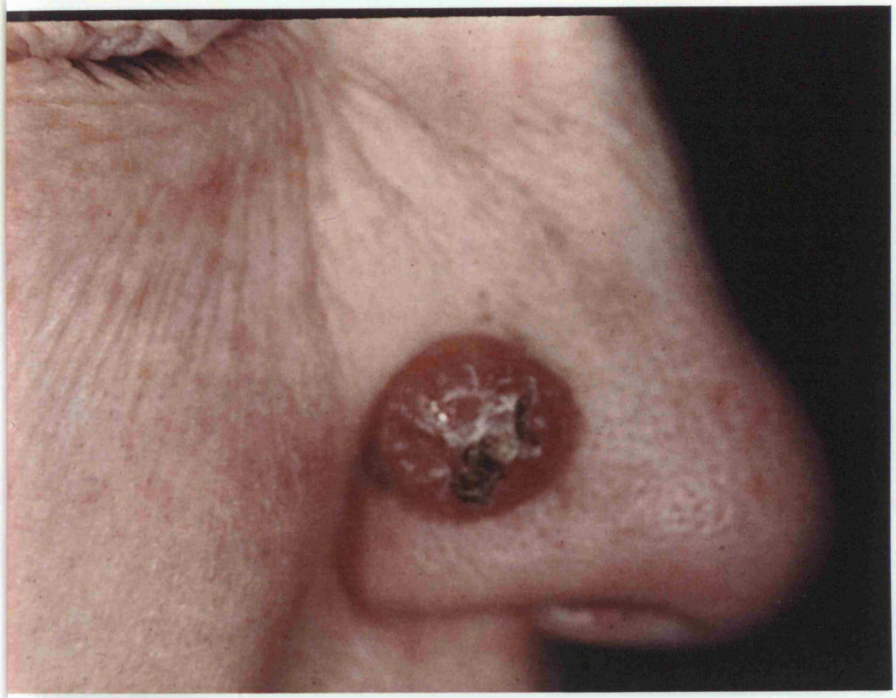


Figure 2.1.1 BCC on the face

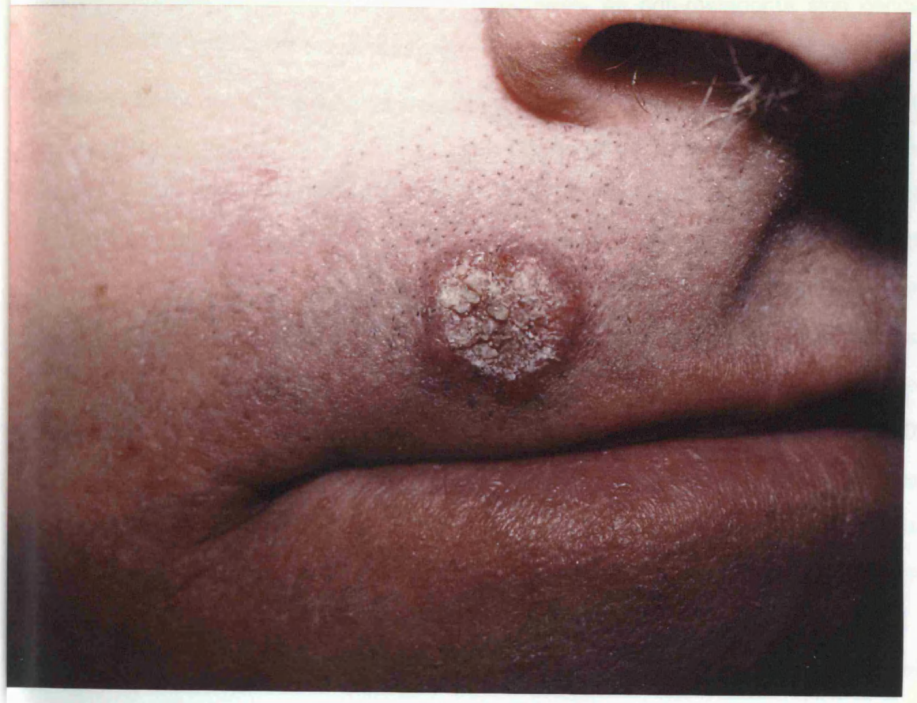


Figure 2.1.2 SCC on sun exposed site

2.2. Incidence/Mortality

The common skin cancers are BCC, SCC and malignant melanoma (MM, see figure 2.2.1.). BCC and SCC are referred to collectively as non-melanoma skin cancer (NMSC). BCC is most common followed by SCC and MM. The ratio of BCC to SCC is approximately 4:1 (Glass and Hoover, 1989). Solar keratoses and Bowen's disease (see figure 2.2.2.) are non-invasive but may be precursors to SCC. A BCC comprises cells similar to those found in the basal layer of the epidermis and appendages, hence its name. It is the most common malignancy in Caucasians, being rare in black skinned individuals. Estimates in 1994 in the USA by the American Cancer Society predicted 532,000 new skin cancers. The total number of all cancers estimated was 1,700,000 showing that skin cancer accounts for one third of all newly diagnosed cancers (Boring et al., 1994). Reported incidence rates of BCC vary with rates of 300 per 100,000 people in 1977 in the USA have been described and up to 1000 per 100,000 seen near the equator in Australia (Miller and Weinstock, 1994). Accurate figures on skin cancer are difficult to obtain as most countries do not register them and thus incidences are often estimates based on sampling. The lifetime risk of a BCC for a child born in 1994 in the USA is 28-33% and 7-11% for SCC. NMSC will affect approximately 1 million people in the USA in 1994 alone leading to predictions that incidence will equal all other malignancies combined (Boring et al., 1993). This illustrates the significant burden placed by BCC on health service provision now and in the future.

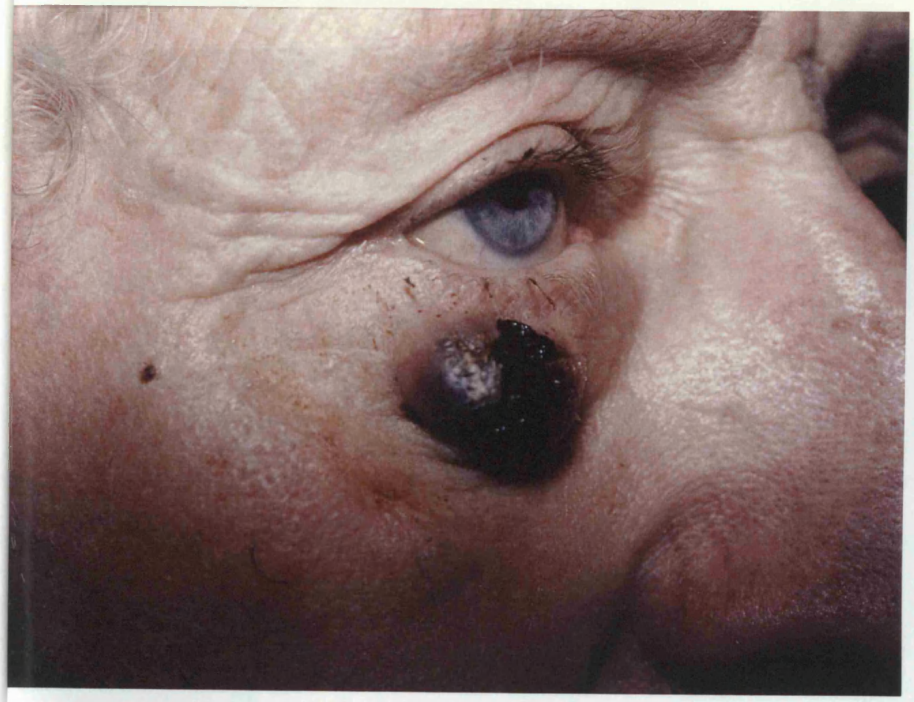


Figure 2.2.1. Nodular MM.

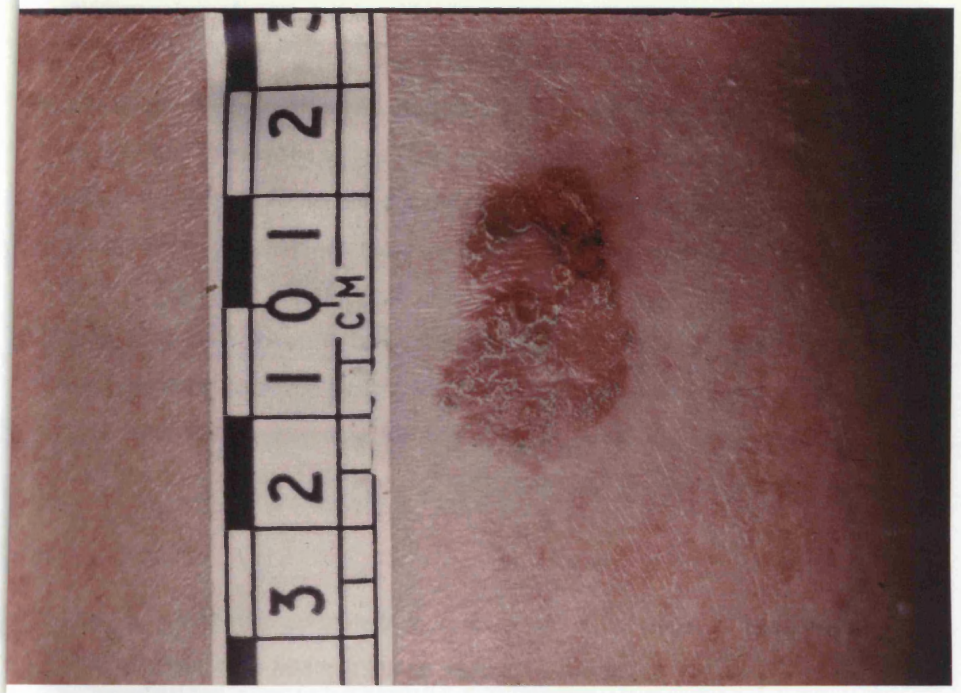


Figure 2.2.2. Bowen's disease

Incidence rates are highest in Australia and are increasing in many countries. Depletion of stratospheric ozone leading to increased UV radiation is predicted to further increase rates (Russell-Jones,1987; Fears and Scotto,1983). There is evidence from four sources, with different ascertainment methods, of a striking increase in incidence of NMSC in North America during the past two decades (Fears and Scotto,1982; Gallagher et al., 1990; Glass and Hoover, 1989, Schreiber et al., 1971). Population based estimates of NMSC in 1977-78 revealed rates are higher for men than women except for the lower leg site where women have higher rates (Scotto et al., 1993). When compared with a previous study by the same group in 1971-72 (Scotto et al., 1974), an increase of 15-20% had occurred. Men over 70 years have been shown to be at particular risk (Serrano et al., 1991). In Australia, BCC incidence increased by 11% and SCC by 51% between 1985 and 1990 (Marks et al, 1993). Similar increases have been shown in Tasmania (Kaldor et al., 1993), U.K. (Ko et al., 1994), Sweden (Dahl et al., 1992) and the Netherlands (Coebergh et al., 1991).

Mortality data is more accurate in most countries (Marks, 1994). In the USA mortality rates in whites from MM in 1985-89 were 2.4 per 100,000 person years. Men had almost twice the rate as women (Hartman,1992). For NMSC, recent estimates (Weinstock,1993) suggest a rate of 0.5 per 100,000 whites/year in 1987-88 (0.67 for men and 0.30 for women). These rates have been dropping with a 20-30% decrease from 1969 through to 1988. Although mortality is lower than MM, morbidity and cosmetic deformity is important for BCC but there is no good quantitative assessment of the degree of disability or handicap. In view of the numbers affected, NMSC places a substantial burden on health service provision throughout the world indicating the need for research in this area. Considerably more research is performed on MM than

NMSC. A Medline CD rom search for 1991-95 revealed approximately 6000 citations for MM, 1000 for BCC and 1000 for SCC.

2.3. Clinical Features/Risk Factors

Early BCC's are translucent or pearly, with raised, rounded areas covered by thin epidermis through which dilated vessels may show. Occasionally pigment can be seen. As they advance they can have a wide variety of patterns (see figures 2.3.1 to 2.3.4) including:

- nodulo-ulcerative (rodent ulcer)
- pigmented (more common in dark skinned people)
- morpheaform (ulceration rare) and keloidal (Requena et al., 1996)
- superficial (found mainly on the trunk)
- premalignant fibroepitheliomas (flesh coloured, sessile lesions)

This makes classification sometimes difficult.

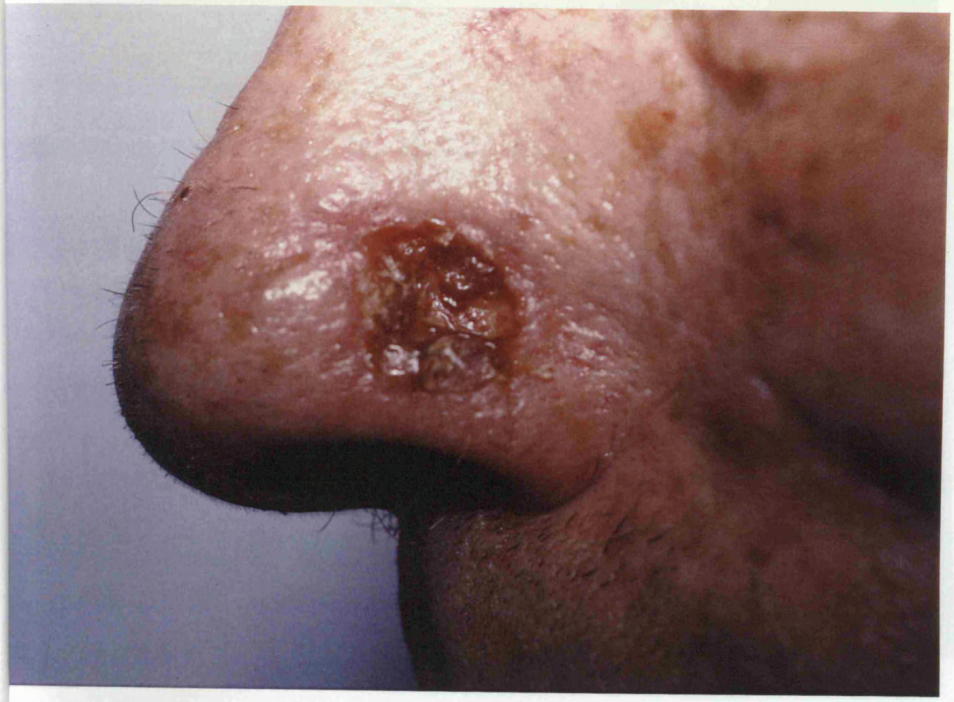


Figure 2.3.1. Rodent ulcer: nodular, ulcerated lesion on the face

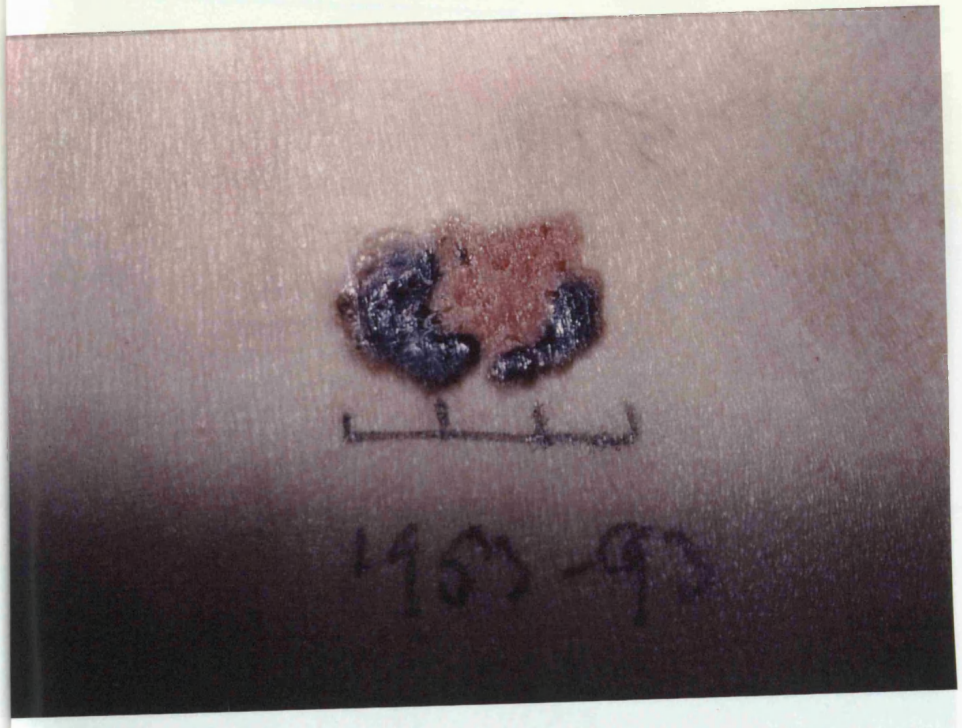


Figure 2.3.2. Pigmented BCC.

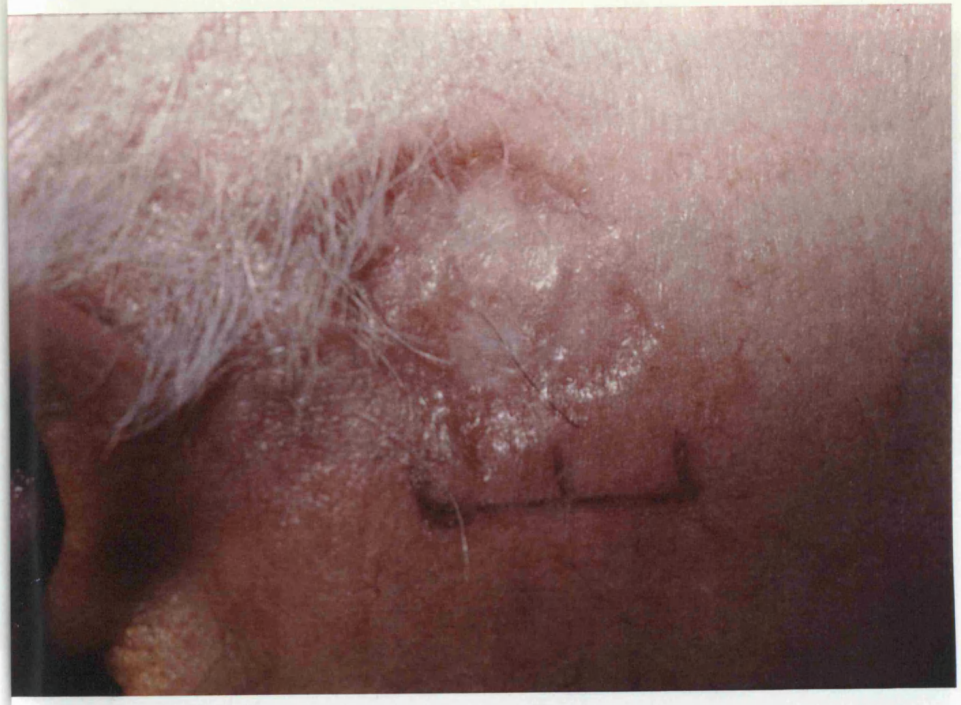


Figure 2.3.3. Morphoeic BCC with indistinct edges and telangiectasia.

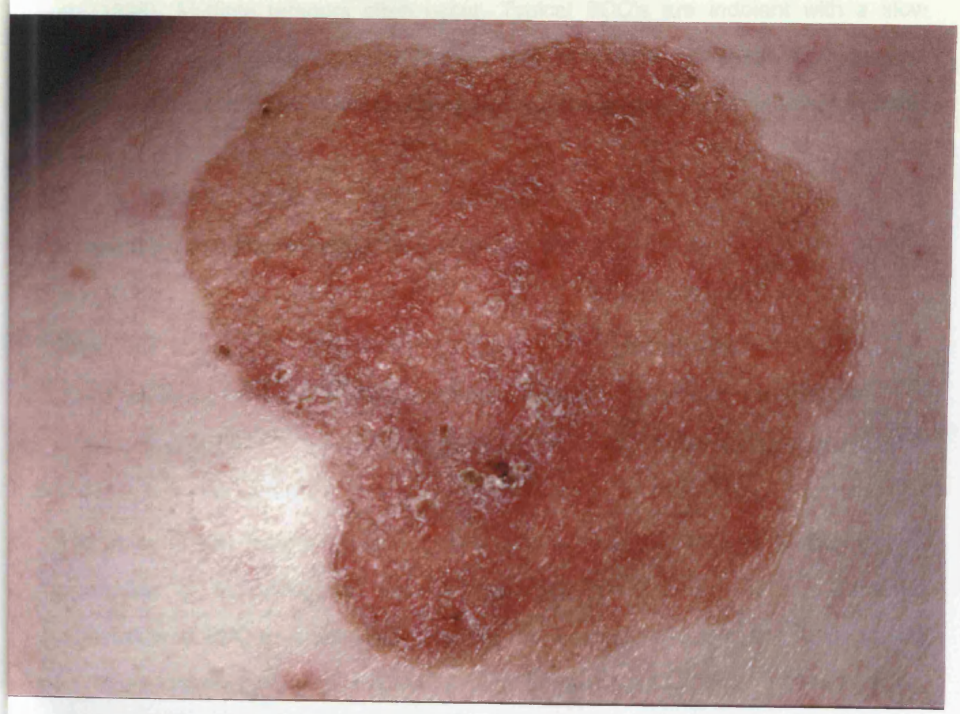


Figure 2.3.4. Superficial BCC on the trunk

Telangiectasia are characteristically seen especially in the morpheaform variety. The majority of tumours (80%) occur on the head and neck particularly the upper central part of the face. The superficial type, however, is found mainly on the trunk (Betti et al., 1995). Multiple tumours often occur. Typical BCC's are indolent with a slow progression. Spontaneous fluctuations in size can occur. Diagnosis is based on histological examination of biopsy specimens but many tumours have a characteristic clinical appearance.

Differential diagnosis (MacKie, 1992) includes:

- SCC
- MM
- solar keratosis
- Bowen's disease
- eczema
- naevi
- sebaceous hyperplasia
- appendygeal tumours
- psoriasis
- seborrhoeic warts
- molluscum contagiosum
- granulomatous reactions

The study of risk factors enables identification of at risk individuals in the hope of preventing or modifying the natural history of the condition. This is of great importance in BCC as smaller tumours are more easily dealt with. In the U.K. there have been relatively few case control studies on risk factors in BCC (McHenry et al., 1995). It was shown that actinic keratoses, freckling and the number of hours spent outdoors after the age of 60 years were important. Other recognised risk factors include increasing age, red/blonde hair, blue green eyes and fair skin (Lin and Carter, 1986). Outdoor occupation and family history may also be important (Hogan et al., 1989). Smoking, although a risk factor for cutaneous SCC, has not been consistently associated with BCC (Karagas et al., 1992). A recent large multicentre south European study identified blonde hair, pale eyes, recreational sun exposure and skin type 1 to be significant BCC risk factors (Zanetti et al., 1996; Rosso et al., 1996).

Patients with albinism and xeroderma pigmentosum are at increased BCC risk. In the naevoid basal cell carcinoma syndrome (Gorlin's syndrome) multiple BCC's at an early age (<30 years) are found together with other manifestations including: milia; punctate hyperkeratosis and circular pits on the hands and feet; dental cysts; spina bifida, bifid ribs; hypertelorism; syndactyly and cataracts. This is inherited in an autosomal dominant fashion with a linked locus on chromosome 9q (Wicking et al., 1994). A strong candidate gene has recently been identified (Johnson et al., 1996). The familial risk in sporadic NMSC is unclear with some supportive data (Czarnecki et al., 1992) and other suggestions that inheritance of complexion may explain familial clustering (Belisario, 1954). Although UV radiation is considered to be the major factor predisposing to BCC, exogenous chemicals have also been implicated. Exposure to arsenic predisposes to the development of multiple BCC particularly the superficial type on the trunk, illustrating exogenous factors can be important, possibly via interactions with UV radiation (Yeh et al.,1968).

Skin type has been studied in relation to cutaneous malignancy. The concept of sun reactive skin typing (SRST) was created in 1975 to classify persons with white skin enabling selection of correct initial doses of UVA in the application of PUVA photochemotherapy (Fitzpatrick, 1986). This need to create SRST arose from the identification of persons who, despite black hair and brown eyes, developed severe phototoxic reactions following oral methoxsalen and exposure to 4-6 J/m^2 of UVA. A simple classification was proposed using an interview to determine what patients say their responses are to an initial sun exposure (45-60 minutes of noon exposure in northern latitudes in early summer). Patients were asked about their responses (burning at 24 hours and tanning at seven days) to three such exposures with the classification into skin types 1 to 4 with skin type 1 (always burn and never tan) and

skin type 4 (never burn and always tan) at each end of the spectrum (Fitzpatrick, 1986). Later brown and black skinned individuals were included in the classification. This skin typing was used as a basis for selection of initial UVA dose in two large PUVA trials (Melski et al., 1977; Roenigk and Martin, 1977). The relationship of erythema dosages to skin typing is unresolved. Some studies have found that the average minimal erythema dose (MED) is lowest for skin type 1 and highest for skin type 4 (Amblard et al., 1982; Sayre et al., 1981; Agin et al., 1985) and others finding no significant correlation (Rampen et al., 1988). More recently SRST and burning tendency have been used by many investigators in studying risk factors to skin cancer. Thus, patients with melanoma tend to burn rather than tan on exposure to sunlight (Beral et al., 1983; Gange et al., 1995). Recently, mutations in the MSH receptor have been described which correlate strongly with skin type 1 (Valverde et al., 1996). Mutations identified in the MSH receptor are present in over 75% of individuals with skin type 1 but less than 5% of individuals with skin types 3 and 4.

The paucity of data on risk factors to BCC in the U.K. indicates that further large studies are needed in the British population. Low socio-economic status and infrequent physician visits have been shown to be associated with very large BCC's. Such patients are less concerned about their general health and pose a significant management problem. Patients with BCC are at high risk of suffering further primary BCC, 40-50% at five years (Kricker et al., 1993; Karagas and Greenberg, 1995). Importantly, this risk depends on the number of lesions present. Thus, in subjects with 1 lesion the 5 year risk is 27%, in those with 10 or more tumours the risk is 90% suggesting accrual of lesions is not just dependent on time but that some subjects have an increased susceptibility. Male gender, age over 60 years, burning easily and sun damaged skin were also associated with an increased risk of subsequent new tumours

(Karagas et al., 1992). Clearly Karagas's observations suggest that patients with more than one tumour at presentation may benefit from closer observation. Research is needed to further define those at risk of a subsequent tumour.

It is interesting to note whether patients with BCC are at increased risk of other malignancies. Such observations have implications for follow up and give insights into the pathogenesis of this tumour. Several studies show that patients with BCC are at an increased risk of both SCC (Karagas, 1994) and MM. One study found a relative risk of 17 for the development of MM in patients with a prior BCC (Margoob et al., 1994). Other studies have found relative risks between 2.8 to 6.6 (Lindelof et al., 1991; Holman and Armstrong, 1984). Differences between these rates may be explained by recruitment bias, histological classification and increasing incidence. The link between these associations is considered to be UV radiation as in both diseases UV is considered to be a major aetiologic factor. Indeed, the presence of this association has been considered by some as evidence that UV is involved in the pathogenesis of MM and BCC (Green and O'Rourke, 1985). Thus patients with BCC are at high risk for the development of MM and could potentially benefit from surveillance, especially as early melanomas in the horizontal growth phase have a much better prognosis than later lesions in a vertical growth phase (Koh et al., 1993). Regular total cutaneous examinations have been advocated as a useful, non-invasive, quick surveillance technique to detect new tumours in at risk individuals (Lookingbill, 1988; Margoob et al., 1994). However, a physician skilled in the detection of skin cancers is needed to give the best results and such people are in relatively short supply in the U.K.

The association with internal malignancy remains unclear with some studies suggesting no association (Sandstrom et al., 1984; Moller et al., 1975) and other studies

suggesting men with BCC have an increased risk of cancer of the lung and thyroid gland and women an increased risk of cancer of the uterine cervix (Lindelhof et al., 1991; Frisch et al., 1996). An association with non-Hodgkin lymphoma has also been described (Frisch et al., 1996). The explanation for these observations is not clear but exposure to carcinogens such as arsenic was suggested. A recent study observed an excess risk of developing SCC and MM in patients with non-Hodgkin's lymphoma (rel. risk 5.5 for SCC, 2.4 for MM) and chronic lymphocytic leukaemia (rel. risk 8.6 for SCC, 3.1 for MM) suggesting a link between haematological malignancies and skin cancer, possibly due to UV radiation (Adami et al., 1995). Renal transplant recipients are at increased risk of both skin cancer, particularly SCC, and lymphomas illustrating the role of the immune system in cancer prevention (Boyle et al., 1984). Further studies, in particular case control studies of which there have been few, are indicated.

2.4. Treatment/Recurrence/Metastasis

Untreated, BCC's can grow to very large sizes, become locally invasive, metastasise and are cosmetically undesirable and so treatment is almost always indicated. The principles of management include: identifying high risk patients for prevention and early detection; complete removal of the lesion; careful follow up to detect local recurrence and detection of new tumours. Available treatment modalities are:

- Curettage
- Primary resection with closure of defect (including flaps and grafts)
- Mohs' micrographic surgery
- Radiotherapy
- Cryotherapy

• Laser excision

The choice of treatment depends on: size and site of tumour; age of patient; efficacy of treatment modality; cosmetic considerations and preference of patient and physician. In general, the smaller the tumour the easier it is to treat, with minimal morbidity and a favourable outcome (Fleming et al., 1995). Cryotherapy and laser excision are rarely used and I will concentrate on surgical methods of treatment and radiotherapy in this review. There have been no large randomised prospective studies comparing one treatment modality with another, though there are many reports of large series of patients treated with a particular modality. An ideal treatment would be one with a high cure and low recurrence rate; quick, cheap and easy to perform that gives good cosmetic results.

Surgical resection is the commonest form of treatment used in the U.K. (Motley et al., 1995). Its advantage is that removal is rapid and can be performed easily, usually in an out-patient setting. 80% of lesions can be removed with primary closure but larger, more complex lesions require grafts or rotational skin flaps to close the defect and may need general anaesthesia. Another advantage of resection is the availability of tissue for histological evaluation and diagnosis. The simplest surgical procedure is curettage where the lesion is scraped off with a specially designed implement. It is quick, sutures are not required (haemostasis is achieved via electrodessication) and cosmetic results can sometimes be better than resection. However, recurrence rates can be higher than resection especially with inexperienced operators. Many physicians prefer to use this technique although it seems most suitable in small lesions (<6 mm) at any site, any lesions on the neck, trunk and limbs and in elderly

patients (Silverman et al., 1991). Cure rates of 90-95% can be achieved with selection of patients who fit into the above categories.

Primary surgical excision is associated with a 90-95% cure rate in most studies. Ideally the margin of resection should be at least 5mm because as closer margins are obtained, local recurrences are more likely (Chandrasekhar and Terry, 1994). Bigger tumours often need more sophisticated techniques such as plastic surgery or Mohs to achieve complete excision. In 1939, Frederick E. Mohs developed a technique to fix skin cancer in situ and a method of systematically excising and pathologically mapping the excised tumour to obtain margins of normal skin. Since 1970, the technique has improved so that chemical fixation is no longer needed (Mohs, 1978). The technique is, however, expensive, slow and tedious but cure rates of 97% can be achieved even in large tumours. It can be performed in stages on an out-patient basis. This method is particularly useful for morphoeic BCC where the margins of the lesion are unclear to the naked eye. Grafts or flaps may be needed after Mohs' resection to reconstruct the defect. Mohs' surgery is a highly specialised technique and there are few trained individuals in the U.K.

Radiotherapy is a useful and effective treatment modality (Fleming et al., 1995). Approximately 4000 cGy must be delivered. Better cosmetic results are achieved by fractionation of dose (4000 cGy in 10-16 fractions for small tumours (<5cm) and 4500-6000 cGY in 15-30 divided doses for larger tumours). Therefore multiple sessions over a period of weeks are required. However, because of time constraints, often 1 to 3 fractions are used. Therefore multiple sessions over a period of weeks are required. Cure rates of 95% can be achieved.

Advantages include:

- No anaesthesia or surgery needed
- 95% effective
- Effective in troublesome areas (nose, eyes, ears)
- Margins not critical
- Useful in elderly patients

Disadvantages of radiotherapy include:

- Time consuming for patient and radiotherapist
- Expensive
- No histological specimen
- Radiation necrosis to skin
- Poor cosmetic result sometimes
- After radiotherapy subsequent surgery can be more difficult.
- ?Carcinogenesis

Some preliminary evidence suggests that vitamin supplementation may reduce risk of basal cell carcinoma (Wei et al., 1994) and that interferon reduces recurrence (Ikic et al., 1995). Further studies are required to confirm these observations. Clinical trials are currently underway to assess the effect of photodynamic therapy in BCC. This makes use of the tumour cells ability to take up a haematoporphyrin derivative in higher concentrations than the surrounding tissue (Svanberg et al., 1994). This makes the cells photosensitive to light of wavelengths 514.5, 488 and 625-30 nm. The tumour is then exposed to laser light which destroys selectively the tumour cells. Further work is needed to establish the role of this therapy in clinical practice.

Intralesional interferon alfa-2b 1.5 million units three times a week for 3 weeks gave a cure rate of 85% with excellent cosmetic results (Cornell et al., 1990). However, large or recurrent lesions were not treated. Oral retinoids can produce regression but do not cure BCC. They have a role in prevention of lesions in patients with xeroderma pigmentosum or Gorlin's syndrome.

Depending on site, size of tumour and treatment modality, up to 10% of tumours recur making the treatment of recurrent lesions a common problem faced by physicians. Surgical resection of recurrent tumours gives a cure rate of 65% whereas Mohs' gives 94% cure rates (Zitelli, 1992). Factors influencing recurrence include tumour size; location (eyes, nose, ears); deep and marginal invasion (Kirihara et al., 1992); resection margin distance and growth pattern (Dixon et al., 1993) and initial treatment modality (Fleming et al., 1995). Treatment of choice for recurrent BCC is therefore Mohs' surgery but access to this facility is extremely limited in the U.K. and recurrences are usually re-excised via non-Mohs' techniques. Radiotherapy has also been shown to be effective (Wilder et al., 1991).

Metastatic BCC is rare. Reported incidence rates range from 0.0028% to 0.55% (Cotran,1961; Scanlon et al., 1980). The typical metastatic tumour begins as a neglected, large, ulcerated locally invasive neoplasm that recurs despite repeated treatment (Amonette et al., 1981). Tumours greater than 3 cm have a higher incidence of metastasis (Snow et al., 1994). The average age of onset is 48 years which is lower than non-metastatic tumours (Lo et al., 1991). In one study, the interval from onset to metastasis ranged from 1 to 45 years with a median of 9 (Von Domarus and Stevens, 1984). Anatomical location of tumour is not different between metastatic and non-metastatic BCC. Morpheaform and adenocystic types of BCC are

more aggressive than other variants (Lo et al., 1991). Survival after metastasis ranges from 1 month to 192 months with 10% surviving 5 years (Von Domarus and Stevens, 1984). Why BCC metastasises so rarely is not clear.

2.5. The Role Of Ultraviolet (UV) Radiation Exposure.

UV radiation has long been considered the major aetiological agent in the pathogenesis of BCC. Risk has traditionally been thought to be related solely to cumulative dose received, with a monotonic relationship between cumulative dose and BCC risk. Unfortunately information on cumulative dose usually relies on the accurate memory of an often elderly patient who must remember exposure events from many years previously. Thus, the possibility of substantial exposure misclassification exists and results therefore, need to be viewed cautiously. The evidence for this montonic relationship has arisen from: animal experiments (de Gruijl et al., 1983); limited observations that the incidence of NMSC is increased in areas of high ambient solar irradiation; incidence is high on sun-exposed body sites; incidence can be related to occupational sun exposure (Kricker et al., 1994). More often cumulative adult exposure has been studied rather than lifetime exposure and little work has focused on the character and timing of exposure that is responsible for BCC risk. However, other research has cast doubts on this monotonic relationship between dose and risk. Two studies have found little evidence of increasing risk of BCC with increasing UV exposure and postulated a plateau in risk at higher doses (Vitasa et al., 1990; Hunter et al., 1990).

The relationship between dose and risk may not be as well supported for BCC as SCC. Animal experiments relate mainly to SCC as BCC has not been reported in UV-

exposed mice and is rare in other UV-exposed animals (IARC, 1992). Dose-response data has been obtained from albino mice which lack the capacity to form melanin making extrapolation to humans questionable and there is evidence of higher rates of BCC in North America and Europe than more southerly regions of these continents (Kricker et al., 1994). Also the evidence for outdoor occupation and risk is weak with relative risks less than 2.0 (Kricker et al., 1994; IARC, 1992). Thus it seems that cumulative dose does not fully account for BCC risk. Further, the anatomical location of BCC is now seen to increasingly favour sites, mainly trunk, that are not regarded as continuously exposed to the sun when outdoors (Kricker et al., 1994).

The effect of intermittent exposure is still unclear but it was found that a significant increase in BCC risk occurred with increased exposure at the weekend, especially in late teenage years (odds ratio 3.9, Kricker et al., 1995). It was suggested that a particular amount of sun exposure delivered in infrequent, probably intense increments will increase risk of BCC more than a similar dose delivered continuously over the same total time period. Another study found an increased risk with recreational sunlight exposure from the ages of 0 to 19 years and no association with mean annual cumulative exposure (Gallagher et al., 1995). These results suggest that childhood may be a critical period for establishing adult risk for BCC. Freckling, light skin colour and severe sunburn in childhood were also associated with an increased risk. Therefore, freckling may be a marker for UV damage to the skin. It was suggested that by extrapolation of these results, sun-avoidance behaviour in adulthood may not markedly reduce risk for this tumour (Gallagher et al., 1995), although these results need to be verified. The hypothesis that a plateau in risk at higher doses of UV exists (Vitasa et al., 1990; Hunter et al., 1990) has been further supported (Kricker et al., 1995) suggesting a point is reached in the dose-response

relationship beyond which risk of BCC does not increase with continuing increases in sun exposure. The reason for this plateau effect is not clear but its implications are that substantial reductions in exposure may be necessary if heavily exposed populations are to reduce their risk of BCC (Kricker et al., 1995). It has been suggested that accumulation of mutations leads to non-viability of cells that could otherwise have become a BCC or that accumulation of mutations renders cells more antigenic and therefore more susceptible to immunological elimination.

UV has been shown to invoke a degree of immunosuppression in both animals and humans (Whitmore and Morison, 1995). Much research has concentrated on UV induced immunosuppression of contact hypersensitivity with polymorphism at the Tnf-α and Lps loci determining whether the immune system in mice is UV susceptible or resistant (Streilein, 1993). Thus, both UV-induced DNA alterations as well as immune modulation are important in cutaneous carcinogenesis. Furthermore, associations between skin cancer and other internal malignancies, especially those seen in immunosuppressed individuals such as non-Hodgkin's lymphoma (Hoover and Fraumeni, 1973), could be expected to occur. Indeed, this has been shown in SCC and MM (Adami et al., 1995). Further studies are needed to assess any such association with BCC. UV-induced immunosuppression can be blocked by the application of sun screens (Whitmore and Morison, 1995).

The usefulness of sun screens and which factor numbers are needed has attracted much attention. There appears to be a biological compensation for very low doses of radiation so that no clinical effect is seen below a certain threshold level (Marks, 1995). This view is supported by data in animals showing no tumours are produced until a threshold dose of UV is reached. A similar phenomenon is also seen in studies

on the relationship between cell killing and radiotherapy (Withers, 1992). Also, reducing the dose of UV in mice reduces the number of tumours and increases the time of onset of the tumour (Blum et al., 1941; Kligman et al., 1980). However, the limitations of applying animal studies are well recognised. Translated into sun-protection (SPF) numbers, if it takes 40 years to develop a tumour, regular use of a SPF 2 product would delay the onset of the tumour until the age of 80 and SPF 4 would delay it until 160 years. It has been suggested that the extra cost involved related to production of the high SPF products was hard to justify in terms of reduced risk of skin cancer (Marks, 1995). As UV is considered the major aetiological agent, prevention focuses on reducing exposure, both in childhood and in later life. Much effort has addressed these issues in public health campaigns. Sunscreens have been advocated but there is no hard evidence that they prevent BCC. They do suppress actinic keratoses, a possible precursor to SCC (Naylor et al., 1995). Trials are currently underway to assess the effect of sunscreens in BCC prevention.

The association between PUVA (8-methoxypsoralen plus UVA radiation) therapy for psoriasis and development of cutaneous malignancy has stimulated much research. In a series of reports BCC and, more commonly, SCC were detected more often than expected on skin and genital regions in patients who had received PUVA (Stern et al., 1984; Stern et al., 1988; Stern et al., 1990). This led to protocols for PUVA administration and the need for careful examination of patients for cutaneous malignancy in those who had received this therapy. The finding that SCC occurs much more commonly than BCC is interesting and not yet explained. It has been suggested this illustrates that UV radiation plays a more important role in SCC pathogenesis than BCC. UV causes mutagenesis in mammalian cells (Jones et al., 1987), induces pyrimidine dimers in human skin (Freeman et al., 1987) and is

photocarcinogenic in mice (Strickland, 1986). 8-methoxypsoralen forms unstable complexes in DNA and subsequent UVA exposure causes DNA cross linking. Cross linking of the psoralens in the presence of DNA is highly mutagenic (Studniberg and Weller, 1993). PUVA also has immunologic effects including:

- Reduction of contact hypersensitivity (Strauss et al., 1980)
- Reduction of Langerhans cells (Ree, 1982)
- Dose dependent changes in lymphocyte function (Morison et al., 1981)
- A pseudopromoter role by inhibition of immune surveillance (Stern 1989)

These mechanisms are thought to account for skin cancer induction. Psoralens themselves are not recognised to be carcinogenic although a recent study shows that 8-methoxypsoralen causes cancer of the kidney and lung in rats but dosages were 75 times higher than those usually used in humans (National Institutes of Health, 1989).

2.6. HLA Associations/Chromosomal Abnormalities/Oncogenes.

The major histocompatibility complex (MHC) genes code for membrane proteins that play an important role in controlling immune responses (Benacerraf, 1981). Alterations in expression of these antigens on tumour cells are frequent in human cancers (Esteban et al., 1990) and appear to be associated with carcinogenesis (Garrido, 1987). *De novo* appearance, reduction or loss of antigens may occur. These alterations may facilitate escape from immunological attack. The association of BCC with human leucocyte antigen (HLA) molecules has been studied with contradictory findings. An association between HLA-DR1 and multiple BCC was reported in North America (Myskowski et al., 1985), Southern Europe (Cerimele et al., 1988) and Southern Australia (Czarnecki et al., 1991). Early onset of BCC was found in HLA-DR1 positive individuals in Australia (Czarnecki et al., 1991). These results suggest

that HLA-DR1 is a susceptibility marker to BCC development and acts synergistically with environmental factors, probably UV radiation. Differences in HLA associations between patients with BCC and those with both BCC and SCC have been described: HLA-DR1, DR4 and DR7 were significant suggesting that different genes are involved in susceptibility to different forms of skin cancers (Czarnecki et al., 1994). Other studies have found no association with class 1, class 2, HLA-A, HLA-B, HLA-DQA1 or HLA DQB1 antigens (Cabrera et al., 1992; Rompel et al., 1993; Emtestam et al., 1996).

A large body of data now exists indicating that acquisition of genetic rearrangements is an important event in carcinogenesis (Heim and Mitelman, 1987). Particularly important are the results of chromosome analysis in the haemopoietic malignancies, in which certain chromosome aberrations are non-randomly associated with different tumour types (Trent et al., 1989). The gene for naevoid basal cell carcinoma syndrome has been mapped to chromosome 9q (Wicking et al., 1994). Chromosome 9 allele loss occurs at high frequency in patients with BCC (Gailani et al., 1992; Quinn et al., 1994) and is in keeping with observations of chromosome loss seen in other cancers such as colon (Kern and Vogelstein, 1991) and breast (Black and Soloman, 1993). These findings in BCC suggest that chromosome 9q contains one or more tumour suppressor genes important in BCC development. Similar loss is seen in SCC (Quinn et al., 1994). Loss of heterozygosity of markers from chromosomes 9p, 13q, 17p, 17q and 3p has also been described in SCC (Quinn et al., 1994). A high level of non-clonal structural rearrangements, mostly balanced translocations, were found in patients with BCC (Martens et al., 1991). This was felt to provide evidence that BCC may have a muticellular origin reflecting field cancerisation of the skin. With tumour development, selection pressure restricts the number of clones that infiltrate

surrounding tissue. 9q loss is felt to be an essential factor for BCC development with an incomplete relationship seen between sunlight exposure and 9q loss, suggesting that BCC incidence may be associated with other factors in addition to UV exposure (Gailani MR et al., 1996).

Ras genes have also been studied in relation to BCC. The ras gene family consists of three related genes (Ha-*ras*, Ki-*ras*, N-*ras*) which encode proteins with intrinsic GTPase activity (Barbacid, 1987). Mutational activation of these genes has been found in a wide variety of tumours at differing frequencies (Barbacid, 1987). Reports of high frequency activation in the Harvey-*ras* protooncogene in 31% of BCC (Piercaell et al, 1991) were not substantiated (Campbell et al., 1993). These conflicting results may represent technical artefacts or differences in molecular epidemiology between areas of high and low sun exposure. In tumours from xeroderma pigmentosum patients, a high frequency of mutations in *ras* genes was detected (Daya-Grosjean et al., 1993). The high mutation frequency was accompanied by a high level of Ha-*ras* and c-*myc* gene amplification and rearrangement. All mutations were opposite hot-spots for UV-induced DNA lesions suggesting a role for unrepaired DNA lesions as an initiating event in human skin tumours on exposed parts of the body (Daya-Grosjean et al., 1993). Increased expression of the c-*fos* oncogene has been found in infiltrative BCC compared with non-infiltrative (Urabe et al., 1994) suggesting that increased expression of the fos oncogene may affect invasiveness of the tumour cells.

2.7. DNA Repair/Apoptosis/ P53.

The ultraviolet component of sunlight induces the formation of covalent bonds between adjacent pyrimidines, generating photoproducts such as cyclodipyrimidine dimers (T/T) and pyrimidine-pyrimidine (6-4) lesions (Setlow, 1966). These photoproducts are mutagenic if not repaired and may lead to carcinogenesis (Setlow, 1978). The relationship of the persistence of UV-induced DNA damage to mutation fixation has been illustrated by activation of the Ha-ras oncogene on sun exposed body sites. As a consequence, efficient DNA repair mechanisms are needed to protect cells. Consequently, there has been much interest in DNA repair mechanisms in skin cancer. Most early research was performed on patients with xeroderma pigmentosum (XP), a rare autosomal recessive syndrome. In this syndrome multiple primary skin tumours, especially BCC, are seen. A relationship was found between reduced capacity to repair DNA and sunlight exposure in the aetiology of skin cancer in XP patients (Cleaver, 1969; Setlow et al., 1969). Early in life homozygote XP patients develop severe photosensitivity and a 2,000-fold increased risk of sunlight-induced skin cancer (Cleaver and Kraemer, 1989) which can be ameliorated by early, lifelong protection from sunlight exposure (Lynch et al., 1977). More recently, data on decreased DNA repair and susceptibility to sporadic BCC has emerged (Athas et al., 1991; Alcalay et al., 1990; Munch-Peterson et al., 1985) giving insights into the link between sunlight exposure and tumour development. DNA repair capacity below the upper 30th percentile of a control population was associated with a 2.3 fold increase in relative risk for BCC occurrence (Wei et al., 1994). Also, the lower the DNA repair capacity, the greater the number of skin tumours in individuals were seen. Interestingly, those taking oestrogen had higher DNA repair capacities suggesting DNA repair levels may be sensitive to hormonal control via an unknown mechanism

(Wei et al., 1994). Females with reduced DNA repair capacity have been shown to have a greater risk of skin cancer, with ten fold increases, compared to five fold increases in men (Wei et al., 1993). However, the measurement of DNA repair has often used cell transfection techniques which have been criticised (Hall et al., 1994) and some studies have reported increased repair in patients with BCC when compared with controls. Thus, batch variability and the effects of age, family history of skin cancer and current sun exposure may possibly confound results (Hall et al., 1994).

The effect of age on DNA repair has also been assessed (Wei et al., 1993). An age-related decline in DNA repair capacity amounting to 0.61% per year has been shown in patients without skin cancer. Young individuals with BCC repaired DNA poorly when compared with controls but the older the BCC patient, the smaller was the difference between cases and controls (Wei et al., 1993). This suggests that the normal decline in DNA repair may account for the increased risk of skin cancer that begins in middle age.

BCC is typically slow growing. Several explanations for this have been proposed (Verhaegh MEJM et al., 1995):

- tumoral prolongation of the S-(DNA synthesis) phase of the cell cycle
- a small percentage of tumour cells which are actively proliferating
- tumour regression in response to host immune factors
- limited enzyme lysis of adjacent connective tissue
- mechanical resistance of the dermis counteracting the expansion of the carcinoma

An alternative explanation for the slow growth was suggested by Kerr: the phenomenon of apoptosis (programmed cell death) which was seen to be prominent

in BCC. (Kerr and Searle, 1972). A fundamental feature of carcinogenesis is deregulation of proto-oncogenes which increase malignant potential by inhibiting programmed cell death (Williams, 1991). B-cell leukaemia/lymphoma-2 (bcl-2) was discovered at the chromosomal translocation point t(14;18) in many follicular lymphomas (Tsujimoto et al., 1985). Bcl-2 functions as a repressor of programmed cell death. The other form of cell death is necrosis. Apoptotic cells show a decrease in volume, clumping of chromatin, nuclear fragmentation and cell membrane blebbing (Cohen, 1993). This mode of physiological cell death appears to be intrinsically programmed and regulates tissue size. Overproduction of bcl-2 in transgenic mice provided evidence for the tumorigenic potential of these proteins in that they prevented cell death as demonstrated by prolonged cell survival and cell accumulation (McDonnell et al., 1989). Recently, bcl-2 has been shown to associate with a further protein, bax (Oltvai et al., 1993). Bax shows amino acid homology with bcl-2 and it appears the ratio of bcl-2 to bax determines survival or death following an apoptotic stimulus. If bcl-2 protein is in excess within a cell, the cell survives (Baranaga, 1994).

Bcl-2 is localised to mitochondria, nuclear membranes, the endoplasmic reticulum and is expressed in the basal cell, but not superficial, layer of skin (Hockenberry et al., 1990) and expression would be expected on BCC tumour cells. The described prominent cell death seen in BCC implies an absence of bcl-2 (Kerr and Searle, 1972). However, the situation is unclear with a study demonstrating positive bcl-2 staining in 15 patients with BCC suggesting BCC is caused by extended cell survival rather than increased cell proliferation and that this type of neoplastic growth may be associated with less aggressive tumour behaviour (Verhaegh et al., 1995). Furthermore, studies with the proliferation-related antigen Ki-67 in BCC demonstrated

immuno-reactivity localised either to the nuclei of 3-5 rows of peripheral cells, or to nuclei scattered in the central as well as peripheral parts of the tumour strands suggesting the majority of cells are not actively proliferating (Baum et al., 1993). A more recent study suggested that a disturbance of bcl-2 or p53 may enhance tumour formation by suppressing apoptosis in human NMSC (Wikonkal et al., 1997). P53 acts as a brake on tumour growth to allow optimal DNA repair. However, it has also been suggested that if this repair is not successful the P53 gene can induce apoptosis. The mutant version of the P53 gene may act in a similar way to bcl-2 in blocking apoptosis (Marx, 1993). The pattern of bcl-2 expression may be of use in distinguishing BCC and trichoepithelioma (a benign follicular neoplasm). The separation of these two tumours can be difficult for the histopathologist. Trichoepitheliomas show bcl-2 expression on the outermost cells whereas BCC, in general, show more diffuse staining (Smoller et al., 1993).

The p53 gene, located on chromosome 17p, encodes a nuclear phosphoprotein which is thought to influence cell proliferation (Lane and Benchimol, 1990; Levine et al, 1991). Several domains of the p53 molecule are highly conserved throughout evolution suggesting functional importance (Soussi et al., 1990). The wild type p53 protein acts as a potent suppressor of cell growth, allowing DNA repair or facilitating apoptosis. It is present at low concentrations and can suppress the transformation of cells transfected with *ras* and mutant p53 genes or with E1a or c-*myc* and *ras* (Finlay et al., 1989; Eliyahu et al., 1989). Deletion or mutation of the p53 gene is one of the commonest genetic abnormalities found in human cancers (Hollstein et al., 1991). In carcinoma of the colon up to 80% of tumours show loss of function of both wild type p53 alleles, one by deletion the other by mutation (Baker et al., 1989). These events result in loss of heterozygosity for polymorphisms in the chromosomal region

surrounding the p53 gene. In BCC, however, it appears there is only a single inactivated p53 allele which is in contrast to other epithelial tumours which usually progress by inactivation of both alleles (Van Der Riet et al., 1994). These observations have led to the suggestion that inactivation of both p53 alleles may lead to aggressive BCC behaviour. Some mutant p53 protein inhibits the function of normal p53 by forming complexes with wild type protein, acting as a dominant negative (Eliyahu et al., 1988). All mutations so far examined have lost the function to suppress transformation (Halevy et al., 1990). Much research has assessed the role and stage of involvement of p53 mutations in SCC. It has been shown that skin appears to have a p53 dependent response to DNA damage which aborts precancerous cells. If this response is reduced in a single cell by a prior p53 mutation, sunburn can select for clonal expansion of the p53 mutated cell into an actinic keratosis (believed to be a precursor of SCC). Thus, sunlight can act both as tumour initiator and promoter (Ziegler et al., 1994).

The low concentration of p53 protein in cells makes it difficult to detect but the mutant protein has a longer half-life and so any detection by conventional methods suggests that mutation has occurred (Lane and Benchimol, 1990). The major problem with this approach is that detectable staining is only suggestive of mutation and not diagnostic. Indeed inaccuracies have been shown (Kubo et al., 1994). The presence of mutant p53 protein may affect tumour behaviour: for example high grade breast tumours are associated with p53 protein detection enabling use of p53 protein detection as a prognostic marker (Ostrowski et al., 1991). In skin cancer, one study showed that 42% of BCC's had detectable p53 protein compared with 3.6% of MM's, suggesting detectable p53 protein is not associated with malignant phenotype or metastatic potential in malignant epithelial lesions (Ro et al., 1993). Greater numbers of UV-

related SCC and actinic keratoses have detectable p53 when compared to UV-unrelated SCC suggesting UV acts as a mutagen for the p53 gene and this may occur at a relatively early stage of carcinogenesis (Naguno et al., 1993). It has also been shown that detectable p53 protein was more common in aggressive BCC than non-aggressive (De Rosa et al., 1993).

Recently, *PTC,* a strong candidate gene for Gorlin's syndrome has been identified on chromosome 9q22.3 (Hahn et., 1996; Johnson et al, 1996). This is a human homologue of a drosophila gene, *patched*, which encodes a protein that acts in opposition to the *hedgehog* signalling protein with effects on cell death, proliferation and differentiation. Heritable mutations in this gene were described in studies on families with Gorlin's syndrome (Hahn et., 1996; Johnson et al, 1996). Mutations have also been described in patients with sporadic BCC with high expression of *PTC* in tumour cells but not normal skin, suggesting mutation of the gene leads to over expression of mutant transcript (Gailani et al., 1996). Interestingly, many observed mutations in patched were not typical of UVB induced mutations, suggesting factors other than UV may be important. It has been suggested that *PTC* mutation may be essential for BCC development and that *PTC* is a putative gatekeeper of human skin (Sidransky, 1996). Furthermore, sonic hedgehog protein (the ligand for *PTC,* which inactivates its function*),* is also important in that this mimics loss of *PTC* function with development of BCC in transgenic mice overexpressing sonic hedgehog protein (Oro et al., 1997). This has also recently been shown in transgenic human skin (Fan et al., 1997). Thus, sonic hedgehog protein is sufficient to induce BCC in mice and *in vivo* in human skin. Many future studies will undoubtedly appear on the role of *PTC* and sonic hedgehog in BCC pathogenesis.

2.8. Detoxifying Enzymes.

UV has pleiotrophic effects on DNA, membrane lipids and amino acids in skin (Kripke 1994) suggesting differences in response to this stress may mediate risk. Thus, the effectiveness of repair of UV-damaged DNA is associated with the risk of multiple BCC (Wei et al 1994). Since exposure to UV constitutes an oxidative stress with the formation of reactive oxygen species (ROS) in skin (Black,1987), the detoxification of these species and their products is also likely to be critical, implicating genes involved in these reactions as susceptibility candidates. Because of the potentially deleterious effects of ROS, cells use various mechanisms to modulate their concentration including widespread expression of the antioxidant enzymes, CuZn- and Mn-superoxide dismutase, catalase and selenium-dependent glutathione peroxidase (Touati 1989). Co-ordinated expression of these genes ensures regulation of the levels of superoxide radical and hydrogen peroxide (H_2O_2) in cells and suppression of formation of the dangerously reactive hydroxyl radical ($HO^{\cdot}$). While appropriate expression of these enzymes protects cells, many cellular molecules demonstrate evidence of oxidative damage even in health under resting conditions. Much interest has focused on the extent of damage to membrane lipid and mitochondrial and genomic DNA. Peroxidation of membrane lipid is continuous, though in healthy tissues levels of oxidised products such as malondialdehyde and alkenals are low, indicating continuous detoxication of these potentially dangerous compounds. Increased lipid peroxidation has been found in various pathologies and while it may be a consequence rather than cause of injury, it is potentially deleterious as it alters membrane fluidity and permeability. Lipid peroxidation results in formation of peroxy radicals which form lipid hydroperoxides. These decompose to a complex mixture that includes cytotoxic aldehydes such as malondialdehyde, 2-alkenals and 4-hydroxyalkenals. DNA strand

breaks and damage to bases and sugars are a further early and significant result of oxidative stress. They result from $HO^{\cdot}$-induced damage or activation of nucleases. $HO^{\cdot}$ induced damage to bases can be identified by end-products such as 5-hydroxymethyluracil, 8-hydroxyadenine and 8-hydroxyguanine (Halliwell and Aruoma, 1991).

The use of oxygen as an electron acceptor requires expression of enzymes that decrease production of $HO^{\cdot}$ and/or allow repair/replacement of damaged lipid and DNA. Although these processes remain poorly understood, peroxidase activity appears central in detoxication of lipid and DNA hydroperoxide. Thus, recent data suggest the enzymes of the glutathione S-transferase (GST) supergene family, via their peroxidase activities are critical in the detoxification of cytotoxic, lipid and DNA products of oxidative stress including DNA hydroperoxides (Figure 2.8.1.) (Hayes and Strange 1995).

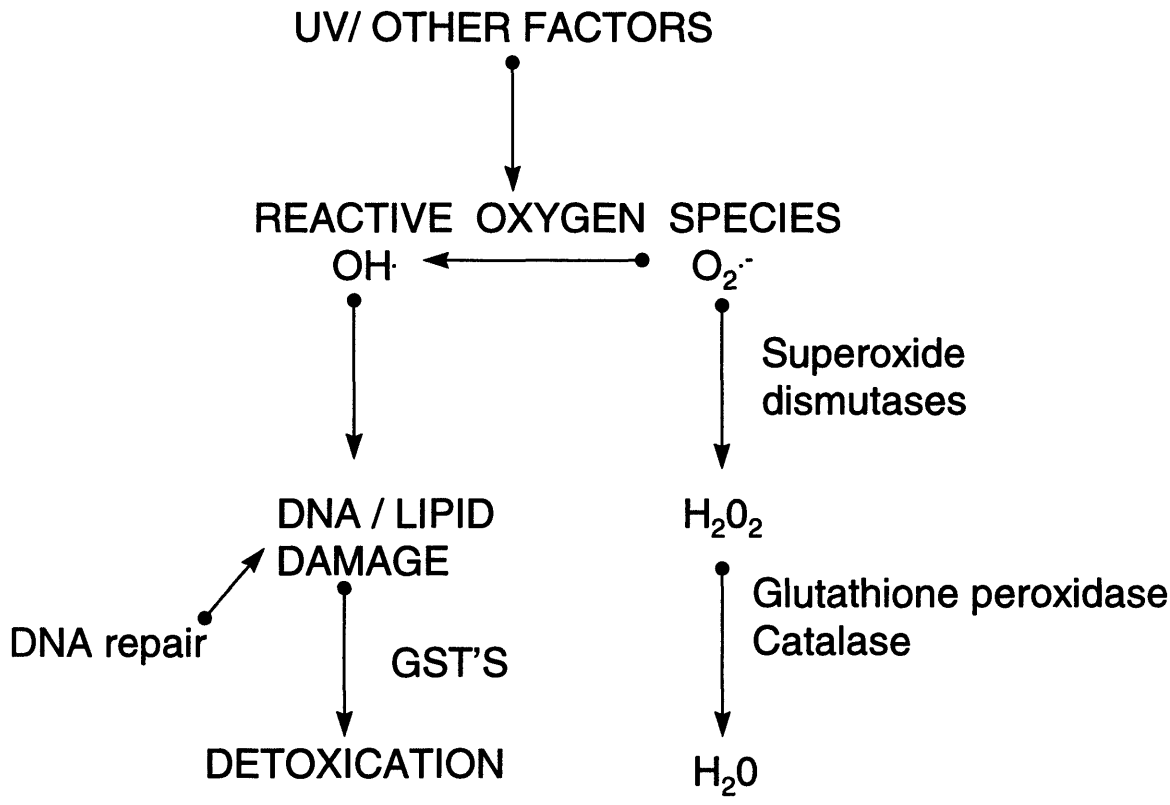


Figure 2.8.1. **Relationship between GST and other antioxidant enzymes**

Glutathione S-transferase (GST) genes encode a family of dimeric phase 2 enzymes (monomer molecular mass 17-28 kDa) that have a pivotal role in catalysing the conjugation of glutathione to a wide range of electrophilic substrates, many of which are formed during phase 1 metabolism (Rushmore and Pickett, 1993). This is an important pathway of protection against chemical toxins and carcinogens. Four cytosolic GST gene families expressed in mammalian tissues have been extensively studied; alpha, mu, theta and pi. Each of these GST families may include multiple genes, with active enzymes comprising homo- and heterodimeric combinations of monomers encoded by genes of the same family. For example, 5 mu class genes are arranged in tandem on chromosome 1. Members of the same gene family show at least 65% amino acid identity (Mannervik et al., 1992). A fifth form, the trimeric microsomal GST, is membrane bound (De John et al., 1988). Table 2.8.1 illustrates the location and some of the functions of the GST family.

Table 2.8.1. Human Glutathione S-transferases.

GST family	No. of genes	Chromosomal localisation	Carcinogen substrates
mu GSTM	5	1p13	DNA peroxidation products
theta GSTT	>2	?	solvents, DNA hydroperoxides
alpha GSTA	>2	6	aromatic amines, lipid peroxidation products, anti-cancer drugs
pi GSTP	1	11	polycyclic aromatic hydrocarbons
Microsomal GST mic	1	12	?

A major function of the cytosolic enzymes is believed to be the detoxication of reactive mutagenic electrophiles (Jacoby, 1978). They are also increasingly considered to have an important role, via their glutathione-dependent peroxidase activities, in free radical scavenging in vivo thus protecting cells from the deleterious effects of oxidative stress (Sies and Ketterer, 1988). Therefore, these enzymes may play a role in protection against tumour promotion. Like cytochrome P450s (CYPs), these enzymes are thought to have evolved as an adaptive response to environmental insult and utilise a wide variety of structurally diverse substrates, many of which induce GST expression (Rushmore and Pickett, 1993). For example, GST expression is significantly increased on exposure to chemoprotective agents such as food preservatives (Smith et al., 1995). This increased expression is mediated in part by an anti-oxidant responsive element present in the promoter region of the GST genes (Rushmore et al., 1990). Unlike the cytochrome P450s, GSTs exhibit higher levels of constitutive expression in a variety of tissues, many of which demonstrate different patterns of GST iso-enzyme expression. Indeed, increased expression of specific GST iso-enzymes in tumour cells, especially those resistant to anti-cancer drugs, has suggested a role for these proteins in resistance to chemotherapy (Hayes and Wolf, 1990). In many cases, GST expression is increased in neoplastic tissues. However, this is not universal as in prostatic tumours GSTP1 mRNA and protein are undetectable (Lee et al., 1994).

Polymorphic loci have been identified in all cytosolic GST gene families but the molecular basis and pathological consequences of such variation, if any, remains largely unclear (Smith et al., 1995). There are well described differences in the phenotypic expression of GSTA in human tissues (Strange et al., 1992) but there is only one report of an RFLP in GSTA2 (Chen and Board, 1987). Similarly, two

polymorphisms at the GSTP1 locus have been identified but their significance remains unknown (Harada et al., 1994). Most interest has focused on the polymorphisms at the GSTM1 and GSTT1 gene loci. One of these, GSTM1 has been intensively studied. Indeed, the observation that GSTM1 can utilise 5'-hydroxymethyluracil led to the suggestion that it is involved in repair of UV-damaged DNA (Ketterer et al 1993). Two cytosolic theta class GST, T1-1 and T2-2, have been isolated from human liver. Importantly, the distribution of enzymes of the various GST gene families expressed in cytosol can be different (Hayes and Strange,1995).

Common null alleles have been identified at GSTM1 and GSTT1 (Ketterer et al 1993). Thus, GSTM1 demonstrates phenotypes that arise from combinations of the GSTM1*0, GSTM1*A and GSTM1*B alleles. GSTM1*0 is a complete gene deletion and homozygotes (GSTM1 null) who comprise about 50% of most populations (Strange 1995), express no protein. So far two alleles have been identified at GSTT1; GSTT1*0 and GSTT1*A (Ketterer et al 1993; Pemble et al 1994). Homozygosity for the deleted GSTT1*0 allele is found in about 20% of control subjects (Strange 1995). Both GSTT1*0 and GSTM1*0 homozygotes appear at greater cancer risk though the specificity (i.e. why some cancers but not others) and sensitivity (i.e. how much extra risk is conferred) remains unclear (Smith 1995; Strange 1995).

GSTM1*A and GSTM1*B differ by a single base in exon 7. In vitro data suggests the effectiveness of these enzymes are similar. GSTM1 has been studied particularly in relation to smoking-related cancers especially lung. Some studies have found the GSTM1 null genotype to be more common (Seidegard et al., 1986; Zhong et al., 1991;McWilliams et al., 1995) while others found no such association (Kerb et al., 1995). The reasons for the discrepancies are unclear. One problem could be the

relatively weak influence of any single gene on disease risk and possible confounding factors such as polymorphisms at other loci. Differences in exposure to carcinogens, diet, gender and control populations may also influence such analyses (Smith et al., 1995). Increased frequency of GSTM1 null has been reported in colorectal and gastric cancer (Strange et al., 1991). In BCC, GSTM1 A/B heterozygotes are protected against multiple tumours suggesting a gene dosage effect is important, with those expressing two alleles having better protection than GSTM1 null heterozygosity (Heagerty et al., 1994). Also, studies in smokers with lung cancer showed that p53 and H- ras mutations were more frequent in GSTM1 null individuals suggesting GSTM1 protects these genes against mutagenesis (Ryberg et al., 1994). It is known that GSTM1 null is associated with photosensitive Systemic Lupus Erythematosus (Ollier et al., 1996) suggesting that detoxication of products of oxidative stress may be important in determining response of the skin to UV radiation. Furthermore, mean MED's have recently been shown to be lower in those with GSTT1 and GSTM1 null genotypes than in those who express the enzymes (Kerb et al., 1997).

Studies in lung cancer suggest that co-ordinated expression of some mu class genes is important: thus, *GSTM1*0* homozygotes express less GSTM3 than subjects with other GSTM1 genotypes (Antilla et al., 1995). The mechanism for this observation is unknown but may be related to the finding GSTM3 is also polymorphic with two alleles, *GSTM3*A* and *GSTM3*B*, identified (Inskip et al., 1995). Importantly, *GSTM3*B* is in linkage disequilibrium with *GSTM1*A* and contains in intron 6, a recognition motif for the YY1 transcription factor which is known to regulate gene expression from intragenic sites (Inskip et al., 1995). The widely expressed YY1 factor influences the expression of many genes (Flanagan, 1995) suggesting *GSTM3*A* and *GSTM3*B* are expressed at different levels.

The identified polymorphism in GSTT1 (Pemble et al., 1994) accounts for the observed phenotypic variation in glutathione related detoxication of halomethanes by human erythrocytes. GSTT1 is an attractive candidate gene for susceptibility to cancer as it metabolises potential carcinogens including monohalomethanes, ethylene oxide (present in cigarette smoke) and epoxides suggesting individuals with null genotypes at both GSTM1 and GSTT1 may be at risk of smoking-related cancers. GSTT1 also catalyses the detoxication of oxidised lipid and DNA, suggesting a role in DNA repair (Ketterer et al., 1993; Pemble et al., 1994).

Cytochrome P450 enzymes are a supergene family of monomeric mixed function mono-oxygenases, responsible for the phase 1 metabolism of a wide range of structurally diverse substrates (Gonzalez, 1990). Table 2.8.2. illustrates some substrates for P450s (Smith et al., 1995). They are haem-containing proteins located in the endoplasmic reticulum or mitochondrial membrane. They are arranged in families on various chromosomes and many members are genetically polymorphic. Some of these polymorphisms have phenotypic consequences whereas the significance of others is still unclear. Cytochrome P450 expression in the liver varies widely between individuals (Forrester et al., 1992; Shimada et al., 1994). However, isozyme expression in extrahepatic tissues with cell specific substrate activation is also seen. These enzymes insert an atom of molecular oxygen into their substrates leading to an increased hydrophilicity and to generate substrates for phase 2 enzymes (Smith et al., 1995). They function as terminal electron acceptors. Certain substrates, including carcinogens, are activated by these enzymes, producing mutagenic, chemically active electrophiles. Most chemical carcinogens require some metabolic activation in order to achieve maximum activity leading to the proposition of the involvement of P450s in the

specificity of tumour formation following exposure to certain xenobiotics (Parke and Ionnides, 1990). This also indicates the need for co-ordinated phase 1 and 2 expression to prevent accumulation of carcinogenic intermediates. It is still unclear which combination of genotypes are associated with increased susceptibility in specific cancers. A wide variety of chemicals are involved in tumour promotion.

Table 2.8.2 P450 Substrates in Humans

Gene Family	Substrates
CYP1A	Benzo(a)pyrene, aflatoxin, anilene, charbroiled meat, phenacetin, theophylline, caffeine, imipramine, tamoxifen, dioxin (TCDD)
CYP2A	Coumarin, aflatoxin, phenobarbitone
CYP2B	Phenobarbitone, nicotine, cyclophosphamide
CYP2C	Retinoic acid, tolbutamide, warfarin, phenytoin, propranolol, diazepam, diclofenac
CYP2D	Haloperidol, debrisoquine, amitryptyline, codeine, ecstasy, lignocaine
CYP2E	Pyridine, nitrosamines, ethanol
CYP3A	Aldrin, aflatoxin, doxrubicin, cyclosporin, vinblastine, gestodene

The human CYP1A sub-family has two members, CYP1A1 and CYP1A2. CYP1A1 is predominantly extra-hepatically expressed whereas CYP1A2 is liver specific. Both CYP1A1 (aryl hydrocarbon hydroxylase) and its ligand-dependent transcription factor, the Ah receptor, are widely expressed in extra-hepatic tissues including skin (Raunio et al., 1995) and though the gene has largely been studied in the context of environmental pollutants (e.g. polycyclic aromatic hydrocarbons (PAH)), there is evidence its products utilise endogenous ligands and participate in defence against oxidative stress (Smith et al., 1995; Nebert, 1994). Thus, aryl hydrocarbon hydroxylase activity in rodent skin is increased by UV presumably because of photoproducts that induce expression. Indeed in vitro studies show UV irradiation of amino acids such as tryptophan, that are present in skin, results in oxidised products that bind to the Ah receptor with high affinity (Rannug et al., 1987). An Msp1 RFLP has been identified in the 3' non-coding region of the CYP1A1 gene (Spurr et al., 1987).The phenotypic consequence of this mutation has been linked to increased inducibility of CYP1A1 expression in response to PAH substrates (Kawajiri et al., 1990). A further point mutation in exon 7 leads to an isoleucine to valine amino-acid substitution. The mutant enzyme is suggested to have greater specific activity towards benzo(a)pyrene (Hayashi et al., 1991). CYP1A1 expression is induced in the airways of lung cancer patients who smoke (Kawajiri et al., 1990) and the relative level of CYP1A1 expression has been linked to the pathogenesis of smoking-induced lung cancer (Kouri et al., 1992). The high inducibility phenotype has been associated with lung cancer in a Japanese population (Hayashi et al., 1991) but not in a Caucasian study (Hirvonen et al., 1992).

The CYP2D gene locus contains three genes arranged on human chromosome 22 (Gough et al., 1993). Two of these contain inactivating mutations (Kimura et al., 1989; Heim and Mayer, 1992) and the third, CYP2D6, is genetically polymorphic (Maghoub et

al., 1977). Many commonly prescribed drugs are substrates for CYP2D6. Polymorphism in this gene lead to profound changes in drug metabolism and can have severe clinical consequences (Kroemer et al., 1994). Genotypes are designated EM (extensive metaboliser), PM (poor metaboliser) and HET (heterozygotes). The PM frequency shows considerable inter-ethnic differences illustrating the importance of ethnicity when studying such genes in disease. Caucasians have a PM frequency of 5-7% (Eichelbaum et al., 1979) whereas blacks have a frequency of 2% (Evans et al., 1993) and Orientals <1% (Lou et al., 1987). Numerous studies have assessed the role of CYP2D6 polymorphisms in the incidence of many cancers. However, it is still unclear as to the effects of these polymorphisms in cancer development. A decrease in PM frequency has been found in lung cancer patients (Wolf et al., 1994) and CYP2D6 metabolises some carcinogenic components of cigarette smoke (Crespi et al., 1991). However, the reduction in cancer risk was small (odds ratio 0.67). Further well constructed case-control studies are needed to obtain a clear consensus (Smith et al., 1995). Other conditions associated with the CYP2D6 PM genotype include leukaemia (Wolf et al., 1992), Parkinson's disease (Smith et al., 1992), Alzheimer's disease (Benitez et al., 1993), meningioma (Wundrack et al., 1994) and rheumatoid arthritis (Benitez et al., 1994).

The N-acetyl-transferases (NATs) are another important group of phase 2 detoxifying enzymes. They catalyse the transfer of an acetyl group from the co-factor acetyl coenzyme A to the amine atom of aromatic amines and hydrazines (Smith et al., 1995). The human NAT gene family is located on chromosome 8 (8p22) and consists of three loci: two of which are functional in humans (Hickman et al., 1994; Kelly and Sim, 1994). Both functional NAT loci are polymorphic (Smith et al., 1995) but the functional significance of the polymorphism in NAT1 is still unknown. However, NAT2

polymorphisms enable classification into fast and slow acetylator phenotypes. There is a clear association between acetylation phenotype and susceptibility to bladder cancer with a preponderance of slow acetylators (Cartwright et al., 1982). Other cancers with possible associations include colorectal (Kadlubar et al., 1992) and laryngeal (Drozdz et al., 1987).

Table 2.8.3. summarises the molecular biological findings described in BCC. Figure 2.8.2 summarises the possible mechanisms in cutaneous carcinogenesis.

Table 2.8.3. Summary of described genetic abnormalities in BCC

	Findings	Authors	Year
UV radiation	Mutagenesis	Jones et al.	1987
	Cyclo-pyrimidine dimers	Setlow.	1966
	Photocarcinogenic	Strickland.	1986
	Immunosuppressive effects	Kripke	1994
DNA repair	Capacity impaired in xeroderma patients	Cleaver.	1969
		Setlow et al.	1969
	Capacity impaired in sporadic BCC	Wei et al.	1994
	Age related decline in capacity	Wei et al.	1993
Detoxifying	GSTM1 A/B protective	Heagerty et al.	1994
enzymes	GSTM1 null/skin type influences susceptibility	Lear et al.	1996
	Genotypes related to number/accrual of BCC	Lear, Yengi et al.	1996
HLA	Multiple BCC/early onset with DR1	Myskowski et al.	1985
		Czarnecki et al.	1991
		Cabrera et al.	1992
	No correlation	Emtestam et al.	1996
Chromosome	High frequency of chromosome 9 allele loss	Gailani et al.	1992
defects		Quinn et al	1993
	High level of balanced translocations	Mertens et al.	1991
Ras genes	High frequency of activation	Piercaell et al.	1991
	Not substantiated	Campbell et al.	1993
Apoptosis	Prominent cell death described	Kerr, Searle	1972
	Positive Bcl-2 staining	Verhaegh et al.	1995
P53	Single, inactivated p53 allele in BCC	Van der Riet et al.	1989
	42% of BCC detectable protein staining	Ro et al.	1993
PTC	Mutations in those with Gorlin's syndrome	Hahn et al.	1996
		Johnson et al.	1996
	Mutations in sporadic BCC	Gailani et al.	1996

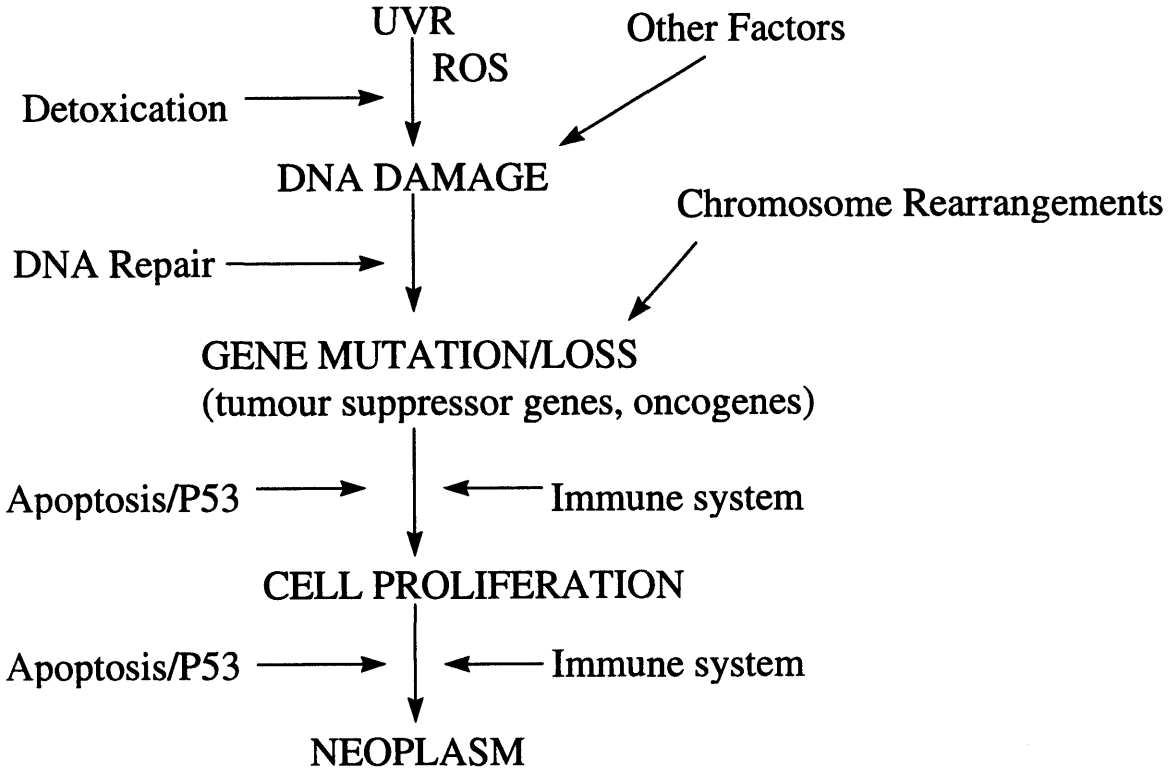


Figure 2.8.2. Cutaneous carcinogenesis

2.9. Aims Of The Study.

1. To identify individual characteristics as risk factors for BCC development in the U.K. and, in particular, factors for multiple BCC development.

2. To assess the influence of polymorphisms in GSTM1, GSTM3, GSTT1, CYP1A1 and CYP2D6 on susceptibility to BCC. In particular to assess effects on total number of primary tumours, rate of lesion accrual, time to next tumour presentation and tumour site.

3. To identify a possible association between BCC and internal malignancy.

4. To compare individual characteristics between individuals with MM, SCC and BCC.

5. To identify risk factors in individuals with skin cancers of different histological types and to compare individual characteristics in this group to those with BCC.

6. To assess the influence of individual characteristics and polymorphisms in GSTM1, GSTM3, GSTT1, CYP1A1 and CY2D6 on skin type.

CHAPTER 3 - METHODS

3.1. Patients and Statistics

3.1.1 Patients: Recruitment and Characterisation.

Patients. Unrelated Northern European Caucasians patients with histologically proven BCC (approximately 850 patients) were identified and recruited from Dermatology clinics in the North Staffordshire Hospital, Stafford District General Hospital and Royal Cornwall Hospitals. These cases constituted a random sample of patients from the participating centres from whom DNA was available and represented cases from a mix of urban and rural environments. None of those approached refused to participate. No patients with basal cell naevus syndrome or xeroderma pigmentosum were included. Patients were examined and interviewed to obtain information on age, sex, hair colour at 21 years of age (blonde/red and brown/black), eye colour (blue/green and brown) at 21 years of age, skin type (types 1-4), occupation (indoor/outdoor) and a smoking history was also obtained allowing subjects to be classified as current-, ex- or never-smokers. Social class was calculated using the Office Of Population Censuses And Surveys, Classification Of Occupations document, 1980 (Her Majesty's Stationery Office, London,1980). Patients were also questioned regarding ingestion of arsenic-containing tonics and use of drinking water from potentially contaminated wells. No exposed patients were identified. All case notes were carefully reviewed by myself (and Dr. W. Bowers for the Cornwall patients n=100) to determine tumour site at presentation, number of BCC at presentation, total tumour numbers, date of presentation with each BCC, date of presentation of subsequent BCC, duration of BCC as determined by the patient, tumour recurrence, site of tumour recurrence, other malignancies (internal and skin), whether the other malignancy occurred before or after the BCC, the type of internal malignancy or other

skin cancer, histological sub-type, presence of invasion. Recurrences were excluded from the total number of primary BCC. General practitioner records were consulted where uncertainty regarding number of tumours occurred. Patients were followed up to ensure accuracy of data on further tumours and recurrences.

A control group of British Caucasians (47.0% males, mean age 70 years) from these centres who were without clinical or histological evidence of any malignancy was also recruited. These hospital in- and out-patients suffered a variety of non malignant diseases including varicose veins, hernias, haemorrhoids, mild iron deficiency anaemia, mild hyperlipidaemia, benign ovarian cysts (about 30% in total), tension headaches (~25%), benign skin papillomas (~20%), benign breast lumps (~5%), cerebrovascular accidents (~20%). Patients suffering inflammatory pathologies such as ulcerative colitis, diabetes or asthma or, receiving blood transfusions within 3 months of blood sampling, were excluded. A number of controls (n=800) were also recruited in dermatology out-patients with various diagnoses including psoriasis, eczema, benign skin lesions, leg ulcers, acne and normal skin. Controls were examined and interviewed to obtain information on age, sex, hair colour at 21 years of age (blonde/red and brown/black), eye colour (blue/green and brown) at 21 years of age, skin type (types 1-4), occupation (indoor/outdoor) and a smoking history was also obtained allowing subjects to be classified as current-, ex- or never-smokers. Social class was calculated using the Office Of Population Censuses And Surveys, Classification Of Occupations document, 1980 (Her Majesty's Stationery Office, London,1980). Blood (5ml) was taken with appropriate Ethics Committee approval, into EDTA and stored at -50^{o}C.

In order to perform a case control study of internal malignancy in patients with multiple BCC, we recruited, examined, interviewed and analysed case notes on a cohort of

patients with BCC from out-patient clinics. Type and date of onset of any internal malignancy was recorded. Age/sex matched controls with either no skin pathology or benign skin conditions (one case to one control) were then recruited. All were interviewed, examined, case notes analysed and followed up in the same way as the BCC patients to determine the number with internal malignancy and which type. The occurrence of internal malignancy was then compared between the two groups.

To enable a comparison of risk factors between BCC, MM and SCC, patients with histologically proven MM (n=215) and SCC (n=188) were recruited from dermatology out-patient clinics. Patients were interviewed to obtain information on age, sex, hair colour at 21 years of age (blonde/red and brown/black), eye colour (blue/green and brown) at 21 years of age, skin type (types 1-4), occupation (indoor/outdoor) and a smoking history was also obtained allowing subjects to be classified as current-, ex- or never-smokers. Social class was then calculated. All case notes were carefully reviewed to determine tumour site at presentation, date of presentation, duration as determined by the patient, tumour recurrence, site of tumour recurrence, other malignancies (internal and skin), histological sub-type, presence of invasion and histological number. General practitioner records were consulted where uncertainty regarding number of tumours occurred. Patients were followed up to ensure accuracy of data on further tumours and recurrences. A subgroup of patients with skin cancers of different histological types (n=150) comprising mostly BCC plus another type of skin neoplasm (8 patients had only MM and SCC) were recruited, interviewed, case notes examined, followed up and general practitioners contacted to determine the above parameters. A comparison of individual characteristics between this multiple skin cancer type group and controls was then undertaken. A comparison with single BCC was also performed.

3.1.2. Statistical Analysis.

The following statistical tests have been used in analysing the results: chi-squared tests; logistic regression; Poisson regression; Poisson regression with a rate multiplier; Cox's proportional hazards regression in survival analysis; McNemar's test and the Armitage trend test. These tests will be described in the appropriate results section.

As a number of investigators recruited patients, there may be some variation in determining some of the parameters (ie. eye/hair colour, skin type). We feel this will have had minimal effect on the results as all investigators recruiting patients are dermatologists and were in regular contact during patient recruitment, when such issues of classification were discussed.

3.2. Overview Of Genotype Determination.

GSTM3 genotypes were identified using primers to exon 6/7 (Inskip et al., 1995). *GSTM3*B* was differentiated from *GSTM3*A* which is 3 bp larger, by digestion with MnlI. DNA from *GSTM3*A* homozygotes, containing the additional MnlI site, gave 11, 51, 86, 125 bp fragments while *GSTM3*B* homozygotes gave fragments of 11, 125, 134 bp. GSTM1 genotypes were identified using ARMS-based PCR with primers to intron 6/exon 7 (Heagerty et al., 1994). GSTT1 null and expressing subjects were also identified using PCR (Heagerty et al., 1996). Two mutant CYP2D6 alleles (G-A transition at intron 3/exon 4, base pair deletion in exon 5) were identified (Smith et al., 1995; Heagerty et al., 1996). Together these assays are 90% predictive of phenotype in British Caucasians (Smith et al., 1995). The 3'-flanking region mutation (m1m1 wild type homozygotes) and the exon 7 Ile/Val mutation (Ile/Ile wild type homozygotes) in CYP1A1 were detected using PCR with restriction digestion (Cantlay et al., 1995).

Enzyme digests were occasionally performed overnight or over a weekend (18-48 hours). Minor products may also be seen on some of the agarose gels due to under or over restriction digestion.

3.3. Phenol/Chloroform Extraction of Genomic DNA from Blood

Part 1

1. Cold lysis buffer was added to 3ml EDTA blood in a 50ml centrifuge to give a final volume of 35ml, mixed by inversion and centrifuged at 2000 rpm for 10 mins.
2. The supernatant was decanted to waste.
3. The white nuclear pellet was re-suspended in 1.5ml cold SE buffer and 170 µl of a solution containing SDS (5% w/v) and proteinase K (see appendix) and incubated for 18h at 37°C.

Part 2

4. 1.5ml phenol (tris-HCR saturated, pH 8.0, containing 8-hydroxyquinoline) was pipetted into glass tubes.
5. 1.5ml chloroform:isoamyl alcohol (see appendix) was pipetted into two further sets of the labelled glass tubes.
6. The proteinase K-treated samples were transferred into the phenol-containing tubes, mixed, placed in Falcon containers and spun at 2000 rpm room temperature for 15 mins in sealed buckets.
7. The viscous aqueous layer was taken off into a chloroform-containing glass tube, mixed well and spun at 2000 rpm, room temperature for 5 mins.
8. The viscous aqueous layer was taken and the chloroform:isoamyl alcohol extraction repeated. The aqueous layer was transferred to a fourth set of tubes and 0.1 volume (150µl) Sodium Acetate (see appendix) and 2 volumes (3.3ml) Ethanol were added.
9. The tube was inverted tube until the DNA precipitates and DNA removed with a glass hook. The DNA was resuspended in 200µl of sterile water and dissolved at 4°C.

3.4. GSTT1 Genotype Determination.

PCR OF GSTT1

Primers

GSTT1

Forward	5'-TTCCTTACTGGTCCTCACATCTC-3'
Reverse	5'-TCACCGGATCATGGCCAGCA-3'

B– globin

Forward	5'-ACACAACTGTGTTCACTAGC-3'
Reverse	5'-CAACTTCATCCACGTTCACC-3'

Setting up the PCR Reaction

1) DNA samples, Taq polymerase and 10x buffer, dNTP's and oligonucleotide primers (stored at -20°C) were allowed to reach room temperature and spun briefly to bring solutions to the bottom of the tube.
2) A 0.5ml microcentrifuge tube was labelled for each test DNA sample, positive control and negative control.
3) A 1.5ml microcentrifuge tube was labelled 'PCR stock'.
4) The following was added, in order, to the stock tube add:

Sterile water	416ul
10x Taq buffer (inc Mg)	100ul
B-globin primer (forward)	100ul
B-globin primer (reverse)	100ul

GSTT1 primer (forward)	100ul
GSTT1 primer (reverse)	100ul
dNTP's	40ul
Taq polymerase	4ul

5) The stock was mixed by inversion and 48ul aliquoted to each 0.5ml tube.

6) 1.5ul sterile water was added to the negative control and 1.5ul known GSTT1 positive DNA sample to the positive control. 1.5ul test DNA was added to the remaining tubes.

7) 2 drops mineral oil was added to all tubes and centrifuged at 12,000rpm for 20 seconds to separate the phases.

8) The tubes were randomly placed in the Thermal Cycler (Omnigene, Hybaid Ltd.) and the PCR was facilitated using the following programme:

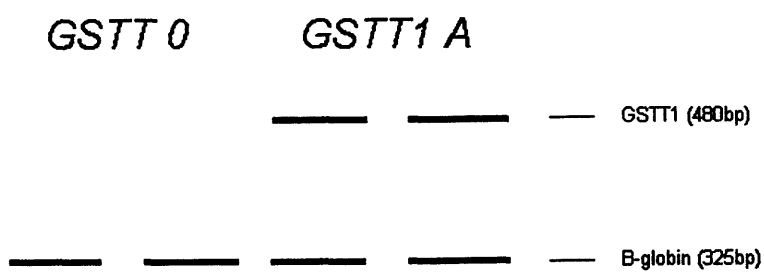
Thermal Cycler Programme:

94°C	150sec	1 cycle
94°C	60sec}	
58°C	60sec}	39 cycles
72°C	60sec}	
94°C	60sec}	
58°C	60sec}	1 cycle
72°C	450sec}	

Agarose Gel Electrophoresis

1) Agarose (0.6g, 2% w/v final concentration) was weighed out in a 50ml sterile conical flask and 3ml 5xTBE and 27ml sterile water added. The suspension was microwaved (approx 90 secs) until dissolved.
2) This was cooled to 60°C using running water and 1ul ethidium bromide added.
3) The agarose was poured into the gel tray and allowed to set.
4) While the gel was setting, the samples were prepared as follows: one 0.5ml microcentrifuge tube was labelled for each PCR sample and 4ul loading buffer added to each tube. 10ul PCR product was added ensuring the sample was taken from beneath the mineral oil layer. This was mixed well and spun at 12,000rpm for 20 secs.
5) 300ml of 0.5xTBE was used as electrophoresis buffer and for the preparation of molecular weight markers. 5ul ethidium bromide was added to the buffer and mixed well.
6) The molecular weight markers were prepared by adding 1ul of pBR322 Hae III digest to 10ul 0.5xTBE and 8ul loading buffer.
7) The 0.5xTBE was poured into the tank so that it covers the gel to a depth of approx. 1-2mm.
8) 5ul of sample was pipetted into the wells.
9) The lid was replaced and the gel run at 120 volts constant voltage for 25min (2% gel).
12) The gel was photographed onto type 667 film using a Wratten 23A filter (1/4sec; f4.7).

Figure 3.4.1. GSTT1 Gene Map and Gel Banding Patterns



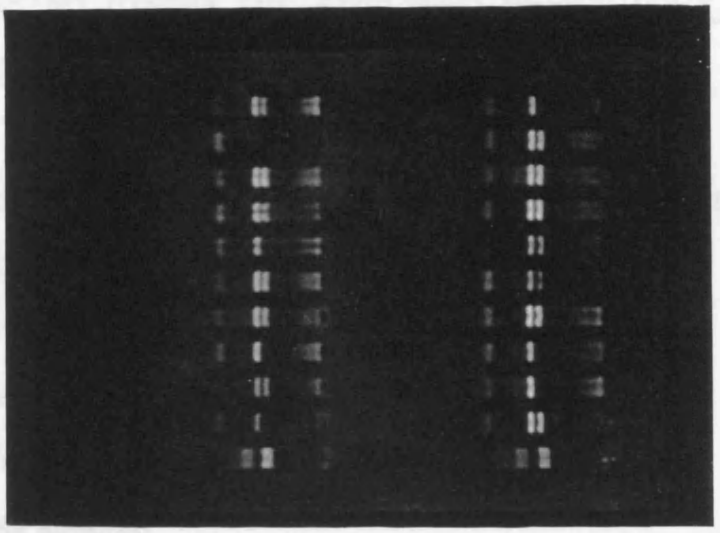


Figure 3.4.2. Photograph of GSTTT1 Gel.

LEFT COLUMN: Lane 1/2- positive/negative control, Lane 3/4- GSTT1A, Lane 5- GSTT1 null, Lane 6/7- GSTT1A, Lane 8- GSTT1 null, Lane 9- GSTT1A, Lane 10- GSTT1 null, Lane 11- size markers

3.5. GSTM1 Genotype Determination.

PCR OF GSTM1 (USING THE AMPLIFIED REFRACTORY MUTATION SYSTEM (ARMS) METHOD)

Determination of the GSTM1 genotype was performed by PCR on ARMS, using the following oligonucleotide primers:

Common GSTM1 specific

Exon 4 forward 5'-CTGCCCTACTTGATTGATGGG-3'

Exon 5 reverse 5'-CTGGATTGTAGCAGATCATGC-3'

GSTM1 allele specific

Intron 6 forward 5'-GCTTCACGTGTTATGAAGGTTC-3'

Exon 7 (A-specific) 5'-TTGGGAAGGCGTCCAAGCGC-3'

Exon 7 (B-specific) 5'-TTGGGAAGGCGTCCAAGCAG-3'

B-globin (internal control)

B-globin (forward) 5'-ACACAACTGTGTTCACTAGC-3'

B-globin (reverse) 5'-CAACTTCATCCACGTTCACC-3'

1.) DNA samples, including known *GSTM1*A* and *GSTM1*B* positive controls, Taq polymerase and 10x buffer(10mM Tris-HCl, pH 9.0, 0.1% v/v Triton X-100, 1.5mM MgCl), dNTP's and primers were removed from the -20^{O}C and allowed to reach room temperature.

2.) Two 0.5 ml microcentrifuge tubes were labelled "A" and "B" respectively for each test DNA sample. One "A Stock" 1.5 ml tube and another "B Stock" were marked. Two

tubes for positive controls for *GSTM1*A* and *GSTM1*B* were labelled, as well as, two other tubes for negative controls.

The "A" and "B" Stock tubes contained the following:

	"A Stock"	"B Stock"
Sterile water	216ml	216ml
10x Taq buffer	100ml	100ml
Exon 4/5 primers	200ml	200ml
Intron 6 primer	100ml	100ml
A-specific primer	100ml	------
B-specific primer	------	100ml
b-Globin primer (forward)	100ml	100ml
b-Globin primer (reverse)	100ml	100ml
d-NTP's	40ml	40ml
Taq polymerase	4ml	4ml

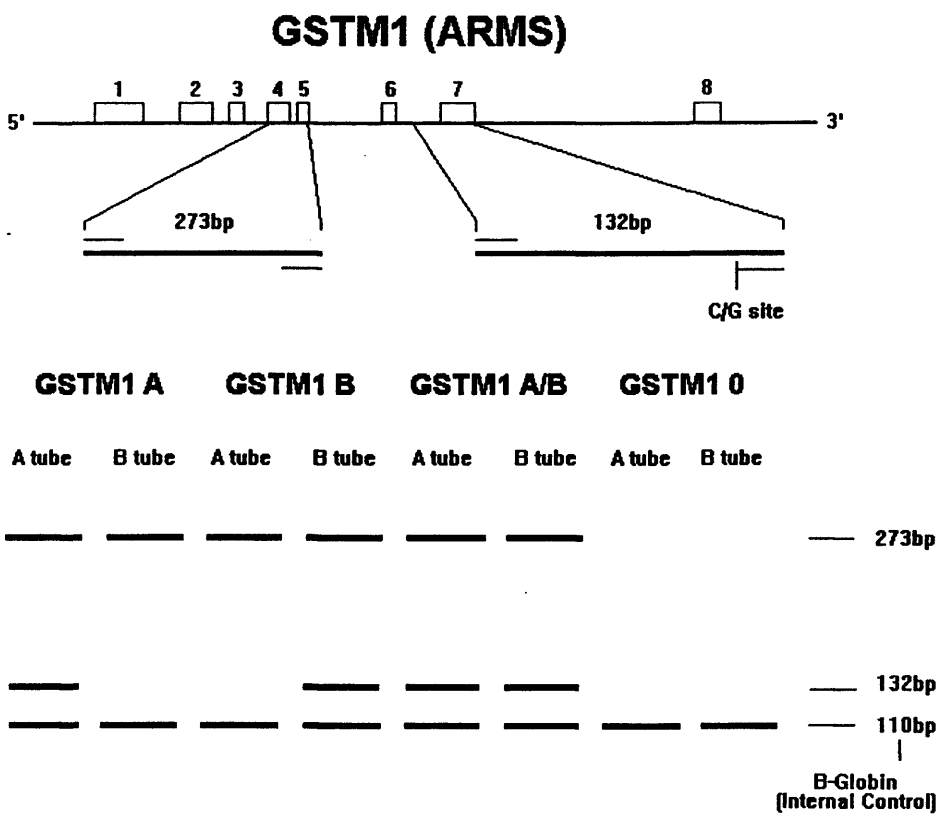
3.) All these components of the "A" and "B" Stock solutions were mixed gently by inversion and 48ml of each was aliquoted respectively to every "A" and "B" labelled tube. To both negative control tubes 1.5ml of sterile water was added, whereas *GSTM1*A* or *GSTM1*B* positive DNA was added to the tubes labelled as *GSTM1*A* and *GSTM1*B* positive control. Each "A" and "B" marked tubes received 1.5ml of test DNA. To every tube two drops of mineral oil were added. Finally these tubes were centrifuged at 12000rpm for 20 sec.

4.) The tubes were randomly placed in a thermal cycler (Omnigene Hybaid Ltd.) and incubated for one cycle of 150 sec at 94^{o}C, followed by five consecutive cycles at 94^{o}C for 45 sec, 60^{o}C for 60 sec and 72^{o}C for 60 sec. Subsequently, thirty cycles at 94^{o}C for

30 sec, 58^{O}C for 30 sec and 72^{O}C for 45 sec. The final cycle consisted of 5 sec at 40^{O}C.

5.) Samples were resolved by electrophoresis (120V., 45 min.) in 4% agarose gels, containing Ethidium Bromide (0.5mg/l) and photographed under U.V. light on a transilluminator.

Figure 3.5.1. GSTM1 Gene Map and Gel Banding Patterns



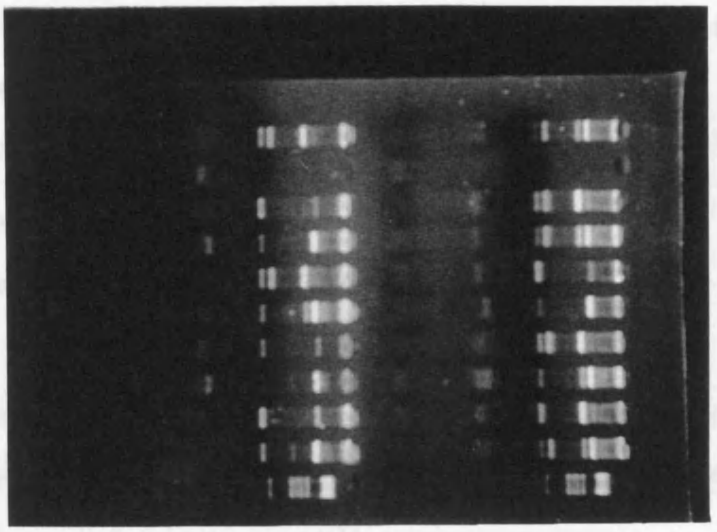


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Figure 3.5.2. Photograph of GSTM1 Gel.

LEFT COLUMN: Lane 1- positive A control, Lane 2- negative control, Lane 3/4- null, Lane 5/6- A, Lane 7/8- null, Lane 9/10- null, Lane 11- size markers.

RIGHT COLUMN: Lane 1- positive B control, Lane 2- negative control, Lane 3/4- A/B, Lane 5/6- null, Lane 7/8- A, Lane 9/10- B, Lane 11- size markers.

3.6. GSTM3 Genotype Determination

PCR OF GSTM3 WITH RESTRICTION DIGESTION

Determination of the GSTM3 genotype was performed by PCR, using the following oligonucleotide primers:

GSTM3 Primers

Exon 6 forward 5'-CCTCAGTACTTGGAAGAGCT-5'

Exon 7 reverse 5'-CACATGAAAGCCTTCAGGTT-3'

1.) DNA samples, including known GSTM3 AA positive control, Taq polymerase and 10x buffer(10mM Tris-HCl, pH 9.0, 0.1% v/v Triton X-100, 1.5mM MgCl), dNTP's and primers were removed from -20^{O}C freezer, allowed to reach room temperature and centrifuged at 12000 rpm for 10 sec. For each test DNA, a 0.5 ml microcentrifuge tube was labelled and additional ones for positive and negative controls.

2.) PCR stock was made up in a 1.5 ml microcentrifuge tube and mixed by inversion:

Sterile water	616ml
10x Taq buffer	100ml
Exon 6 primer	100ml
Exon 7 primer	100ml
d-NTP's	40ml
Taq polymerase	4ml

3.) 48ml PCR stock were aliquoted to each, previously labelled, 0.5 ml microcentrifuge tube. To the negative control tube 1.5ml of sterile water were added, whereas in the positive control tube 1.5ml of known positive GSTM3 AA DNA were added. In the

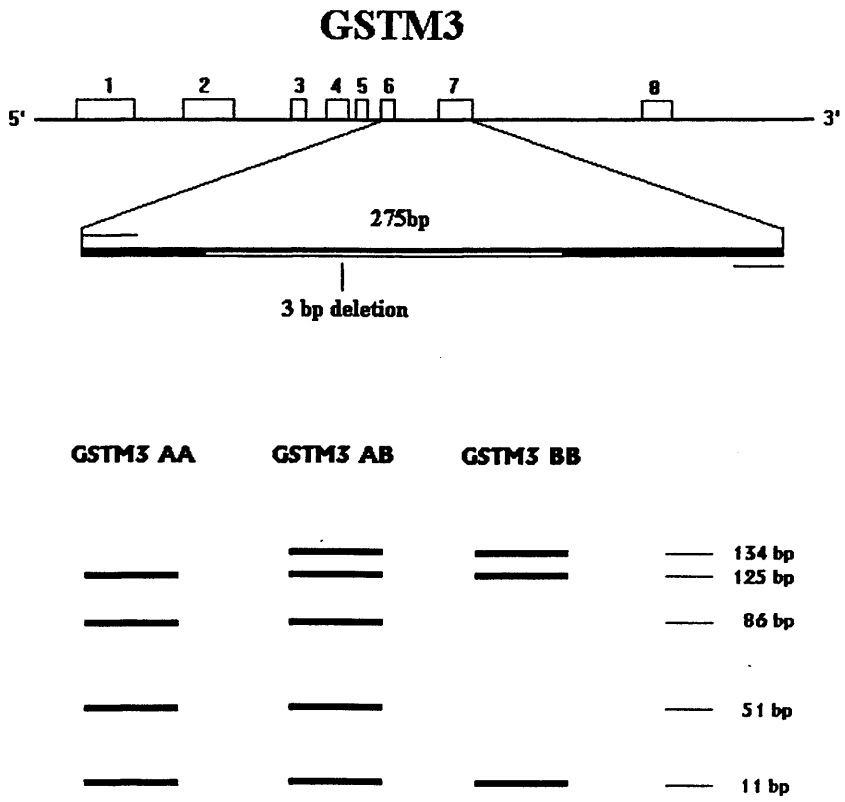
remaining tubes 1.5ml of test DNA was added. Two drops of mineral oil were put in all the tubes. These tubes were centrifuged at 12000 rpm for 20 sec to separate the phases.

4.) The tubes were randomly placed in a thermal cycler (Omnigene Hybaid Ltd.) and underwent one cycle of 180 sec at 94^{o}C, followed by thirty five consecutive cycles at 94^{o}C for 45 sec, 58^{o}C for 30 sec and 72^{o}C for 45 sec. Subsequently, a cycle at 94^{o}C for 45 sec, 58^{o}C for 30 sec and 72^{o}C for 480 sec.

5.) Samples were digested for 48 h at 37^{o}C with MnlI restriction enzyme (10 ml PCR product, 10 ml enzyme stock solution, containing: 160 ml sterile water, 40 ml 10x restriction enzyme buffer, 5 ml MnlI restriction enzyme, 8 ml Bovine Serum Albumin-BSA). The MnlI restriction enzyme recognises and cuts the sequences CCTC/GAGG.

6.) Samples were resolved by electrophoresis (120V., 45 min.) in 4% agarose gels, containing Ethidium Bromide (0.5mg/l) and photographed under U.V. light on a transilluminator.

FIGURE 3.6.1. GSTM3 Gene Map and Gel Banding Patterns



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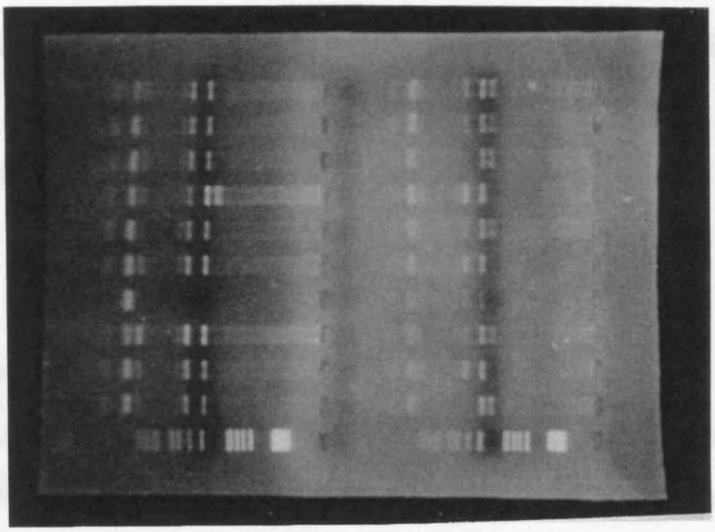


Figure 3.6.2. Photograph of GSTM3 Gel.

LEFT COLUMN: Lane 1- positive AA control, Lanes 2/3- AA, Lane 4- AB, Lanes 5/6- AA, Lane 7- negative control, Lanes 8/9/10- AA, Lane 11- size markers.

RIGHT COLUMN: Lanes 1/2- AB, Lane 3- BB, Lane 4- AA, Lane 5-AB, Lanes 6/7- AA, Lane 8- AB, Lane 9- AA, Lane 10- BB, Lane 11- size markers.

3.7. CYP1A1 3' Msp1 Site Genotype Determination.

PCR OF CYP1A1 3' Msp1 SITE WITH RESTRICTION DIGESTION

To determine this genotype, a PCR assay was used with the following oligonucleotide primers:

Forward - 5'-CAGTGAAGAGGTGTAGCCGCT-3'

Reverse - 5'-TAGGAGTCTTGTCTCATGCCT-3'

Setting up the PCR Reaction

1) DNA samples (including a known CYP 1A1 m2m2 genotype as a positive control), Taq polymerase and 10x buffer, dNTP's and oligonucleotide primers were removed from the -20°C freezer and allowed to reach room temperature. Tubes were labelled for each test DNA sample, positive and negative control.

2) A 1.5ml microcentrifuge tube was labelled PCR 'stock' and the following was added:

Sterile water	616ul
10x Taq buffer (inc Mg)	100ul
Forward primer(1F,5uM)	100ul
Reverse primer(1R,5uM)	100ul
dNTP's (4x5uM)	40ul
Taq polymerase	4ul

The stock was mixed by inversion and 48u aliquoted to each 0.5ml tube. 1.5ul sterile water was added to the negative control and1.5ul known CYP 1A1 m2m2 sample to

the positive control. 1.5ul of test DNA was added to the labelled tubes. 2 drops mineral oil was added to all tubes which were then centrifuged at 12,000rpm for 20 seconds to separate the phases.

3) The tubes were randomly placed in the Thermal Cycler and the PCR facilitated using the following programme:

94°C	2.5min}	x1 cycle
94°C	1min}	
64°C	1min}	x30 cycles
72°C	1min}	

Restriction Enzyme Digestion

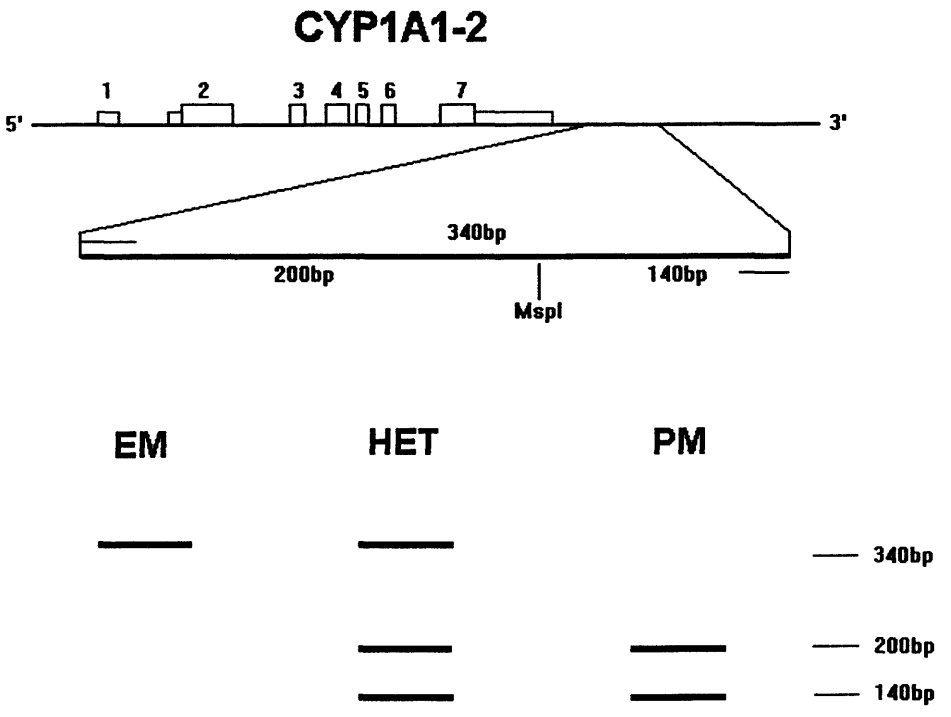
1) The restriction enzyme (Msp 1) and its 10x buffer were defrosted. Microcentrifuge tubes were labelled for each sample and control. A 0.5ml microcentrifuge tube was labelled 'Stock' and the 10x restriction enzyme buffer and restriction enzyme pipetted.

2) 10ul of the stock was aliquotted into each of the labelled tubes and 10ul of the relevant PCR product was added, ensuring that the sample was taken from beneath the mineral oil layer. This was mixed, spun at 12,000rpm for 20 secs and incubated at 37°C for 18 hours.

Agarose Gel Electrophoresis

3) Samples were resolved by electrophoresis (85V., 35 min) in 2% agarose gels, containing ethidium bromide and photographed under U.V. light on a transilluminator.

FIGURE 3.7.1. CYP1A1 3' Msp1 Site Gene Map and Gel Banding Patterns.



Banding Patterns for Cyp1A1- MspI Site

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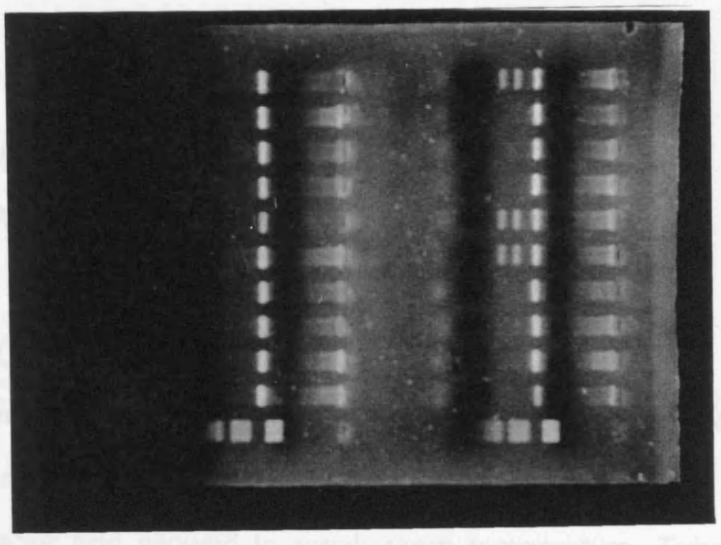


Figure 3.7.2. Photograph of CYP1A1 3' Msp 1 Site Gel.

LEFT COLUMN: Lanes 1 to 10- all m1m1 (EM), Lane 11- size markers.

RIGHT COLUMN: Lane 1- m1m2 (HET), Lanes 2/3/4- m1m1, Lanes 5/6- m2m2 (PM), Lanes 7 to 10- all m1m1, Lane 11- size markers.

3.8. CYP1A1 Exon 7 Genotype Determination

PCR OF CYP1A1 Exon 7 Mutation with Restriction Digestion.

To determine this genotype, a PCR assay was used with the following oligonucleotide primers:

Forward 5'-AAAGGCTGGGTCCACCCTCT-3'

Reverse 5'-AAAGACCTCCCAGCGGGCCA-3'

Setting up the PCR Reaction

1) DNA samples (including a known CYP1A11 Het as a positive control), Taq polymerase and 10x buffer, dNTP's and oligonucleotide primers were removed from the -20°C freezer and allowed to reach room temperature. Tubes were labelled for each test DNA sample, positive and negative control.

2) A 1.5ml microcentrifuge tube was labelled PCR 'stock' and the following was added:

Sterile water	616ul
10x Taq buffer (inc Mg)	100ul
Forward primer(1F,5uM)	100ul
Reverse primer(1R,5uM)	100ul
dNTP's (4x5uM)	40ul
Taq polymerase	4ul

The stock was mixed by inversion and 48u aliquoted to each 0.5ml tube. 1.5ul sterile water was added to the negative control and1.5ul known HET sample to the positive

control. 1.5ul of test DNA was added to the labelled tubes. 2 drops mineral oil was added to all tubes which were then centrifuged at 12,000rpm for 20 seconds to separate the phases.

3) The tubes were randomly placed in the Thermal Cycler and the PCR facilitated using the following programme:

94°C	2.5min}	x1 cycle
94°C	1min}	
58°C	1min}	x30 cycles
72°C	1min}	

Restriction Enzyme Digestion

1) The restriction enzyme (Nco1) and its 10x buffer were defrosted. Microcentrifuge tubes were labelled for each sample and control.

2) A 0.5ml microcentrifuge tube was labelled 'Stock' and the following pipetted:

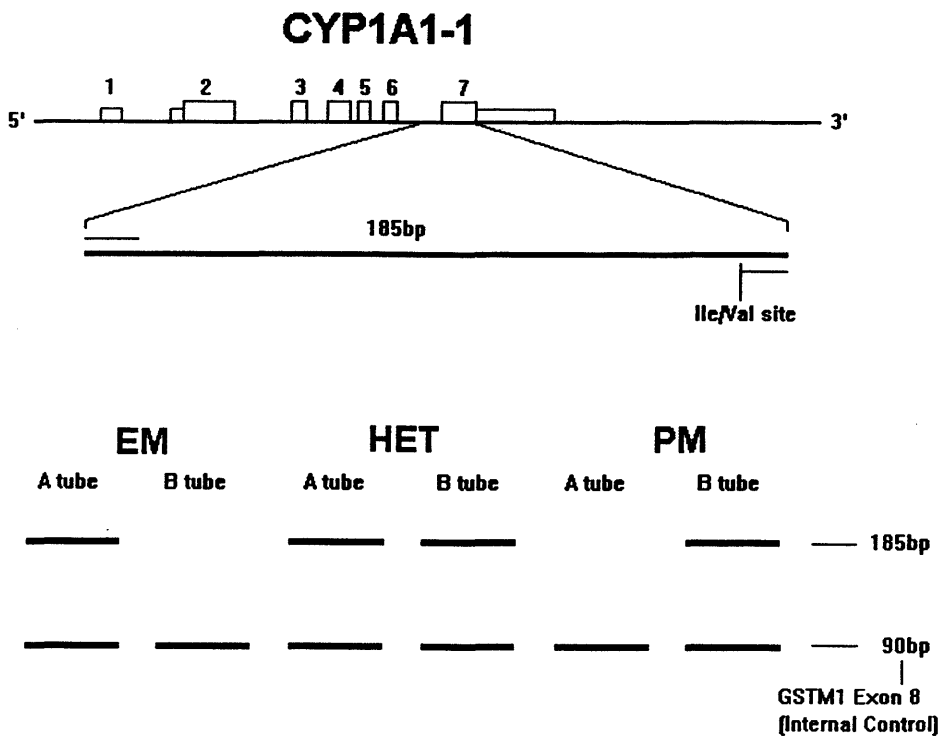
Sterile Water	160ul
10x restriction enzyme buffer	40ul
Restriction enzyme	5ul

3) 10ul of the stock was aliquotted into each of the labelled tubes and 10ul of the relevant PCR product was added, ensuring that the sample was taken from beneath the mineral oil layer. This was mixed, spun at 12,000rpm for 20 secs and incubated at 37°C for 18 hours.

Agarose Gel Electrophoresis

4) Samples were resolved by electrophoresis (85V., 35 min) in 4% agarose gels, containing ethidium bromide and photographed under U.V. light on a transilluminator.

FIGURE 3.8.1. CYP1A1 Exon 7 Mutation Gene Map and Gel Banding Patterns.



Banding Pattern for Cyp1A1 Ile/Val Site

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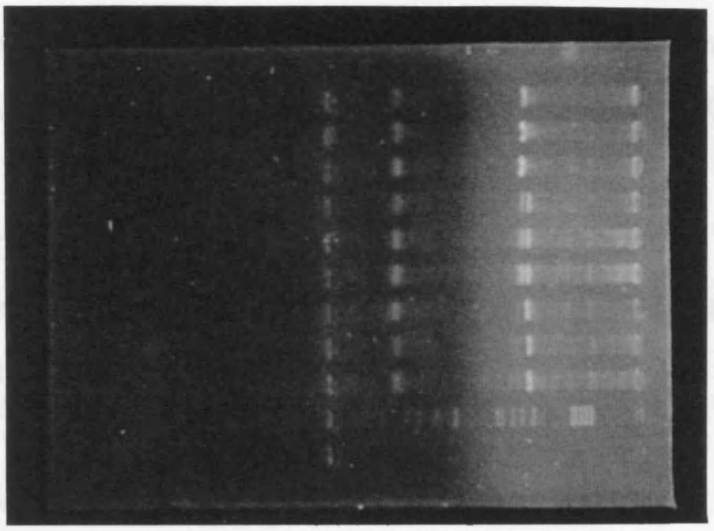


Figure 3.8.2. Photograph of CYP1A1 Exon 7 Gel.

Lanes 1/2/3- all ile/ile (EM), Lanes 4/5/6- all ile/val (HET), Lanes 7/8/9- all val/val (PM), Lane 10- size markers, Lane 11- negative control.

3.9. CYP2D6 Genotype Determination.

Two mutant CYP2D6 alleles were identified in separate PCR assays: the G-A transition (CYP2D6B) using intron3/exon4 primers followed by BstN1 digestion and the exon 5 deletion (CYP2D6A) using primers to exon5/intron5 followed by HpaII digestion. Together these assays are ~90% predictive of the phenotype in Caucasians.

PCR OF CYP2D6-B WITH RESTRICTION DIGESTION

To determine this genotype, a PCR assay was carried out using the following oligonucleotide primers:

Exon 3 forward	5'-GCCTTCGCCAACCACTCCG-5'
Intron 4 reverse	5'-AAATCCTGCTCTTCCGAGGC-3'

1.) DNA samples including sample of a known EM genotype as a positive control, Taq polymerase, 10x Taq buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% v/v Triton X-100, 1.5 mM MgCl),dNTP's and oligonucleotide primers were removed from -20^{O}C and allowed to reach room temperature. They were centrifuged at 12000 rpm for 10 sec. For each test DNA, a 0.5 ml microcentrifuge tube was labelled, and also for positive and negative controls.

2.) PCR stock was mixed by inversion in a 1.5 ml microcentrifuge tube and contained:

Sterile water	616 ml
10x Taq Buffer	100 ml
Intron 3 primer (forward)	100 ml

Exon 4 primer (reverse)	100 ml
dNTP's	40 ml
Taq polymerase	4 ml

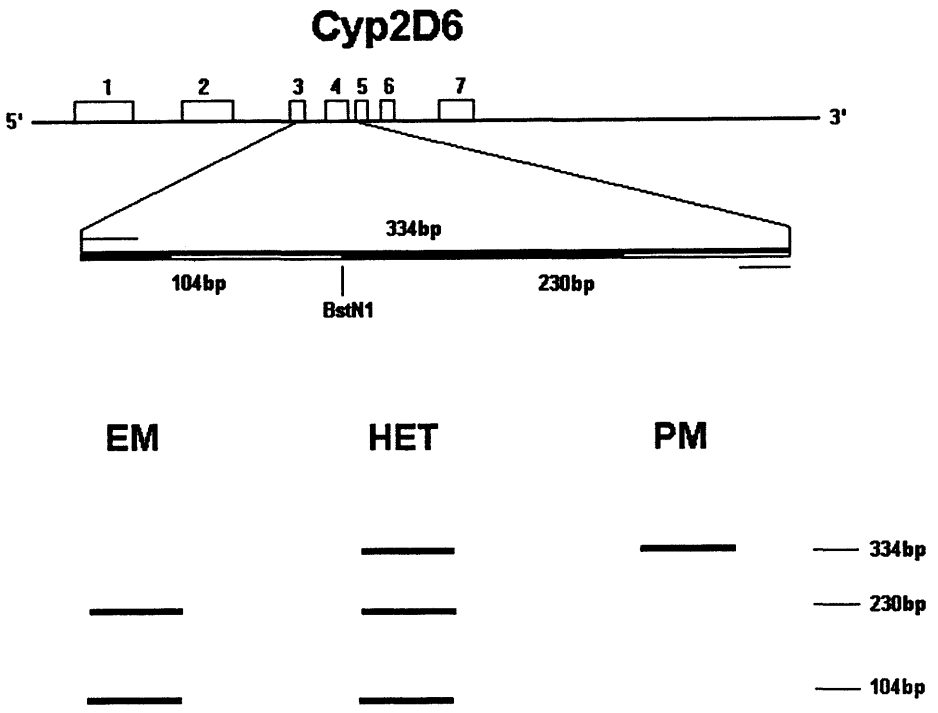
3.) 48 ml PCR stock were aliquotted to each previously labelled 0.5 ml microcentrifuge tube. To the negative control tube 1.5 ml of sterile water were added, while in the positive control tube 1.5 ml of known EM positive DNA were added. To the rest of the tubes 1.5 ml of corresponding test DNA were added. Two drops of mineral oil were put in all the tubes. These were centrifuged at 12000 rpm for 20 sec to separate phases.

4.) Tubes were put in a Thermal Cycler (Omnigene, Hybaid, Ltd.) and underwent one cycle at 94^{o}C for 180 sec followed by 34 cycles at 94^{o}C (30 sec), 60^{o}C (60sec) and 72^{o}C (60 sec) and one final cycle at 94^{o}C (30 sec), 60^{o}C (60 sec) and 72^{o}C (480 sec).

5.) Samples were digested for 18 h at 60^{o}C with BstN1 restriction enzyme (10 ml PCR product, 10 ml enzyme stock solution, containing: 160 ml sterile water, 40 ml 10x restriction enzyme buffer, 5 ml BstN1 restriction enzyme, 10 ml Bovine Serum Albumin-BSA).

6.) Samples were resolved by electrophoresis (85V., 35 min) in 2% agarose gels, containing ethidium bromide and photographed under U.V. light on a transilluminator.

Figure 3.9.1. CYP2D6-B Gene Map and Gel Banding Patterns.



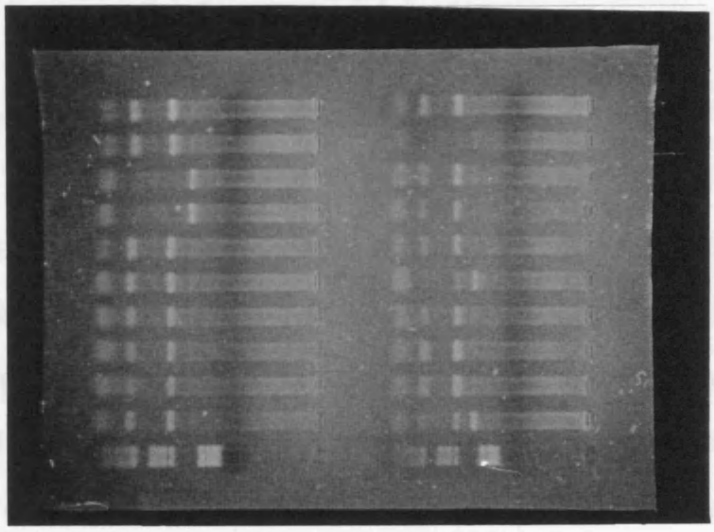


Figure 3.9.2. Photograph of CYP2D6-B Gel.

LEFT COLUMN: Lane 1- positive EM control, Lane 2- EM, Lanes3/4- both PM, Lanes 5 to 10- all EM, Lane 11- size markers.

RIGHT COLUMN: Lane 1- EM, Lane 2- PM, Lanes 3/4/5- all EM, Lane 6- HET, Lanes 7/8- both EM, Lane 10- HET, Lane 11- size markers.

3.10. CYP2D6-A Genotype Determination.

PCR OF CYP2D6-A WITH RESTRICTION DIGESTION

For this assay the following oligonucleotide primers were used:

Exon 5 forward 5'-GATGAGCTGCTAACTGAGCCC-3'

Intron 5 reverse 5'-CCGAGAGCATACTCGGGAC-3'

1.) DNA samples including a sample with a known PM genotype as a positive control, Taq polymerase, 10x Taq buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% v/v Triton X-100, 1.5 mM MgCl),dNTP's and oligonucleotide primers were removed from -20^{O}C and allowed to reach room temperature, following which they were centrifuged at 12000 rpm for 10 sec. For each test DNA, a 0.5 ml microcentrifuge tube was labelled, and also for positive and negative controls.

2.) PCR stock was mixed by inversion in a 1.5 ml microcentrifuge tube and contained:

Sterile water	616 ml
10x Taq Buffer	100 ml
Exon 5 primer (forward)	100 ml
Intron 5 primer (reverse)	100 ml
dNTP's	40 ml
Taq polymerase	4 ml

3.) 48 ml PCR stock were aliquotted to each previously labelled 0.5 ml microcentrifuge tube. To the negative control tube 1.5 ml of sterile water was added, while in the positive control tube 1.5 ml of known PM positive DNA was added. To the rest of the

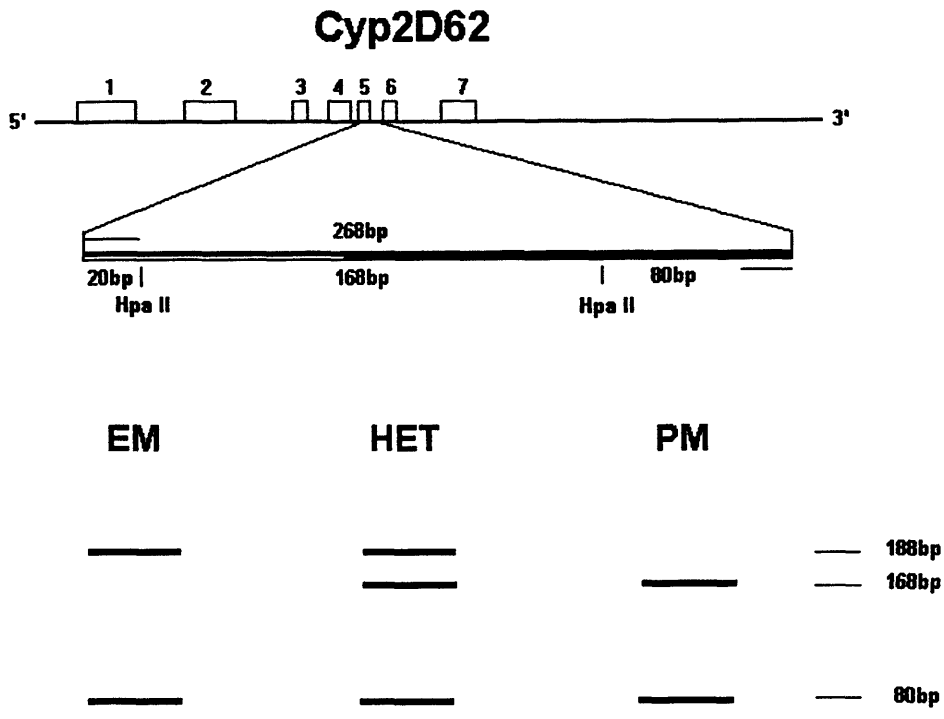
tubes 1.5 ml of corresponding test DNA were added. Two drops of mineral oil were put in all the tubes. These were centrifuged at 12000 rpm for 20 sec to separate phases.

4.) Tubes were put in a Thermal Cycler (Omnigene, Hybaid, Ltd.) and underwent one cycle at 94^{o}C for 180 sec followed by 34 cycles at 94^{o}C (30 sec), 60^{o}C (60sec) and 72^{o}C (60 sec) and one final cycle at 94^{o}C (30 sec), 60^{o}C (60 sec) and 72^{o}C (480 sec).

5.) Samples were digested for 18 h at 37^{o}C with Hpa II restriction enzyme (10 ml PCR product, 10 ml enzyme stock solution, containing: 160 ml sterile water, 40 ml 10x restriction enzyme buffer, 5 ml Hpa II restriction enzyme).

6.) Samples were resolved by electrophoresis (120V., 60 min.) in 4% agarose gels, containing ethidium bromide and photographed under U.V. light on a transilluminator

FIGURE 3.10.1. CYP2D6-A Gene Map and Gel Banding Patterns.



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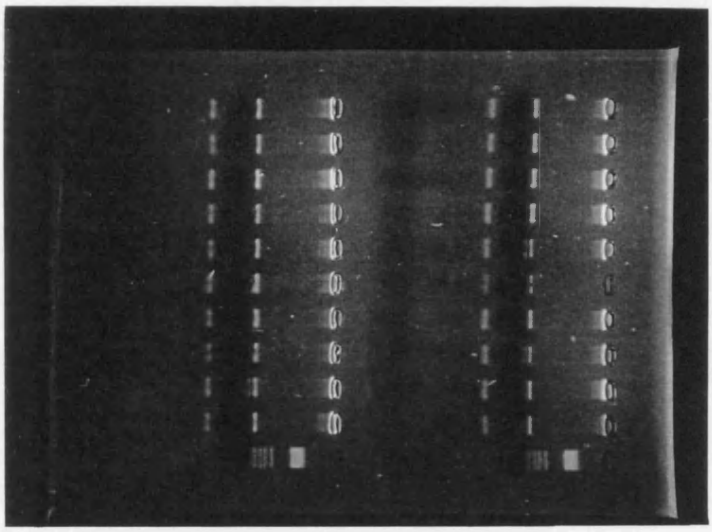

Figure 3.10.2. Photograph of CYP2D6-A Gel.

LEFT COLUMN: Lane 1- positive EM control, Lanes 2 to 8- all EM, Lane 9- HET, Lane 10- EM, Lane 11- size markers.

RIGHT COLUMN: Lanes 1 to 4- all EM, Lane 5- HET, Lanes 6/7- both EM, Lane 8- HET, Lanes 9/10- both EM, Lane 11- size markers.

CHAPTER 4 - RESULTS

4.1. RISK FACTORS FOR BCC: A MATCHED CASE CONTROL STUDY IN 806 PATIENTS.

4.1.1 Introduction/Statistical Approach

By studying risk factors, individuals with high susceptibility may be identified with consequent opportunity for primary and secondary prevention. However, there have been relatively few large case-control studies of risk factors in BCC in the U.K. with many studies grouping BCC and cutaneous squamous cell carcinoma under the title non-melanoma skin cancer. Few studies have addressed whether patients with a single lesion have a different risk factor profile to those with multiple lesions. In order to address these issues, we describe a case-control study to determine the risk factor profile in patients with BCC in the U.K. We have also compared risk factor profiles between patients with single and multiple BCC.

Patients with BCC (n=600) and a control group (n=503) were recruited. The control diagnoses included benign skin lesions (n=353) and eczema (n=150). Cases were then age and sex matched with controls. Due to differences in age and gender between cases and controls, we were only able to match 403 cases to controls. 278 patients with multiple BCC were then age/sex matched to those with a single BCC. The various parameters were compared within the groups. Conditional logistic regression was used to identify differences between cases and controls and in the comparison of single and multiple BCC cases.

4.1.2. RESULTS

i. BCC case control study

403 matched pairs were analysed. Table 4.1.1 illustrates the individual characteristics of the study groups. Table 4.1.2 shows significant differences between the two groups. Red/blonde hair (p=0.037), skin type 1 (p=0.024), blue/green eyes (p=0.001) and social class 1 and 2 (p=0.007) were significantly associated with BCC, social class having the biggest odds ratio (2.36). We analysed the social class data for males and females separately and found a similar strength of effect between the two genders (odds ratio 2.1 for men and 3.5 for women). Due to the reduction in numbers of matched pairs when sub-dividing the social class data in this way, the statistical significance was reduced (p<0.03). Smoking history and outdoor occupation were not significant. No significant two-factor interactions were identified.

Table 4.1.1. Characteristics of the BCC and control groups.

	Cases (%)	Controls (%)
Ever smoked	245 (62.5)	238 (59.6)
Blue/green eyes	289 (74.3)	248 (63.8)
Red/blonde hair	61 (24.3)	60 (16.4)
Skin type 1	57 (14.5)	36 (9.3)
Social class 1 and 2	38 (21.7)	36 (9.9)
Outdoor occupation	24 (12.6)	38 (10.5)

Table 4.1.2. Significant differences between BCC cases and age/sex matched controls.

	p value	Odds ratio	95% confidence interval
Social class 1 and 2	=0.007	2.36	1.26-4.40
Skin type 1	=0.024	1.66	1.07-2.57
Red/blonde hair	=0.037	1.61	1.03-2.553
Blue/green eyes	=0.001	1.71	1.23-2.36

ii. Comparison of patients with single BCC against those with multiple BCC.

278 patients with multiple BCC were matched to those with a single BCC. Patients with multiple lesions were more likely to have a truncal tumour at presentation (p=0.002, odds ratio 4.03, 95%C.I. 1.64-9.89). No significant associations between multiple, as compared to single, BCC and smoking history, eye/hair colour, skin type, social class and outdoor occupation were identified.

4.2. SUSCEPTIBILITY TO MULTIPLE BCC: INTERACTIONS BETWEEN GSTM1 GENOTYPES, SKIN TYPE AND MALE GENDER.

4.2.1. INTRODUCTION/STATISTICAL APPROACH

The role of predisposing or protective genes in the genesis of sporadic, multiple BCC is not clear. Karagas et al (1994) showed risk of further lesions increased with age, male gender and intermittent high exposures to UV. We used a case-control approach to determine the relevance of GSTM1, GSTT1 and CYP2D6 genotypes in mediating susceptibility to single and multiple BCC. 737 unrelated Caucasian patients with BCC were recruited. 481 patients (52.2% males, mean age 67 years) suffered a single tumour and 256 patients (61.3% males, mean age 70 years) more than one tumour (between 2-50 tumours/patient). 563 British Caucasians (47.0% males, mean age 70 years) from these centres who were without clinical or histological evidence of any malignancy was also recruited.

Chi-squared tests were used to examine for homogeneity between cases and controls. Since some genotype frequencies were small, the StatXact-Turbo statistical package was used to obtain exact p values. As various factors (CYP2D6 EM, GSTT1 null, GSTM1 null, skin type, gender etc.) were studied, the influence on susceptibility of each (alone and in combination in pairs and triplets) was studied by comparing frequency distributions over the resulting mutually exclusive categories. The advantage of this approach is that it allows identification of those factors (alone and in combination) that contribute most to observed differences between cases and controls. p values for the main comparisons (GSTM1, skin type 1, gender) were not adjusted for multiple comparisons as they were sufficiently small to remain significant if adjusted using the Bonferoni correction.

4.2.2. RESULTS

Genotype frequencies in cases and controls. Table 4.2.1. shows the frequencies of GSTM1 genotypes in controls, the total BCC group and, patients with single and multiple BCC. The frequencies of the null, A and B genotypes were not different though the frequency of GSTM1 A/B was significantly lower in the multiple BCC group than in the controls (odds ratio 0.29, 95% conf int 0.055-0.098).

We found no differences in the frequencies of GSTT1 genotypes in controls and the BCC case groups (Table 4.2.1.). The frequencies of the CYP2D6 EM and HET genotypes in controls and case groups were also not different though the difference between the frequency of the PM genotype in controls and single BCC cases approached significance (Table 4.2.1.).

Table 4.2.1. CYP2D6, GSTM1 and GSTT1 genotype frequencies in patients with single and multiple basal cell carcinomas of skin.

GSTM1	null	A	B	A/B
total BCC (n=699)	376 (53.8%)	197 (28.2%)	108 (15.5%)	18 (2.6%)
single BCC (n=454)	236 (52.0%)	128 (28.2%)	75 (16.5%)	15 (3.3%)
multiple BCC (n=245)	140 (57.1%)	69 (28.2%)	33 (13.5%)	3 (1.2%)
controls (n=561)	306 (54.5%)	158 (28.2%)	74 (13.2%)	23 (4.1%)

GSTT1	null	positive
total BCC (n=584)	97 (16.6%)	487 (83.4%)
single BCC (n=384)	57 (14.8%)	327 (85.2%)
multiple BCC (n=200)	40 (20.0%)	160 (80.0%)
controls (n=484)	90 (18.6%)	394 (81.4%)

CYP2D6 genotypes:	EM	HET	PM
total BCC (n=599)	375 (62.6%)	181 (30.2%)	43 (7.2%)
single BCC (n=396)	243 (61.4%)	121 (30.6%)	32 (8.1%)
multiple BCC (n=203)	132 (65.0%)	60 (29.6%)	11 (5.4%)
controls (n=310)	194 (62.6%)	99 (31.9%)	17 (5.5%)[b]

a: frequency GSTM1 A/B in controls and multiple BCC $X^2_1=4.52$; exact p=0.048.

b: frequency CYP2D6 PM in controls and single BCC $X^2_1=3.57$, Yates corrected p=0.059

Interactions between GSTM1, GSTT1 and CYP2D6 genotypes. Comparison of the frequency distributions for combinations of the putatively high risk GSTM1 null, GSTT1 null, CYP2D6 EM genotypes (i.e. GSTT1 null/GSTM1 null/CYP2D6 EM and GSTT1 null/GSTM1 null) showed no significant differences between the controls and patients in the BCC groups.

Corresponding interactions between GSTM1 A/B and the putatively protective CYP2D6 PM and GSTT1 positive genotypes were also examined. Thus, multinomial frequency distributions for combinations of GSTT1 expressers/GSTM1 A/B and, CYP2D6 PM/GSTM1 A/B in patients with single and multiple BCC and, controls and patients with multiple tumours were not significantly different. The differences between frequency distributions of the three genotypes combined (CYP2D6 PM/GSTT1 expressers/GSTM1 A/B) in the multiple BCC and single BCC cases and, multiple BCC and controls approached significance (X^2_6=11.24, exact p=0.055 and X^2_5=10.06, exact p=0.067 respectively). These differences largely resulted from differences in the proportion of subjects with the combination GSTT1 positive/GSTM1 A/B; thus, the frequency of this combination was significantly lower (X^2_1=6.83, exact p=0.011) in the multiple BCC group than in controls. Frequency distributions of GSTM1 A/B/CYP2D6 EM in controls and multiple BCC were significantly different (Table 4.2.2.). This difference largely resulted from the reduced frequency of subjects with CYP2D6 EM/GSTM1 A/B in the multiple BCC group compared with controls (Table 4.2.2.).

Table 4.2.2. Multinomial frequency distributions of GSTM1 A/B and CYP2D6 EM

	controls	single BCC	multiple* BCC
GSTM1 A/B+CYP2D6 EM	13 (3.4%)	8 (2.0%)	1 (0.5%)**
GSTM1 A/B only	7 (1.8%)	5 (1.3%)	0 (0%)
CYP2D6 EM only	235 (61.7%)	235 (59.5%)	131 (64.5%)
neither	126 (33.1%)	147 (37.2%)	71 (35.0%)
total	381 (100%)	395 (100%)	203 (100%)

* frequency distributions in controls and multiple BCC; $X^2_3=8.75$, p=0.033

** frequency GSTM1 A/B+CYP2D6 EM in controls and multiple BCC; $X^2_1=4.82$, exact p=0.042

Interactions between gender and GSTM1, GSTT1 and CYP2D6 genotypes. The proportion of males in the multiple BCC group (61.3%) was significantly greater than in controls (47.0%) (X^2_1=11.85, Yates corrected p=0.0006) and single BCC (52.2%) (X^2_1=5.29, Yates corrected p=0.0214). Interactions between GSTM1 null and male gender were examined by comparing multinomial frequency distributions (Table 4.2.3.); distributions in single and multiple BCC were significantly different. In particular, the frequency of the combination GSTM1 null/male gender was significantly greater in patients with multiple tumour.

Table 4.2.3. Interactions between male gender and GSTM1 null

	Controls	single BCC	multiple* BCC	total BCC
GSTM1 null+male	79 (31.7%)	115 (25.9%)	90 (37.5%)**	205 (30.0%)
male only	51 (20.5%)	117 (26.4%)	57 (23.8%)	174 (25.4%)
GSTM1 null only	62 (24.9%)	114 (25.7%)	47 (19.6%)	161 (23.5%)
neither	57 (22.9%)	98 (22.1%)	46 (19.1%)	144 (21.1%)
total	249 (100%)	444 (100%)	240 (100%)	684 (100%)

* frequency distributions in single and multiple BCC; $X^2_3=10.49$, p=0.015

** frequency GSTM1 null+male in single and multiple BCC; $X^2_1=9.44$, Yates corr p=0.002

Interactions between other patient characteristics and genotypes. The proportions of patients in the single and multiple BCC groups with brown, blue or green eyes were not significantly different.

Frequency distributions of skin types 1-4 in the single and multiple BCC cases were also not significantly different (Tables 4.2.4 and 4.2.5.). Considering skin type in terms of no protection (type 1) and variable protection to UV (types 2-4), we compared multinomial frequency distributions of GSTM1 null with skin type 1 in the patients with single and multiple BCC. The proportion of subjects with these factors was significantly greater in the multiple BCC group than in those with a single BCC (Tables 4.2.4. and 4.2.5.). Thus, the frequency distributions of GSTM1 null/skin type 1 were significantly different and, the proportion of subjects who were GSTM1 null with skin type 1 was significantly greater (Table 4.2.4; odds ratio 3.25, 95% conf int 1.30-8.27) in the multiple BCC group. We examined the data for three-way interactions between GSTM1 null/skin type 1/male gender by comparing multinomial frequency distributions of these factors in the single and multiple BCC groups. The distributions were almost significantly different (Tables 4.2.4. and 4.2.5.). No significant interactions between GSTT1 null or CYP2D6 EM and skin type 1 were identified.

Interactions between smoking and genotypes. The proportion of cases who were current smokers or ever smokers was not significantly different in the single and multiple BCC groups. Comparisons of multinomial frequency distributions of smoking with each of the GSTM1 null, GSTT1 null and CYP2D6 EM genotypes identified no differences between patients with single and multiple tumours.

Table 4.2.4. **Interactions between skin type, GSTM1 and male gender in single and multiple BCC.**

Skin type:	1	2	3	4
single BCC	34	94	98	76
(n=302)	(11.3%)	(31.1%)	(32.5%)	(25.1%)
multiple BCC	26	50	54	32
(n=162)	(16.0%)	(30.9%)	(33.3%)	(19.8%)

Frequency distributions of GSTM1 null and skin type 1:

	controls	single BCC[a]	multiple BCC
GSTM1 null+ type 1	2 (4.4%)	9 (3.3%)	15 (10.0%)[b]
type 1 only	0 (0%)	20 (7.4%)	7 (4.7%)
GSTM1 null only	24 (53.0%)	131 (48.2%)	66 (44.0%)
neither	19 (42.2%)	112 (41.2%)	62 (41.3%)
	45 (100%)	272 (100%)	150 (100%)

a: frequency distributions in single and multiple BCC; $X^2_3=9.06$; p=0.0285

b: frequency GSTM1 null+skin type 1 in single v multiple BCC;

$X^2_1=6.87$,p=0.009

TABLE 4.2.5. **Interactions between GSTM1 null, skin type 1 and male gender.**

	single BCC[a]	multiple BCC
GSTM1 null+type 1+male	5 (1.9%)	7 (4.8%)
GSTM1 null+type 1 only	5 (1.9%)	7 (4.8%)
type 1+male only	10 (3.7%)	3 (2.0%)
GSTM1 null+male only	60 (22.2%)	44 (29.9%)
male only	63 (23.3%)	34 (23.1%)
skin type 1 only	10 (3.7%)	4 (2.7%)
GSTM1 null only	69 (25.6%)	22 (15.0%)
none	48 (17.8%)	26 (17.7%)

a: frequency distributions in single and multiple BCC; $X^2_7=13.88$, exact p=0.051

4.3. GLUTATHIONE S-TRANSFERASE (GSTM1, GSTT1) AND CYTOCHROME P450 (CYP2D6, CYP1A1) POLYMORPHISMS INFLUENCE BCC NUMBERS AND ACCRUAL.

4.3.1. Introduction/Statistical Approach

BCC is of particular importance in that many patients develop multiple tumours at different rates. We examined the influence of allelism at CYP1A1, CYP2D6, GSTM1, GSTT1 and physical characteristics (gender, skin type, hair, eye colour) on susceptibility assessed in terms of total tumour numbers and their accrual (number of tumours/year from first presentation). The influence of allelism on numbers of BCC has been studied in a cross-sectional study. We also used a longitudinal approach to study the influence of genotypes, characteristics, patient age and number of lesions at first presentation on accrual of BCC.

Cross-sectional study. 827 patients with BCC were recruited. 565 patients suffered one tumour and 262 patients between 2-35 tumours. Cross-sectional analysis. Poisson regression was used to model count data using the EGRET statistical package (SERC, Seattle, 1993). The model assumes the Poisson rate parameter (mean number BCC) may be expressed as a function of a set of covariates; i.e. age, skin type 1, eye colour, hair colour, gender, genotypes. A rate ratio, defined as the multiplicative effect of a change of a covariate by 1 was calculated. Thus, the rate ratio for males [1] (1.43) against females [0] is mean number BCC in males (2.38)/mean number BCC in females (1.66), when gender alone (i.e. not in the presence of other covariates) is considered. In the Poisson regression this will change in the presence of other covariates.

Longitudinal study. The influence of genotypes and characteristics on tumour accrual was studied in 169 of the patients described above selected because accurate data on the time between first presentation and August 1995 and, the number of primary lesions could be recovered. Longitudinal analysis. Poisson regression with a rate multiplier was used to adjust for the number of person years at risk since the increase in the number of BCC is likely to change depending on the number of years between first presentation and August 1995. The rate multiplier was corrected for the different numbers of person years at risk in different patients. This is not present in the cross-sectional study.

4.3.2. RESULTS

Cross-sectional study: Patient characteristics. Table 4.3.1. shows the characteristics of the study group and numbers of BCC in these patients; 62 subjects had 5 or more tumours and 23, had 10 or more lesions. As expected the proportion of males (55.8%) was greater than in age-matched controls (47%) from the study centres (Heagerty et al., 1996). Table 4.3.2. shows the frequencies of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes in these patients. These were not significantly different from controls. Comparison of the frequencies of homo- and heterozygotic combinations of the exon 7 and 3'-mutations in CYP1A1 showed, as reported (Hayashi et al., 1991; Hirvonen et al., 1992), they were in linkage disequilibrium (X^2_4=56.12; p<0.0005).

Table 4.3.1. **Patient demographics.**

(i) Cross-sectional analysis: Influence of genotypes and physical characteristics on BCC numbers

number of BCC	1-35 tumours (n=827)
	>5 tumours (n=62)
	>10 tumours (n=23)
mean age (years)	67.3±12.4 (SD) (n=823)
males/females	55.8%/44.2% (n=813)
blue and green eyes	72.8% (n=418)
brown eyes	27.2% (n=156)
skin type 1	14.2% (n=78)
skin type 2-6	85.8% (n=473)
ever smoker	62.5% (n=367)
never smoker	37.5% (n=220)
brown and black hair	74.9% (n=242)
blonde and red hair	25.1% (n=81)

(ii) Longitudinal analysis: Influence of genotypes and physical characteristics on accrual of BCC

number of patients	169
median number of BCC	3 (range 1-30)
median number of new tumours/year	0.31 (range 0-18.7)
median follow up (years)	4.54 (range 0-28.5)
median no. of new tumours during follow-up	1 (range 0-29)

Table 4.3.2. Frequency of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes in patients with basal cell carcinoma

GSTM1	null	A	B	A/B	n
	392	213	114	22	741
	(52.9%)	(28.7%)	(15.4%)	(3.0%)	

GSTT1	null	expresser	n
	122	579	701
	(17.4%)	(82.6%)	

CYP2D6	EM	HET	PM	n
	429	205	43	677
	(63.4%)	(30.3%)	(6.4%)	

CYP1A1	Ile/Ile	Ile/Val	Val/Val	n
exon 7 mutation	572	94	7	673
	(85.0%)	(14.0%)	(1.0%)	

	m_1m_1	m_1m_2	m_2m_2	
Msp1 3'-flanking region	561	119	4	684
	(82.0%)	(17.4%)	(0.6%)	

Factors influencing the numbers of BCC. Table 4.3.3. shows the genotypes and individual characteristics associated with increasing numbers of BCC. The data is adjusted for age since, as expected, this was a risk factor for multiple tumours (p=0.013, rate ratio 1.005, 95% CI 1.001-1.009, n=769) (Kricker et al., 1993; Karagas and Greenberg, 1995). Neither GSTM1 null, GSTT1 null nor smoking were significant risk factors. CYP1A1 Ile/Ile did not influence tumour numbers though both the CYP1A1 m_1m_1 and the CYP2D6 EM genotypes were associated with increased numbers of primary tumours (Table 4.3.3). Male gender, skin type 1 and blue/green eyes were also significantly associated with increased numbers of BCC.

Interactions between genotypes/patient characteristics influencing BCC number. Table 4.3.3. shows significant, age-corrected interactions in which the interaction term was more significant than either of the main effects. Thus, the rate ratio for the interaction between GSTM1 null and skin type 1 (2.702) was the highest identified. Significant interactions between CYP2D6 EM and male gender and, this genotype and blue/green eyes as well as between CYP1A1 m_1m_1 and blue/green eyes were also identified. Significant interactions between male gender and GSTM1 null (p=0.045), gender and skin type 1 (p<0.001) and, skin type 1 and CYP1A1 m_1m_1 (p=0.012) were identified but appeared to result from the strength of the main effects. No significant interactions between genotypes were identified though the interaction between CYP2D6 EM and CYP1A1 m_1m_1 approached significance (p=0.095; rate ratio 1.326).

Table 4.3.3. Age-corrected factors and interactions influencing number of BCC

	p	rate ratio	95% CI
(i) individual factors			
male gender (n=755)	<0.001	1.487	1.342-1.648
Skin type 1 (n=529)	<0.001	1.393	1.205-1.609
Blue+green eyes (n=522)	0.001	1.361	1.187-1.559
CYP2D6 EM (n=623)	<0.001	1.266	1.125-1.425
CYP1A1 m_1m_1 (n=635)	0.004	1.242	1.071-1.441
(ii) **interactions between factors**			
GSTM1 null.skin type 1(n=463)	<0.001	2.702	1.964-3.717
CYP1A1 m_1m_1.blue+green eyes (n=509)	0.002	1.917	1.279-2.873
CYP2D6 EM.blue+green eyes (n=429)	0.046	1.388	1.005-1.915
CYP2D6 EM.male gender	0.049	1.279	1.001-1.633

Longitudinal study: Patient characteristics. Table 4.3.1. shows the details of the 169 patients in whom information on the time between first presentation and August 1995 and the number of BCC was obtained. The median follow up time (i.e. time between first presentation and August 1995) and median number of new lesions are recorded.

Factors influencing accrual of new BCC. Table 4.3.4. shows the factors that influenced the rate of appearance of new BCC. As expected male gender and, the number of lesions and age at first presentation were significant factors. Male gender appeared the single most influential factor (rate ratio 2.260) though both CYP2D6 EM and GSTT1 null were significantly associated with the rate at which new tumours appeared. Current and ex-smoking (ever smokers) was also significantly associated with more rapid appearance of further tumours. The influence of GSTM1 null and CYP1A1 Ile/Ile on the rate of accrual of tumours also approached significance. Table 4.2.4. also shows that GSTT1 null, CYP2D6 EM, male gender and skin type 1 were associated with faster appearance of new tumours after adjustment for the effect of age and the numbers of BCC at presentation. Comparison of the rate ratios indicated GSTT1 null was the most influential. The CYP1A1 Ile/Ile genotype was also found to be a significant factor. The rate ratio (0.690) indicated it was associated with slower accrual of new tumours and that it was relatively less influential than GSTT1 and CYP2D6 EM. No significant interactions between genes were identified. Except for the interaction between CYP2D6 EM and male gender, significant interactions between male gender and GSTM1 null ($p=0.035$) and skin type 1 ($p=0.009$) appeared to result from the strength of the main effect.

Table 4.3.4. **Factors influencing accrual of BCC**

	p	rate ratio	95%CI
(i) unadjusted			
male gender (n=143)	<0.001	2.260	1.793-2.848
BCC number at presentation (n=146)	<0.001	1.213	1.167-1.260
age at presentation (n=140)	0.002	1.014	1.005-1.022
CYP2D6 EM (n=128)	<0.001	2.398	1.880-3.059
GSTT1 null (n=126)	<0.001	2.321	1.821-2.959
ever smoking (n=110)	0.048	1.282	1.002-1.639
(ii) **adjusted for number of BCC and age at presentation**			
GSTT1 null (n=121)	<0.001	2.677	2.075-3.452
male gender (n=135)	<0.001	2.174	1.720-2.749
CYP2D6 EM (n=122)	<0.001	2.154	1.653-2.807
skin type 1 (n=94)	0.035	1.386	1.024-1.877
CYP1A1 Ile/Ile (n=115)	0.008	0.690	0.524-0.909
(iii) **interactions adjusted for number of BCC and age at presentation**			
male gender.CYP2D6 EM (n=117)	<0.001	2.633	1.510-4.593

4.4 POLYMORPHISM AT THE GSTM3 LOCUS: INTERACTIONS WITH CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE GENOTYPES AS RISK FACTORS FOR MULTIPLE BCC.

4.4.1 Introduction/Statistical Approach

While GSTM1 null may be a susceptibility marker for BCC, data from some studies on the role of GSTM1 (eg. lung cancer) are conflicting (Strange, 1991, Smith et al., 1995). The reason for these discrepancies is unclear but may reflect the influence on detoxification, of interactions between GSTM1 and other allelic loci encoding detoxifying enzymes. Thus, a model based only on the hypothesis that GSTM1 null confers increased risk may be too simplistic (Strange, 1991). Studies in lung suggest co-ordinated expression of some mu class genes; thus, *GSTM1*0* homozygotes express less GSTM3 than subjects with other GSTM1 genotypes (Antilla et al., 1995). We propose therefore, that assessment of the contribution of GSTM1 genotypes as susceptibility markers for cancer risk needs to take account of interactions with GSTM3. Polymorphism in GSTM3 has recently been described (Inskip et al., 1995). To assess the importance or otherwise of this polymorphism, we used firstly, immunohistochemical techniques to identify GSTM3 in skin and secondly, a case-control approach to compare GSTM3 genotype frequencies in controls and patients with BCC.

In a pilot study, 286 English Caucasians suffering BCC were studied. These cases were divided into two groups; firstly, 92 patients who suffered one BCC (mean time of follow-up 1.8 years, range 8 months-11.5 years). The second group comprised 194 patients with 2-35 tumours (mean follow up 7.5 years, range 1.1-28.9 years). 300 Caucasian controls (mean age 62.0±17.7 SD years; males 41.1%) were also recruited. Normal skin from 11 patients was provided at operation for breast reduction. Skin from 9 patients suffering BCC was obtained from archive material in the Department of

Histopathology. GSTM3 genotypes were identified using primers to exon 6/7 (Inskip et al., 1995).

X^2-tests were used to examine for homogeneity between cases and controls. As some genotype frequencies were small, the StatXact-Turbo statistical package was used. The influence of genotypes and characteristics alone and in combination, was studied by logistic regression. Analysis of the effect of genotypes on BCC numbers was studied by applying a Poisson regression model (EGRET statistical package; SERC, 1993) to count data in the cases with 2-35 primary tumours. The data was transformed to take account of all counts >2. This analysis was exploratory and not intended to be predictive, our objective being to identify covariates associated with the difference in transformed counts. A rate ratio, defined as the multiplicative effect of a change of a covariate by 1 was calculated (usually a change from 0 to 1).

4.4.2 RESULTS.

Immunohistochemical identification of GSTM3 The GSTM3 subunit was found in all 11 skin samples from controls though the extent of positivity varied markedly within sections and between subjects. Generally, the intensity of staining increased from the basal layer towards the stratum granulosum though in some subjects or, other regions of the same section, the basal layer demonstrated no immunoreactivity. Thus, in one control subject, positivity was greatest in the basal layer in one part of the section with other parts showing increasing positivity from the basal layer to the stratum granulosum. In a further control, only a few cells of the outer stratum spinosum were weakly positive. In all samples examined, sebaceous glands, sweat glands and arrector pili muscles gave strongly positive results. GSTM3 expression was also found in the tumours from

9 patients with BCC though in 6 of these patients, only a few cells were positive. In 8 cases, perilesional skin was available; 3 samples demonstrated increasing GSTM3 positivity from the basal layer to the stratum granulosum. In the remaining 5 subjects, expression was confined to parts of the stratum spinosum.

Influence of GST genotypes on susceptibility. Table 4.4.1 shows GSTM3 genotype frequencies in controls and BCC cases. Frequency distributions and individual genotype frequencies in these groups were not different. Similarly, frequency distributions and genotype frequencies for GSTM1, GSTT1, CYP2D6 were not significantly different in cases and controls. The proportion of males and the mean age of the BCC group was significantly greater than in controls ($p<0.001$ in both cases). Logistic regression was used to show the age and gender-corrected proportions of GSTM3 AA in the BCC group and controls were not different. Similarly, the age- and gender-corrected proportions of GSTM3 AA in combination with either GSTM1 A, GSTM1 null, GSTT1 null, CYP2D6 EM or CYP1A1 m1m1 in cases and controls were not significantly different.

Table 4.4.1. GSTM3, GSTM1 and GSTT1 genotype frequencies in controls and patients with BCC.

	GSTM3 AA	GSTM3 AB	GSTM3 BB
single BCC (n=92)	68 (73.9%)	17(18.5%)	7(7.6%)
multiple BCC (n=194)	140(72.2%)	49(25.3%)	5(2.6%)
total BCC (n=286)	208(72.7%)	66(23.1%)	12(4.2%)
Controls (n=300)	221(73.7%)	64(21.3%)	15(5.0%)

	GSTM1 null	GSTM1 A	GSTM1 B	GSTM1 A,B
single BCC (n=92)	52 (56.5%)	21(22.8%)	13(14.1%)	6(6.5%)
multiple BCC (n=153)	88 (57.5%)	42(27.5%)	21(13.7%)	2(1.3%)
total BCC (n=245)	140(57.1%)	63(25.7%)	34(13.9%)	8(3.3%)
Controls (n=211)	121(57.3%)	56(26.5%)	25(11.8%)	9(4.3%)

	GSTT1 null	GSTT1 A
single BCC (n=92)	18(19.6%)	74 (80.4%)
multiple BCC (n=167)	35(21.0%)	132(79.0%)
total BCC (n=259)	53(20.5%)	206(79.5%)
Controls (n=284)	56(19.7%)	228(80.3%)

To determine whether GSTM3 genotypes were associated with risk of multiple tumours, the BCC cases were divided into patients with 1 lesion and those with 2-35 tumours (Table 4.4.1). The frequencies of GSTM3 BB and GSTM1 A/B (Table 4.3.1) in the group with between 2-35 tumours were almost significantly lower (exact p=0.0601, X^2_1=3.930; exact p=0.055, X^2_1=4.946 respectively) than in the group with 1 lesion. Within the cases with 2-35 tumours only, Poisson regression was used to identify genotypes, individual characteristics and interactions between genotypes and characteristics associated with increasing numbers of primary tumours. Thus, male gender and age were associated with a significant increase in the number of BCC ($p<0.001$). Neither GSTM3 AA nor GSTM1 A, GSTT1 null, CYP1A1 Ile/Ile alone or, after correction for age and gender, were significantly associated with an increased number of BCC (Table 4.4.2). However, after correction for age and gender, skin type 1, CYP1A1 m1m1, CYP2D6 EM and GSTM1 null were significantly associated with increased numbers of tumours (Table 4.4.3). Significant, age- and gender-corrected interactions in which the interaction term was more significant than either of the main effects were also identified; between GSTM3 AA/skin type 1, GSTM3 AA/GSTM1 null and GSTM3 AA/CYP1A1 m1m1 (Table 4.4.3).

Table 4.4.2. CYP2D6/CYP1A1 genotype frequencies in controls and patients with BCC.

	CYP2D6 EM	CYP2D6 HET	CYP2D6 PM
single BCC (n=88)	53 (60.2%)	31(35.2%)	4 (4.5%)
multiple BCC (n=156)	104(66.7%)	46(29.5%)	6 (3.8%)
total BCC (n=244)	157(64.3%)	77(31.6%)	10(4.1%)
Controls (n=201)	129(64.2%)	67(33.3%)	5 (2.5%)
	CYP1A1 I/I	CYP1A1 I/V	CYP1A1 V/V*
single BCC (n=91)	76(83.5%)	15(16.5%)	0(0.0%)
multiple BCC (n=167)	137(82.0%)	27(16.2%)	3(1.8%)
total BCC (n=258)	213(82.6%)	42(16.3%)	3(1.2%)
Controls (n=84)	77 (91.7%)	6 (7.1%)	1(1.2%)
	CYP1A1 m1m1	CYP1A1 m1m2	CYP1A1 m2m2
single BCC (n=91)	76 (83.5%)	13(14.3%)	2(2.2%)
multiple BCC (n=166)	135(81.3%)	31(18.7%)	0(0.0%)
total BCC (n=257)	211(82.1%)	44(17.1%)	2(0.8%)
Controls (n=82)	71 (86.6%)	11(13.4%)	0(0.0%)

* I, Ile; V, Val

Table 4.4.3. Age- and gender corrected factors and interactions influencing number of BCC:

	p	rate ratio	95% CI
(i) individual factors			
Skin type 1 (n=141)	<0.001	2.019	1.616- 2.523
CYP1A1 m_1m_1 (n=156)	<0.001	1.978	1.424- 2.747
CYP2D6 EM (n=146)	<0.001	1.608	1.246- 2.074
GSTM1 null (n=143)	0.002	1.436	1.144- 1.802
(ii) interactions between factors			
GSTM3 AA.skin type 1 (n=141)	<0.001	2.058	1.628- 2.601
GSTM3 AA.GSTM1 null (n=143)	<0.001	1.606	1.287- 2.004
GSTM3 AA.CYP1A1 m_1m_1	<0.001	1.470	1.184- 1.827

4.5. TRUNCAL SITE AND DETOXIFYING ENZYME POLYMORPHISMS REDUCE TIME TO PRESENTATION OF NEXT BCC.

4.5.1. Introduction/Statistical Approach.

Exposure to ultraviolet radiation (UV) is recognised as a critical factor in the pathogenesis of BCC (Kricker et al., 1993; Karagas and Greenberg, 1995; Kripke, 1994) though the relationship between amount, timing and nature of exposure and risk of multiple tumours is complex and poorly understood. The implications for risk of truncal lesions compared with those on the more sun-exposed head and neck are unclear. The concept of susceptibility in the context of BCC is complex as genetic factors appear to influence both tumour numbers and accrual (Lear et al., 1996). The latter concept is important as an understanding of the factors that influence time to next lesion would be useful if it allowed clinicians to focus on susceptible individuals who are likely to present with further tumours quickly (i.e. within 2 years). We have used a survival analysis approach to investigate the influence of genetic and individual characteristics on the time to presentation of a new lesion. The influence of allelism in GSTM1, GSTT1, CYP2D6 and CYP1A1 has also been studied. 856 Caucasians with BCC were recruited.

Statistical analysis. Methods of survival analysis were used to study factors that influence time from first presentation to presentation of a subsequent tumour. We used time to presentation of next BCC as our main outcome measure. In our clinical experience this is a more reliable endpoint than time to tumour appearance determined by the patient, since the latter parameter relies on accurate memory in an often elderly population. Where patients presented with a single tumour, or with more than one tumour but did not suffer a further BCC by November 1995, their times were

considered to be censored. For these patients the time of appearance of the next tumour obviously exceeds the study period or their survival times must be at least the time to the cessation of follow-up. These censored times are incorporated into the calculation of the estimates of the survival probabilities. For all other multiple BCC patients, the time interval between first presentation and presentation of the next tumour (not necessarily the second lesion) was used in the analysis. Cox's Proportional Hazards Regression was used to determine which factors, alone and in combination, significantly influenced the time from first presentation to presentation of a subsequent tumour. Since number of tumours at presentation, categorised as single versus multiple, was highly significant alone, all subsequent calculations were carried out by correcting for this factor. The results are presented as the following summaries; corrected hazard ratios with 95% confidence intervals and significance levels, and uncorrected median time to next BCC and proportions of patients with further tumours at 3 and 5 years.

4.5.2. RESULTS

Patient characteristics. Table 4.5.1. shows the characteristics of the study group. The proportion of males was greater than in 561 age-matched controls (47%) from the study centres. Frequencies of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes were not significantly different from those in these controls.

Table 4.5.1. Patient demographics.

Number of BCC	1-35 tumours (n=856)
Mean age (years)	67.5±12.2 (SD) (n=856)
Males/females	56.2%/43.8% (n=843)
Blue and green eyes	73.0% (n=435)
Brown eyes	27.0% (n=161)
Skin type 1	14.0% (n=80)
Skin type 2-4	86.0% (n=491)
Ever smoker	63.1% (n=383)
Never smoker	36.9% (n=224)
Brown and black hair	75.0% (n=249)
Blonde and red hair	25.0% (n=83)
Outdoor occupation	11.7% (n=25)
Indoor occupation	88.3% (n=189)
First tumour on trunk	10.8% (n=36)
First tumour on head/neck, upper limbs or lower limbs	89.2% (n=297)
Median number BCC	1 (range 1-35)
Median number BCC at presentation	1 (range 1-10)

Single factors influencing time to presentation of next BCC. Table 4.5.2. shows the genotypes and characteristics associated with time to presentation of the next BCC. Data were adjusted for number of tumours at presentation since, as expected (Kricker et al., 1993; Karagas and Greenberg, 1995), the presence of more than one tumour was significantly associated with a decreased time to next tumour ($p<0.0001$, hazard ratio 2.72, 95% CI 1.84,4.03, median time to next tumour: single tumour at presentation 5.75 years, multiple tumours at presentation 3.58 years). The association of a truncal tumour at presentation with a decreased time to next lesion approached significance (Table 4.5.2.). Male gender also appeared to be associated with a shorter time to presentation of further tumours though the effect failed to achieve significance ($p=0.0815$, hazard ratio 1.38). Skin type, hair colour, eye colour, smoking status and occupation were not significant. GSTT1 null was associated with a significantly reduced time to presentation of next tumour whereas GSTM1 null, CYP2D6 EM, CYP1A1 Ile/Ile and CYP1A1 m1m1 were not significant.

Interactions between genotypes, characteristics and tumour site. Table 4.5.2. shows significant two factor interactions, corrected for number of tumours at first presentation, in which the interaction term was more significant than either of the main effects. Only two-factor interactions were considered. Thus, highly significant two-factor interactions between the presence of the first tumour on the trunk and each of male gender, the GSTM1 null and CYP2D6 EM genotypes were identified. These interactions appeared particularly significant as all of the cases with these combinations demonstrated further tumours within 5 years whereas only half of the cases without these combinations suffered a further lesion during this time (Table 4.5.2. and figures 4.5.1. to 4.5.3.). Indeed, 3 years after first presentation, 78% of the cases with GSTM1 null and a first truncal tumour had suffered a further BCC (see

figure 4.5.1.). No significant two-factor interactions between pairs of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes were identified. Other interactions between genotypes and patient characteristics, including skin type 1 and male gender were not significant.

Table 4.5.2. **Factors influencing time to presentation of next BCC (corrected for number of tumours at presentation)**

	p value	hazard ratio (95 %CI)	median time (years)	% with further tumour 3y/5y
1st tumour on trunk	0.096	1.58 (0.92,2.70)	3.00	41/54
non-truncal 1st tumour			5.04	26/47
GSTT1 null	0.0208	1.74 (1.09,2.79)	5.07	38/59
GSTT1 expresser			6.12	16/35
Interactions (corrected for tumour numbers at presentation)				
GSTM1 null/1st tumour on trunk				
	0.0009	3.82 (1.73,8.42)	1.63	78/100
other cases			5.03	27/48
CYP2D6 EM/1st tumour on trunk				
	0.0031	3.09 (1.46,6.50)	1.88	53/100
other cases			5.14	27/46
Male gender/1st tumour on trunk				
	0.0002	3.33 (1.76,6.30)	1.53	60/100
other cases			5.04	25/47

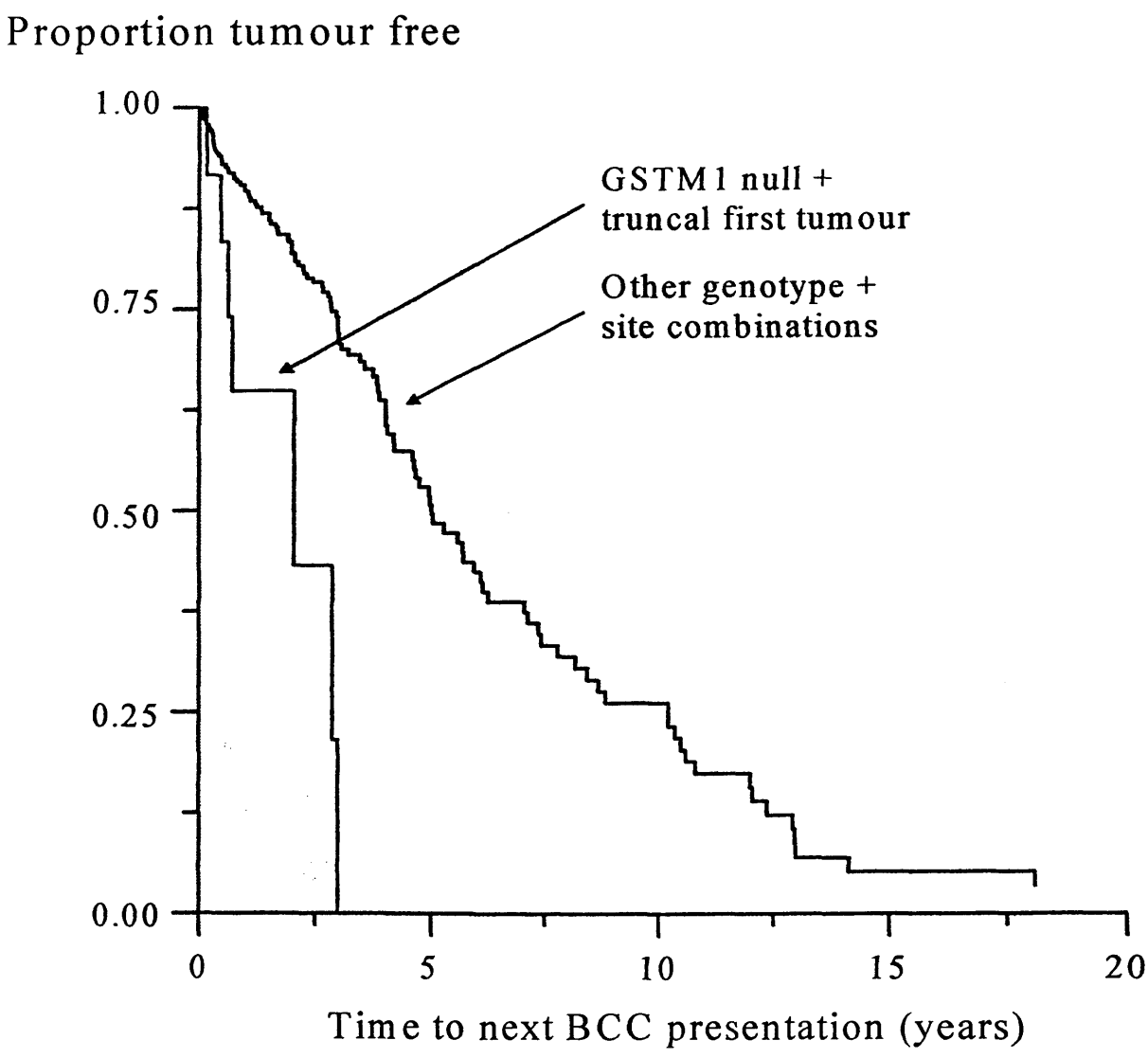

Figure 4.5.1. Time to next BCC curve in those GSTM1 null and with a first truncal tumour.

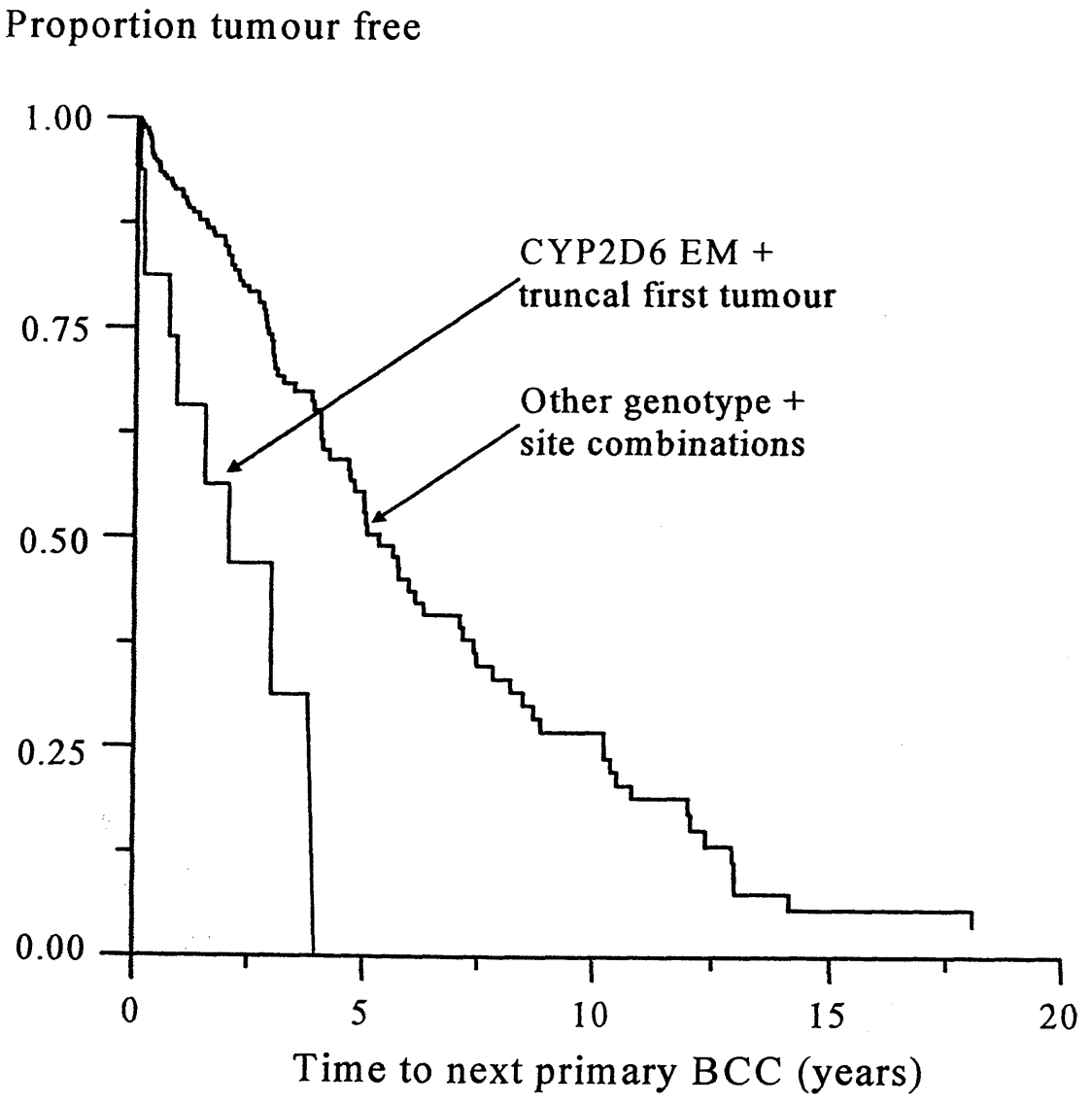

Figure 4.5.2. Time to next BCC curve in those CYP2D6- EM and with a first truncal tumour

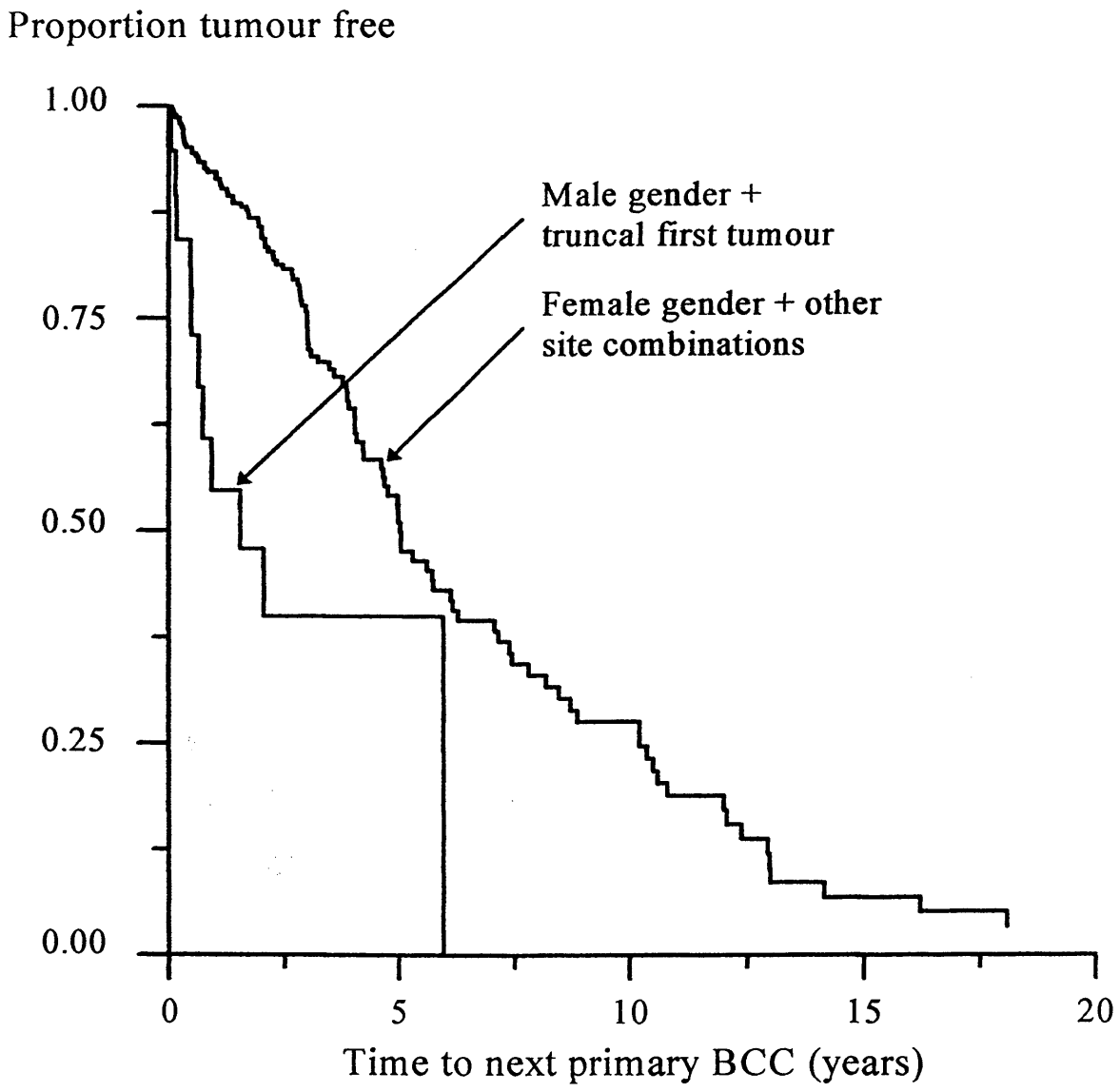

Figure 4.5.3. Time to next BCC curve in males with a first truncal tumour

4.6. TUMOUR SITE IN BCC: INFLUENCE OF GSTT1 AND CYP1A1 GENOTYPES.

4.6.1. Introduction/Statistical Approach

While the incidence of BCC is increasing, it is the proportion of tumours on the trunk that demonstrates the greatest increase (Kricker et al., 1993). Since the trunk is less exposed, or indeed intermittently exposed, to UV, it could be hypothesised that patients with truncal tumours represent a high risk group because they are less able to handle the products of UV-induced damage. We hypothesise that the presence of truncal tumours is more strongly associated with genetic susceptibility. 345 BCC patients with tumour site information were recruited. Patients were divided according to whether they had truncal BCC (either first tumour or at any time) or not. T-tests and Mann-Whitney U test were used to assess differences in ages and number of BCC's, respectively, between the two groups. χ^2-tests were used to examine for homogeneity of genetic and individual characteristics between cases with and without at least one truncal tumour. As various factors were studied, the influence on tumour site of each alone and in combination was studied by logistic regression analysis. Combinations of genotypes and characteristics were studied in the presence of the main effects and only those where the interactive term was more significant than either of the main effects were included. Since age and gender were significant confounding factors, analysis of the influence of genotypes and other patient characteristics were corrected for these factors. Since patients with at least one truncal tumour suffer more BCC, the associations with tumour site were further analysed by correction for BCC number as well as age and gender.

4.6.2. RESULTS

Patient characteristics. Table 4.6.1. shows the characteristics of the study group. Patients with at least one truncal tumour (mean±SD = 66.2±11.9 years) were significantly younger than those without truncal tumours (mean±SD = 68.3±12.2 years; p=0.0170) and the mean time (±SD) between appearance and presentation in the truncal group (2.02±1.80 years) was not different from the non-truncal group (2.03±2.93 years). The proportion of males was greater though this did not reach significance (p=0.0925, χ^2_1=2.83, odds ratio=1.571, 95% CI=0.928,2.659). As expected, the number of BCC in patients with at least one truncal tumour was greater than the non-truncal tumour group (Table 4.6.1.) since the larger the number of tumours, the greater the probability that one will be truncal. However, the median number of primary tumours in patients whose *first* tumour was truncal (n=40; median tumour number=2, interquartile range=1-4) was also significantly greater than those whose *first* tumour was not truncal (n=312; median tumour number=1, interquartile range=1-3; p=0.024). Other patient characteristics (skin type, eye colour, smoking, occupation, hair colour) were not significantly different between the two groups, though the presence of red or blonde hair, corrected for age and gender, approached significance (p=0.0974).

Table 4.6.1. Patient demographics.

	Patients with no truncaltumours	Patients with a truncal tumour
Mean age (years) (n=74)	68.3±12.2 (SD) (n=263)	66.2±11.9(SD)
Males	54.1% (n=268)	64.9% (n=77)
Mean no. BCCs (n=77)	1.99±1.81 (SD) (n=275)	5.40±6.03(SD)
Blue and green eyes	70.1% (n=148)	75.8% (n=47)
Brown eyes	29.9% (n=63)	24.2% (n=15)
Skin type 1	16.4% (n=34)	16.9% (n=10)
Skin type 2-4	83.6% (n=173)	83.1% (n=49)
Ever smoker	65.1% (n=142)	62.9% (n=39)
Never smoker	34.9% (n=76)	37.1% (n=23)
Brown and black hair	74.8% (n=101)	64.1% (n=25)
Blonde and red hair	25.2% (n=34)	35.9% (n=14)
Outdoor occupation	16.7% (n=13)	19.2% (n=5)
Indoor occupation	83.3% (n=65)	80.8% (n=21)

Single factors associated with the presence of truncal tumours. Table 4.6.2. shows the genotypes, corrected for imbalances in patient age and gender, associated with tumour site. Using logistic regression, the age-corrected proportion of GSTT1 null and CYP1A1 Ile/Ile genotypes were significantly greater in patients with at least one truncal tumour. The importance of GSTT1 and CYP1A1 genotypes is further emphasised by the increased significance of the interaction (the combination of both GSTT1 null and CYP1A1 Ile/Ile). In order to further assess the importance of these genotypes as determinants of tumour site, the associations with tumour site were also corrected for BCC number as well as age and gender, since patients with at least one truncal tumour suffer more BCC (Table 4.6.1.). This demonstrated that individually, both GSTT1 null and CYP1A1 Ile/Ile remained significant but with reduced odds ratios ($p=0.0316$, odds ratio=1.44, 95%CI 1.24,1.66 and $p=0.0130$, odds ratio=1.51, 95% CI 1.29-1.77, respectively). The combination of these genotypes (both GSTT1 null and CYP1A1 Ile/Ile) however, remained highly significant with a decreased p value and increased odds ratio ($p=0.0035$, odds ratio=3.56, 95% CI 1.52-8.34) despite this rigorous correction. These factors act synergistically, since correction for the contribution of the individual genotypes showed that the effect of them combined remained significant. None of the other genotypes examined (GSTM1 null, GSTM3 AA, CYP2D6 EM and CYP1A1 m1m1) were associated with tumour site, either alone or in combination with other genotypes or patient characteristics.

Table 4.6.2. **Factors, alone and in combination, demonstrating significant differences between patients with at least one truncal tumour and those with no truncal tumours (Corrected for age & gender).**

	p value	χ^2	Odds ratio	95% CI
GSTT1 null	0.0245	5.06	2.24	1.11-4.53
CYP1A1 Ile/Ile	0.0386	4.28	2.86	1.06-7.72
GSTT1 null+CYP1A1 Ile/Ile	0.0059	18.70	2.95	1.37-6.39

4.7. MULTIPLE BCC AND INTERNAL MALIGNANCY

4.7.1. Introduction/Statistical Approach

There are conflicting reports regarding a link between BCC and internal malignancy (Sandstrom et al., 1984; Moller et al., 1975; Lindelhof et al., 1991). More recently an association was reported between haematological malignancy and MM and SCC (Adami et al., 1995). BCC was not assessed in that study. We therefore studied whether there was an association between BCC and internal malignancy in our population, with particular emphasis on haematological malignancy. Many patients with BCC develop multiple lesions and it might be presumed that these subjects represent a high susceptibility group, that is at greatest risk of internal malignancy. We therefore report findings from a case-control study to identify an association between multiple BCC and haematological malignancy.

141 patients (mean age 71 years; 62 women) with multiple BCC (range 2-35 tumours per patient) were recruited and followed up for approximately 3 years. Age and sex matched controls (one case to one control) with benign skin conditions were recruited. The presence of any histologically proven internal/haematological malignancy in cases or controls was noted. McNemar's test was used to analyse the results.

4.7.2. RESULTS

Thirteen cases, compared to 7 controls, had an internal malignancy (Table 4.7.1.). This was not significant, though that between the number of cases (5) and controls (0) with a haematological malignancy approached significance ($p=0.0625$). Four of the five cases developed the haematological malignancy after developing their first BCC.

Table 4.7.1. Comparison of internal malignancies between multiple BCC cases and controls.

Malignancy	Number of cases	Number of controls
Haematological*	5	0**
Breast	3	1
Gastro-Intestinal Tract	1	2
Genito-Urinary Tract	4	4
Total	13	7

* 4 non-Hodgkin's lymphoma, 1 chronic myeloid leukaemia

** p=0.0625, McNemar's test, StatXact Turbo® statistical package.

4.8. A COMPARISON OF RISK FACTORS FOR MM, SCC AND BCC IN THE UNITED KINGDOM.

4.8.1. Introduction/Statistical Approach

MM, SCC and BCC are clinically distinct with very different natural histories and it may be expected that significant differences would exist between risk factors for these cancers. However, few studies have directly compared risk factors for MM, SCC and BCC between individuals from a single population. This has necessitated comparisons drawn from studies in different populations in different countries (Marks, 1995). Such a direct comparative study might give valuable insights into the pathogenesis of these diseases and be of help in follow-up strategies. We have previously investigated risk factors for BCC in a case-control study in the U.K. (see chapter 4.6.). We therefore assessed firstly, in a case control manner, the associations of individual characteristics with MM and SCC. We then compared risk factors in those with SCC and MM against those with BCC and also to compare those with MM against SCC.

Patients with MM, SCC and BCC were recruited. A control group of patients with benign skin lesions were recruited from dermatology clinics. The various groups were then age/sex matched. Due to differences in age and gender between the groups we were unable to match all the patients within all the groups. Conditional logistic regression was used to identify differences between cases and controls.

4.8.2. RESULTS

MM and SCC compared to controls.

The mean age of the MM group was 55.3 years (SD 16.5) and SCC, 70.5 years (SD 10.5). 170 age/sex matched case-control pairs were analysed for MM and 121 for SCC. Table 4.8.1. lists the associations of individual characteristics with MM and SCC when compared to the control group. Patients with MM were more likely than controls to have red/blonde hair and be of social class 1 or 2, social class having an odds ratio (OR) of 10.0. Outdoor occupation and blue green eyes approached significance. Skin type and smoking history was not significant. Patients with SCC were more likely than controls to have blonde/red hair with outdoor occupation, skin type 1 and blue/green eyes approaching significance. Social class and smoking history showed no significant differences.

Table 4.8.1. Risk factors for MM and SCC compared with age/sex matched controls.

	MM			SCC		
	p value[a]	OR[b]	95%C.I.[c]	p value	OR	95%C.I.
Red/blonde hair	0.004	3.43	1.48-7.96	0.003	3.11	1.47-6.59
Blue/green eyes	0.061	1.53	0.98-2.39	0.075	1.85	0.94-3.63
Social class 1 or 2	0.028	10.0	1.28-78.12	NS[d]		
Outdoor occupation	0.099	3.00	0.81-11.08	0.099	3.00	0.81-11.08
Skin type 1	NS			0.065	2.11	0.96-4.67

[a]by logistic regression, [b]OR = odds ratio, [c]C.I. = confidence interval,

[d]NS = not significant

MM and SCC compared to those with BCC.

Table 4.8.2. lists comparisons of individual characteristics between those with MM and SCC against those with BCC. 144 age/sex matched case-BCC pairs were analysed for MM and 121 for SCC. Patients with MM were more likely to have a tumour on the lower/upper limbs or a truncal tumour at presentation when compared to those with BCC. Skin type 1, outdoor occupation, eye/hair colour, smoking history and social class were not significantly different between the two groups. Patients with SCC were more likely to have a tumour on the lower or upper limbs at presentation when compared to those with BCC. No differences were seen in truncal site at presentation, outdoor occupation, smoking history, eye/hair colour, social class and skin type.

MM compared to those with SCC.

73 age/sex matched pairs were analysed. Patients with MM, when compared to those with SCC, were more likely to have a truncal tumour at presentation ($p=0.012$, OR 7.14, 95% C.I. 1.53-33.44). No significant differences were seen in other tumour sites, outdoor occupation, eye/hair colour, social class and smoking history.

Table 4.8.2. Risk factors for MM and SCC compared to age/sex matched patients with a single BCC.

	MM			SCC		
	p value[a]	OR[b]	95% C.I.[c]	p value	OR	95%C.I.
Lower limb tumour[d]	<0.001	12.05	4.00-36.31	0.004	30.30	2.95-311.4
Upper limb tumour[d]	<0.001	14.13	3.38-59.07	0.018	22.48	1.71-296.4
Truncal tumour[d]	0.0017	4.38	1.74-10.98	NS[e]		

[a]by logistic regression, [b]OR = odds ratio, [c]C.I.= confidence intervals, [d]site of tumour at presentation, [e]NS = not significant

4.9. RISK FACTORS IN INDIVIDUALS WITH SKIN CANCERS OF DIFFERENT HISTOLOGICAL TYPES.

4.9.1. Introduction/Statistical Approach

Few studies have addressed risk factors in those with skin cancers of different histological types and, in particular, compared risk factors to those with a single histological type. Patients with histologically confirmed skin cancers of different histological types (n=150, all with BCC and either SCC or MM) were recruited. We wanted to assess risk factors in those patients; firstly, using a case control approach and secondly directly comparing them to those with a single BCC (as all those in the multi-cancer group had a BCC). Identification of those patients who may develop skin cancers of different histological types would be of use in follow up strategies.

Logistic regression was used to investigate the effects of single factors in the presence of other factors. As the controls were younger and more likely to be female than cases, all subsequent analyses were age and gender corrected. A further, similar analysis was then performed comparing parameters between patients in the multi-cancer group against those with a single BCC. As those in the multi-cancer group were older than the single BCC group and were more likely to be male, all analyses were age and gender corrected.

4.9.2. RESULTS

Those in the multi-cancer group were older than the single BCC group ($p<0.0001$, mean 73.3 (SD 10.3) and 66.4 (SD 12.6) respectively) and were more likely to be male ($p=0.0098$, OR 1.64, 95% C.I. 1.13-2.4). Table 4.9.1. illustrates the influence of

risk factors in the multi-cancer group, firstly against controls and secondly compared to those with a single BCC. When compared to controls, patients in the multi-cancer group were more likely to be skin type 1, have blue/green eyes and be of social class 1 or 2. Social class had the strongest influence (p=0.003, OR 3.79). Smoking history and outdoor occupation had no significant effect. When compared to those with a BCC, the only significant differences were in truncal site of tumours and skin type 1 (OR 3.52 and 2.56 respectively). Hair/eye colour, social class, outdoor occupation and smoking history exhibited no significant differences.

Table 4.9.1. Risk factors for multiple skin cancers of different types (age and gender corrected):

i. compared to controls

	p value*	Odds ratio	95% C.I.**
Social class 1 or 2	0.003	3.79	1.57 - 9.14
Skin Type 1	<0.0001	3.44	1.82 - 6.50
Red/blonde hair	<0.009	2.65	1.28 - 5.47
Blue/green eyes	<0.015	1.97	1.14 - 3.39
Outdoor occupation	<0.077	2.46	0.91 - 6.64

*by logistic regression, ** C.I.= confidence interval

ii. compared to patients with a single BCC

	p value*	Odds ratio	95% C.I.**
Truncal site	<0.025	3.52	1.18 - 10.53
Skin type 1	<0.002	2.56	1.43 - 4.60
Blue/green eyes	<0.092	1.60	0.92 - 2.75

*by logistic regression, ** C.I.= confidence interval

4.10. THE INFLUENCE OF CYP2D6 AND CYP1A1 POLYMORPHISMS, GENDER, EYE AND HAIR COLOUR ON SUN-REACTIVE SKIN TYPE.

4.10.1 Introduction/Statistical Approach

The concept of sun reactive skin typing (SRST) was created in 1975 to classify persons with white skin enabling selection of correct initial doses of UVA in the application of PUVA photochemotherapy (Fitzpatrick, 1988). Variants in the melanocyte stimulating hormone receptor gene are associated with skin type 1 (Valverde et al., 1996). It is known that GSTM1 null is associated with photosensitive Systemic Lupus Erythematosus (Ollier et al., 1996) suggesting that detoxication of products of oxidative stress may be important in determining response of the skin to UV radiation. Furthermore, mean MED's have recently been shown to be lower in those with GSTT1 and GSTM1 null genotypes than in those who express the enzymes (Kerb et al., 1997). We have therefore assessed the influence of detoxifying enzyme polymorphisms and patient characteristics on SRST.

1392 patients were recruited: basal cell carcinoma (n=582), seborrhoeic warts (n=102), malignant melanoma (n=145), psoriasis (n=81), eczema (n=107), benign skin lesions (n=73), acne (n=22), squamous cell carcinoma of the skin (n=107) and other conditions such as alopecia, pruritus, nail dystrophy and normal individuals. The Armitage trend test was used to analyse possible associations between SRST, genotypes, gender and eye and hair colour. This assessed for any significant trend in a variable from SRST 1 to 4 giving either an increasing or decreasing trend depending on the proportion of the variable present in each skin type group.

4.10.2. RESULTS

Of the 1392 patients recruited, 666 were women (47.8%) and 726 (52.2%) men. 194 patients were SRST 1, 465 SRST 2, 407 SRST 3 and 326 SRST 4. 69.3% of patients had blue/green eyes and 23.6% had blonde/red hair. GSTM1 null, GSTT1 null, CYP1A1 ile/ile and indoor/outdoor were not significantly associated with SRST. Table 4.10.1. illustrates the trend analysis for the patient characteristics and genotypes. As expected, blue/green eyes and red/blonde hair showed a decreasing trend from SRST 1 to 4 ($p<0.0001$). Female gender was also associated with a decreasing trend in relation to SRST ($p=0.0202$). CYP1A1 m1m1 showed an increasing trend ($p= 0.0080$) and CYP2D6 EM approached significance with a decreasing trend ($p=0.0518$).

Table 4.10.1:Influence of demographics/genotypes on SRST.

SRST	Female gender(%)*	Blue/green eyes (%)*	Blonde/red hair (%)*	CYP1A1 m1m1(%)*	CYP2D6 EM (%)*
1	105 (54.1)	163 (86.2)	59 (49.6)	100 (76.3)	87 (69.6)
2	224 (48.2)	344 (76.1)	66 (29.3)	241 (79.5)	188 (65.1)
3	194 (47.7)	253 (63.6)	60 (20.2)	202 (80.2)	142 (55.7)
4	143 (43.9)	187 (57.0)	36 (12.2)	132 (88.6)	103 (64.8)
p value**	= 0.0202	<0.0001	<0.0001	=0.008	=0.0518

*Percentages reflect proportion within the particular skin type

**Armitage trend test

We then assessed two-factor interactions between variables and the significant results are illustrated in table 4.10.2. Female gender+CYP2D6 EM was associated with a decreasing trend (p=0.0350), male gender+CYP1A1 m1m1 showed an increasing trend from SRST 1 to 4(p=0.0002) and CYP2D6 EM+CYP 1A1 m1m2/m2m2 had a decreasing trend (p=0.0151). These appeared to result from the strength of the main effects, suggesting they are independent. All trends were reanalysed with the skin cancer patients removed from the analysis to assess whether the trends were the in the same direction in the non skin cancer group. In this analysis, the trend directions were similar between patients with cancer than in those without, although the results were not significant due to the smaller numbers in the non skin cancer group.

Table 4.10.2: Interactions between demographics and genotypes.

SRST	Female + CYP2D6-EM (%)*	Male + CYP1A1 m1m1 (%)*	CYP2D6 EM + CYP 1A1 m1m2/m2m2 (%)*
1	43 (34.7)	43 (33.1)	92 (77.3)
2	85 (29.9)	117 (39.1)	193 (70.7)
3	69 (27.2)	103 (40.9)	146 (61.9)
4	40 (25.3)	82 (55.8)	96 (69.1)
p value**	=0.0350	=0.0002	=0.0151

*Percentages reflect proportion within the particular skin type

**Armitage trend test

CHAPTER 5 - DISCUSSION

5.1. RISK FACTORS FOR BCC: A MATCHED CASE CONTROL STUDY IN 806 PATIENTS.

The life-time risk of BCC for a north American child born in 1994 is estimated to be 28-33% (Miller and Weinstock, 1994). Further, patients with a BCC often develop more than one tumour, tumours may recur or they may be unaware of lesions (Koh et al., 1989; Lookingbill, 1988). Follow-up therefore, places considerable demands on clinicians. However, there is surprisingly little research into BCC. In particular, few large case control studies have been undertaken, with only one in the U.K (McHenry et al., 1995). In the present study, we have reported data from the largest ever case control study of risk factors to BCC in the UK. We have shown that social class 1 and 2, skin type 1, red/blonde hair and blue/green eyes are all significant risk factors associated with the development of BCC. Social class had the strongest effect with an odds ratio of 2.36. Other studies have found age (Lin and Carter, 1986), red hair (Healy et al., 1995), blue/green eyes (Hogan et al., 1989), skin type 1 (Healy et al., 1995), freckling (McHenry et al., 1995) and outdoor occupation (Hogan et al., 1989) to be significant risk factors. A recent large multicentre south European study identified blonde hair, pale eyes, recreational sun exposure and skin type 1 to be significant BCC risk factors (Zanetti et al., 1996).

The finding that higher social classes are at greater risk of BCC is interesting and has not been reported in the UK before. There is no absolute definition of social class and therefore, all methods of determining social class are estimates and are open to criticism and are more limited especially with regard to females and, for this reason, the use of gender-matching in our study was used to eliminate potential bias. We based our assessment on occupation. It is noteworthy that the social class effect was

of similar strength in males and females when compared to controls, illustrating that gender did not significantly affect our social class results. In general higher social classes are associated with more highly paid occupations and we postulate the increased risk of BCC may be due to more frequent overseas travel with resultant increased intermittent sun exposure. This hypothesis has also been proposed to explain the association of malignant melanoma with higher social class (Cooke et al., 1984). However, another possible hypothesis is that higher social class may be associated with increased health seeking behaviour leading to increased reporting of BCC. This hypothesis is supported by the finding that low socio-economic status and infrequent physician visits have been shown to be associated with very large lesions (Robinson et al., 1995). Such patients are less concerned about their general health and pose a significant management problem. Further research is needed to clarify these issues. The relationship of skin type 1, red hair and blue/green eyes with BCC has been shown previously although they are not consistently associated in all studies. Reasons for this may be ethnic differences between studies. In our study outdoor occupation was not a significant factor suggesting that intermittent, recreational exposure may be more important than chronic ultraviolet exposure. Indeed, recent research has supported this hypothesis (Kricker et al., 1995).

Identification of risk factors enables recognition of those most susceptible and also gives insights into the pathogenesis of the disease. It also helps target preventative measures to those most at risk. In BCC susceptibility is complex as patients are not only at risk of developing the disease, they may also develop multiple tumours. This has major implications for follow up. It is therefore important to define those at risk of multiple lesions. The second aim of our study was to address this question. Little research has focused on this area. A study in the U.S.A. found that age over 60 years

and more than one tumour at presentation was associated with a greater risk of further BCC development (Karagas, 1994). We found that patients with a truncal tumour were more likely to have more than one lesion with an odds ratio of 4.03. Clearly, in this country, the trunk is least likely to be chronically exposed to UV and it could be hypothesised that in a UV mediated disease those with a BCC on the trunk are most susceptible and therefore, likely to develop multiple lesions. Alternatively, UV independent mechanisms predisposing to truncal and multiple lesions may be involved. Our data suggests those with truncal BCC are at increased risk of further tumours and patients should be informed of this. If follow up were to enable earlier detection of tumours, patients with truncal lesions would benefit from more meticulous follow up as, in general, smaller lesions are easier to treat. In view of the known association of arsenic ingestion with multiple BCC (Yeh et al., 1968), we enquired about possible exposure but some patients may have been exposed without their knowledge. However, It is noteworthy that the proportion of patients in our study with truncal BCC (10.2%) is similar to that reported in other studies on sporadic BCC.

We have identified risk factors to BCC and to multiple lesions in a British population. Such factors may be helpful in primary and secondary prevention of this common cancer and give insights into the pathogenesis of this disease. Thus, the associations identified here suggest BCC results from an interaction between environmental exposure (UV) and genetic factors (skin type, hair colour, gender). This is consistent with our previous studies identifying the importance of genetic variation in the ability to detoxify UV-induced reactive oxygen species in mediating susceptibility to BCC (Heagerty et al., 1994) and to multiple lesions (Lear et al., 1996).

5.2. SUSCEPTIBILITY TO MULTIPLE BCC: INTERACTIONS BETWEEN GSTM1 GENOTYPES, SKIN TYPE AND MALE GENDER

The role of factors other than UV, in the pathogenesis of BCC is evident from work using a variety of experimental approaches (Heagerty et al 1994; Karagas et al 1994; Wei et al 1994; McHenry et al 1995). We have described further studies into the influence of allelism at loci encoding phase I and II detoxifying enzymes on susceptibility to this tumour. Genotype frequencies in controls have been compared with those in the total BCC group and, patients with single and multiple carcinomas. Interactions with other relevant factors such as skin type, gender and smoking have also been studied.

The present study confirms that the heterozygote GSTM1 A/B genotype is associated with a reduced risk of multiple BCC (Heagerty et al., 1994). The mechanism for this is unclear but is presumably related to the ability of these enzymes to catalyse the metabolism of a variety of products of oxidative stress formed after exposure to UV and/or constituents of cigarette smoke and other environmental pollutants (Ketterer et al 1993). The finding that protection is associated with GSTM1 A/B but not GSTM1 A or GSTM1 B (largely GSTM1*0 heterozygotes) suggests a gene dosage effect that is specific to multiple BCC but not other skin malignancies such as squamous cell cancer or malignant melanoma. No protective effect for GSTT1 was identified though the genotyping assay used cannot differentiate GSTT1*A/GSTT1*A homozygotes and GSTT1*0/GSTT1*A heterozygotes. It is possible the minority of subjects (about 30%) with two expressed alleles are protected but this effect is diluted by the larger number of GSTT1*0 heterozygotes.

Recent studies showing the interactive effects of GSTM1 and CYP1A1 genotypes suggest the influence of detoxifying enzymes in mediating cancer risk will depend on allelism at other relevant loci (Nakachi et al 1993; Warwick et al 1994). We identified no significant interactions between the putatively poor detoxification genotypes, GSTM1 null, GSTT1 null and CYP2D6 EM but did find significant differences between controls and patients with multiple BCC in the frequency of the combinations GSTM1 A/B with CYP2D6 EM and, GSTM1 A/B with GSTT1 expressers.

The importance of GSTM1 was emphasised by the finding that the frequency of the combination GSTM1 null/skin type I was significantly increased in patients with multiple BCC compared with those with a single tumour. Skin type is an arbitrary and subjective classification of individual response to UV. Our results show GSTM1 null alone is not a significant determinant of development of multiple BCC but the influence of skin type 1 is synergistic, such that in combination they are a significant predisposing factor to multiple BCC possibly because these individuals are relatively less able to cope with the chemical products of UV and/or those of the resulting inflammation.

Significant interactions between GSTM1 null and male gender were also identified. The incidence of non melanoma skin cancer is higher in men than women and Karagas et al (1994) showed that in males with a prior tumour, the risk of a further BCC is 50% greater than in women. We also found a greater proportion of men in the multiple tumour group than in the single BCC or control groups. The mechanism for the observed interactions between GSTM1 null and gender and skin type 1 is unclear. Females may be relatively protected because oestrogens appear to stimulate melanin production both in vivo and in vitro (McLeod et al 1994).

Previous studies have failed to demonstrate an association between smoking and BCC or, smoking and risk of further tumours (Hunter et al 1990; Karagas et al 1994). As the number of controls from whom a reliable smoking history could be obtained was limited, we did not compare the proportions of ever/never smokers in the case groups with those in controls. However, it is noteworthy that the proportion of smokers in our BCC case group was significantly greater ($p<0.0002$) than that found by the Health Promotion Service of the North Staffordshire Hospital during a survey of 1,957 unmatched, local adults (465, 23.9%) questioned during 1993. In agreement with previous findings our data showed smoking alone did not increase the risk of multiple tumours (Karagas et al 1994). We have now shown that smoking does not influence risk of multiple tumours even in combination with putatively poor detoxification genotypes.

A better understanding of factors that predispose to single and multiple BCC will help devise preventative strategies for what is an increasing public health problem. While we identified few factors that influence the development of a single BCC, factors that mediate susceptibility to multiple tumours were found. The importance of GSTM1 has been emphasised, both the protective effect of GSTM1 A/B and the increased risk associated with the combination of skin type 1 and male gender with GSTM1 null. The influence of GSTT1 and CYP2D6 appeared less significant except in combination with GSTM1 A/B. We believe our results are compatible with the view that development of multiple tumours is not merely determined by time but rather, certain patients have a genetically-mediated increased susceptibility (Karagas et al 1994). We also presume that our data have underestimated the differences between patients with single and multiple tumours as some patients with single BCC are likely to eventually develop further tumours. There are no data from British patients though local clinical experience

suggests the frequency of multiple tumours is lower than that found in American studies. The significant interaction between GSTM1 and skin type 1 indicates other polymorphic genes that influence this phenotype such as those determining melanin production are promising candidates.

5.3. GLUTATHIONE S-TRANSFERASE (GSTM1, GSTT1) AND CYTOCHROME P450 (CYP2D6, CYP1A1) POLYMORPHISMS INFLUENCE BCC NUMBERS AND ACCRUAL.

We have described a cross-sectional analysis of the influence of GST and CYP genotypes on BCC numbers and a longitudinal analysis of the effect of these genotypes on tumour accrual. The first study allowed recruitment of large numbers of cases though, because follow-up was variable, the influence of genotypes may be underestimated as further lesions develop in some patients. The expected influence of gender, age and skin type was found indicating the value of this approach. The second study includes the variables shown to influence BCC numbers (Kricker et al., 1993; Karagas and Greenberg, 1995) however, since data on the time of accrual (or lack of accrual) was not always available the number of cases was smaller. The biochemical processes that determine BCC numbers and accrual are largely unclear because the relationship between each lesion and exposure to UV, a tumour initiator and promoter, is unknown. These parameters must reflect complex interactions between an initiating event(s) (UV and/or other factors), the extent of damage to host targets, their repair, immune surveillance and time. Thus, while the genetically-mediated response to, and detoxification of, products of UV-induced oxidative stress will be important in determining numbers and accrual of lesions, some common and some different biochemical activities and polymorphisms may mediate these endpoints. We have confirmed the expected importance of male gender, skin type and, age and number of lesions at presentation (Kricker et al., 1993; Karagas and Greenberg, 1995). Further, the cross-sectional analysis showed CYP1A1 m_1m_1 and CYP2D6 EM are associated with increased numbers of BCC and the longitudinal study, after adjustment for age and tumour number at presentation, that GSTT1 null, CYP2D6 EM and CYP1A1 Ile/Ile were significant.

While there is no apparent association between exposure to environmental pollutants and disease risk, CYP2D6 EM was associated with both increased numbers and accrual. The role of this enzyme is unclear, indeed it has been proposed it is functionless and associations with disease susceptibility the result of its closeness of the locus to the immunoglobin gene complex (Ingelman-Sundberg et al., 1995). Further, its expression is mainly hepatic and though found in brain and intestine, it has not been detected in skin (Raunio et al., 1995). However, the association between CYP2D6 PM and susceptibility to Parkinson's disease suggests in vivo substrates include endogenous neurotoxins and possibly molecules containing amine or guanidino- groups (Smith et al., 1995; Guengerich, 1995). Further, as systemic agents such as arsenic predispose to multiple BCC (Yeh et al., 1968), CYP2D6-mediated hepatic detoxification of photosensitising agents may be important.

Data showing UV-oxidised tryptophan binds to the Ah receptor and UV effects increased enzyme expression in skin suggest a role for CYP1A1 in skin carcinogenesis (Raunio et al., 1995; Rannug et al., 1987; Gonzalez, 1995; Jubert et al., 1994) though studies in Caucasians have generally failed to demonstrate an association between allelism at this locus and disease susceptibility (Cantlay et al., 1995; Hirvonen et al., 1992). This failure may in part result from the uncommonness of allelic variants at this locus relative to the small case groups studied. We found CYP1A1 genotypes were associated with altered risks of both BCC numbers and accrual though m_1m_1 influenced numbers and the Ile/Ile genotype was associated with slower accrual. While these results appear contradictory and difficult to explain given uncertainty regarding the consequences of these mutations it is noteworthy that the extent of altered risk associated with CYP1A1 genotypes was only moderate compared with other genes. If the 3'-mutation is associated with greater inducibility, m_1m_1 may confer less effective

detoxification of an unknown carcinogen. Similarly, it is suggested the Val/Val genotype is associated with greater activity towards carcinogens such as benzo(a)pyrene. However, as argued for GSTT1 (Pemble et al., 1995), the influence of a genotype must depend on causative substrates and it is possible the two CYP1A1 mutations effect different responses on expression of CYP1A1 and other phase 2, Ah-dependent genes (Nebert, 1994).

GSTM1 and GSTT1 had different effects on numbers and accrual of lesions. GSTT1 null did not influence the numbers of lesions possibly because its effect is diluted in a group that includes subjects who accrue tumours quickly, slowly or not at all. We speculate the influence of GSTM1 results from its ability to metabolise the products of UV-induced oxidative stress in skin, a view supported by the finding that GSTM1 null is also associated with increased production of anti-Ro (but not anti-La) antibodies in patients with systemic lupus erythematosus (Strange, 1995). Importantly, production of anti-Ro antibodies in the absence of anti-La antibodies is associated with photosensitivity. Available data is less clear regarding the function of GSTT1. Thus, GSTT1 null but not GSTM1 null is associated with increased risk of astrocytoma and meningioma (Elexpuru-Camiruaga et al., 1995), pathologies not associated with inflammation, as well as total ulcerative colitis (Duncan et al., 1997). These data emphasise current uncertainty of the role of GSTT1 and therefore, the significance of the polymorphism. This appears to depend on substrate; while GSTT1 utilises monohalomethanes and expressers are protected against the sister chromatid exchange induced by these compounds (Pemble et al., 1994), GST5-5, the rat GSTT1 homologue, has dehalogenase activity against dichloromethane giving rise to formaldehyde. Thus, expression of GST5-5 in the Ames test leads to enhanced mutagenicity of dichloromethane (Pemble et al., 1994). It is noteworthy that that those

with the GSTT1 null genotype have lower MED's than GSTT1 expressers (Kerb R et al., 1997). Generally studies in Caucasian lung cancer cases have not identified the interactions between GSTM1 and CYP1A1 found in Japanese (Nakachi et al., 1993; Raunio et al., 1995; Hirvonen et al., 1992; Ingelman-Sundberg et al., 1995), though in Scandinavians, different combinations of GSTM1 genotypes and CYP1A1 inducibility appear to influence lung tumour type (Antilla et al., 1994).

As expected, skin type 1 and male gender were associated with increased numbers of BCC (Kricker et al., 1993; Karagas and Greenberg, 1995). These characteristics are related as women have higher skin reflectance's and, oestrogen's stimulate melanin production (McLeod et al., 1994). While blue eyes have been linked with increased risk of cutaneous malignancy (Vitaga et al., 1990), it has been argued blue and green eyes are not independently related to risk of BCC (BCC (Kricker et al., 1993; Karagas and Greenberg, 1995). We found eye colour was associated with increased numbers of lesions presumably because it is associated with skin complexion.

We have identified genetic traits associated with susceptibility to increased numbers of BCC and, for the first time, that influence tumour accrual. Previous studies have shown the importance of DNA repair (Wei et al, 1994), indeed the influence of GSTM1 may result from its ability to utilise 5'-hydroxymethyluracil (Smith et al., 1995). However, the finding that CYP2D6 and CYP1A1 influence tumour numbers and accrual, while not predictable, is in keeping with the view these genes are susceptibility markers for many cancers. The mechanism of their effect is unclear but may involve detoxification of other, unknown molecules possibly containing amino or guanidino groups, indole or steroid ring structures. Indeed, our finding that ever-smoking is associated with faster

accrual also suggests the importance of carcinogen-metabolising enzymes such as members of the CYP 1 and 2 families.

5.4. POLYMORPHISM AT THE GSTM3 LOCUS: INTERACTIONS WITH CYTOCHROME P450 AND GLUTATHIONE S-TRANSFERASE GENOTYPES AS RISK FACTORS FOR MULTIPLE BCC.

We have described the influence of the recently described polymorphism at GSTM3 on susceptibility to BCC. This is the first assessment of a polymorphism in a mu class gene, other than GSTM1, as a candidate for cancer susceptibility. Our initial, immunohistochemical experiments showed specific expression of the gene in skin from cases and controls. The influence of GSTM3 on susceptibility was studied firstly, by comparing genotype frequencies in controls with those in cases with BCC. Secondly, the frequency of genotypes in patients with 1 tumour was compared with that in patients with 2-35 lesions, a group who appear at increased risk of BCC. Thirdly, a Poisson regression model was used in the cases with 2-35 tumours to identify genotypes and characteristics that influence tumour numbers.

Patients with a BCC are at high risk of suffering further lesions (Kricker et al., 1993; Karagas and Greenberg, 1995). Importantly, this risk depends on the number of tumours present. Thus, in subjects with 1 lesion the 5 year risk is 27%, in those with 10 or more tumours the risk is 90% (Kricker et al., 1993; Karagas and Greenberg, 1995) indicating accrual is not just dependent on time but rather some subjects have an increased susceptibility (Karagas and Greenberg, 1995). Mu class GST are attractive candidates for susceptibility to skin cancers including BCC as recent studies from our laboratory suggest GSTM1 contributes to detoxification of the products of UV-induced oxidative stress. For example, GSTM1 null is associated with anti-Ro (but not anti-La) antibodies in patients with systemic lupus erythematosus (Ollier et al., 1996). If the influence of GSTM1 results from its metabolism of products of UV-induced oxidative

stress, GSTM3 is also a candidate as it utilises lipid peroxides (Hayes and Pulford, 1995). Our finding that GSTM3 is expressed in the epidermis and basal layer is compatible with the view this enzyme is part of local antioxidant defences. Thus, the basal layer includes the stem cells believed to be the targets for UV-induced damage from which BCC arise.

Our analysis did not identify differences in the frequencies of GSTM3 genotypes in the total BCC group and controls or, between cases with 1 tumour and those with 2-35 lesions though it is noteworthy that the difference in the frequency of GSTM3 BB in these two case groups approached significance suggesting this relatively uncommon genotype is protective and worthy of further study. Within the cases with 2-35 tumours, a Poisson regression model was used to study the association between genotypes and number of lesions. This analysis showed GSTM3 AA alone was not associated with an increased number of tumours. However, combinations of GSTM3 AA with skin type 1, GSTM1 null or CYP1A1 m1m1 were significantly associated with increased tumour numbers. Indeed, the rate ratio for the interaction between GSTM3 AA and skin type 1 (2.058) was the highest identified. Presumably individuals with skin type 1 and GSTM3 AA or GSTM1 null are less able to metabolise the products of the oxidative stress associated with UV exposure. The finding of a significant interaction between GSTM3 AA and GSTM1 null compliments studies showing lower levels of immunohistochemical positivity for GSTM3 in the lungs of subjects with GSTM1 null (Antilla et al., 1995) and suggests a similar effect in skin. These data may reflect the linkage of *GSTM3*B* with *GSTM1*A* (Inskip et al., 1995) and the consequent association of *GSTM3*A* with the other GSTM1 alleles, the majority of which will be *GSTM1*0.* Our finding of a significant interaction between CYP1A1 m1m1 and GSTM3 AA provides further support for the view that polymorphism in CYP genes influences susceptibility to multiple BCC

(Heagerty et al., 1996). Thus, CYP1A1 is expressed in skin and is induced by UV exposure (Gonzalez, 1995). The consequences of the 3'-downstream mutation are unclear but may confer increased inducibility suggesting CYP1A1 m1m1 provides less effective detoxification on exposure to carcinogens (Gonzalez, 1995; Nebert, 1994). Our finding of an interaction between CYP1A1 and a mu class GST is compatible with data showing GSTM1 null is associated with high inducibility of CYP1A1 transcription (Vaury et al., 1995). The mechanism for the increased susceptibility to BCC conferred by CYP2D6 EM is also unclear though recent studies indicate the importance of this gene in skin and brain tumours (Heagerty et al., 1996; Lear et al, 1996; Elexpuru-Camiruaga et al., 1995).

Our data suggest GSTM3 AA confers increased risk of multiple BCC. While the mechanism is unclear we speculate the YY1 transcription factor acts as a GSTM3 inducer in skin via the 5'-AAGATA-3' motif in *GSTM3*B*. YY1 expression is altered by many molecules including growth factors (Flanagan, 1995) suggesting *GSTM3*B* homozygotes are better able to induce expression of the gene following UV exposure. Our results provide further evidence for the importance of effective metabolism of potential carcinogens in skin and support the view that factors in addition to UV exposure, are determinants of disease risk.

5.5. TRUNCAL SITE AND DETOXIFYING ENZYME POLYMORPHISMS REDUCE TIME TO PRESENTATION OF NEXT BCC.

We have reported the use of survival analysis techniques to study the influence of GST and CYP genotypes, patient characteristics and tumour site on the time to presentation of next BCC. In view of the high and increasing BCC incidence and, because mortality is low, it is predicted the prevalence of this tumour will be greater than that for all other cancers combined (Boring et al., 1994). Further, patients with a BCC often develop more than one tumour, tumours may recur and they may be unaware of lesions (Koh et al., 1989; Lookingbill, 1988). Follow-up therefore, places considerable demands on clinicians. Data identifying which patients will develop further lesions and at what rate would be useful if it allowed earlier detection of lesions as, in general, smaller tumours are more easily and cost-effectively removed with better cosmetic outcome (Fleming et al., 1995).

We have identified in 856 patients, factors associated with time to presentation of next tumour. As expected, patients with more than one lesion at first presentation demonstrated a decreased median time to next tumour (Kricker et al., 1993; Karagas and Greenberg, 1995). We also report, for the first time, that truncal site at presentation in combination with other factors is associated with a reduced median time to next tumour and a increased proportion of patients suffering a further tumour within 3 and 5 years. Thus, male patients with a truncal tumour at presentation demonstrate a decreased time to next tumour with all cases suffering a further tumour within 5 years. Since consumption of arsenic is a potential confounding factor for truncal tumours, it is noteworthy the proportion of our cases with truncal tumours was similar to that reported in other studies of sporadic BCC (Yeh et al., 1968).

Polymorphic variants associated with a decreased time to next tumour were also identified; thus, GSTT1 null was a significant factor with about two thirds of these cases suffering a further tumour within 5 years compared with only one-third of patients who expressed the gene. GSTM1 and CYP2D6 genotypes alone did not influence subsequent tumour presentation time though significant interactions with truncal site were identified. Thus, cases with a truncal tumour at presentation and GSTM1 null demonstrated a decreased time to next lesion such that all patients with this combination suffered a further tumour within 5 years. We speculate the influence of GSTM1 and GSTT1 results from their ability to metabolise the products of UV-induced oxidative stress in skin. However, unlike GSTM1 null, no interaction between GSTT1 null and tumour site was found, a finding that complements data showing the products of these loci have some different substrate preferences.

While there is no known association between environmental pollutants and risk of BCC, CYP2D6 EM in combination with tumour site was associated with decreased time to presentation of subsequent BCC. The role of this enzyme is unclear and though expressed in liver, brain and intestine, it has not been detected in skin (Rannug et al., 1995). As systemic agents such as arsenic predispose to multiple BCC, CYP2D6-mediated hepatic detoxification of unknown carcinogens may be important. Data showing UV-oxidised tryptophan binds to the CYP1A1 ligand-dependent Ah receptor transcription factor and UV induces CYP1A1 expression in skin suggest a role for allelism at this locus in skin carcinogenesis (Rannug et al., 1995). However, while polymorphism at this locus appears to influence tumour numbers (Lear et al., 1996), the present study shows that neither

the mutation in exon 7 nor that in the 3' region influence time to next tumour presentation.

Why patients with more than one lesion or a truncal tumour at first presentation, or are male, demonstrate a shorter time to next tumour is unclear. Indeed, factors that influence rate of presentation of a further tumour may differ from those that influence susceptibility. Thus, we found GSTT1 null influences accrual but not susceptibility to BCC while GSTM1 null in combination with skin type 1 influences susceptibility to multiple tumours but not tumour accrual (Heagerty et al., 1996; Lear et al., 1996). The susceptibility factors that determine tumour site are unclear though patients who demonstrate lesions on less exposed sites such as trunk, might be considered the most susceptible and consequently likely to suffer further tumours. The finding that males with truncal lesions demonstrated decreased time to next tumour presentation is compatible with data showing males are at increased risk of multiple BCC. Outdoor occupation was not a significant factor in our analysis suggesting cumulative UV-exposure alone is not the explanation.

The biochemical processes that determine rate of appearance of further tumours in BCC are unclear. Our data indicate patients with a truncal tumour at presentation, especially males and those presenting with more than one lesion should receive meticulous and more frequent follow-up to expedite early diagnosis. Our data show inter-individual differences in the efficiency of detoxification reactions also determine susceptibility. Thus, the highly significant influence of GSTM1 null or CYP2D6 EM on time to next tumour presentation suggests a possible use of genetic markers in a follow-up strategy

5.6. TUMOUR SITE IN BCC: INFLUENCE OF GSTT1 AND CYP1A1 GENOTYPES.

We have studied the influence of detoxifying enzyme genotypes and patient characteristics on the development of truncal BCC by comparing these factors in patients with at least one truncal BCC and those with no truncal tumours. Although exposure to UV is a recognised risk factor, our data shows around 20% of patients with BCC develop at least one tumour at sites generally believed to suffer relatively little exposure. The mean age at first presentation of patients with at least one truncal tumour was lower than in those with no truncal tumours. Indeed, in our patients the mean time between appearance and presentation of tumours in the truncal group was not different from the non-truncal group. Although the time of appearance of tumours is difficult to define with accuracy since it depends on patient recollection, these data suggest patients with truncal tumours suffer their first tumour earlier than those with non-truncal BCC. Furthermore, patients whose first tumour is truncal are at increased risk of further tumours. Thus, patients with truncal tumours represent a high risk group. The increased prevalence of males in the truncal compared with the non-truncal tumour group supports our local clinical impression, though this just failed to achieve significance. Outdoor occupation was not a significant factor in our analysis. This suggests males being more likely to have increased chronic UV-exposure, due to a greater proportion with outdoor occupation, is not the explanation for the influence of gender.

Since both age and gender were significant confounding factors, subsequent data on the influence of genotypes and patient characteristics were corrected for imbalances in these factors. Following correction, none of the patient characteristics studied were associated with tumour site. In particular, skin type 1 was not significantly different

between the two groups suggesting patients with truncal tumours are no more likely to burn on UV exposure than patients with non-truncal tumours.

Analysis of the role of detoxifying enzymes in determining tumour site showed GSTT1 and CYP1A1 are important, even after correction for BCC number as well as age and gender. Although the level of significance of these factors was relatively low, the influence of the highly significant interactive term (both GSTT1 null and CYP1A1 Ile/Ile) suggests the effects are real. The data presented here supports the view that GSTT1 null genotypes are associated with faster appearance of further tumours and the presence of truncal tumours. Thus, it appears that this genotype exerts its effect on the rate of BCC appearance because it predisposes to tumours on both chronically and intermittently exposed sites. These data suggest individuals deficient in the ability to repair oxidative stress-induced damage to DNA and/or lipids are genetically predisposed to BCC and are more likely to develop subsequent tumours. Since UV constitutes an oxidative stress, GSTT1 null individuals may be more susceptible to UV-induced BCC following relatively little UV exposure. This may result in an increased number of tumours at a younger age and the development of lesions on intermittently exposed sites such as the trunk. However, unlike GSTT1 null, no GSTM1 null effect was identified, complementing data showing the products of these loci have some differences in substrate specificities (Norpa et al., 1995). Data showing UV-oxidised tryptophan binds to the CYP1A1 ligand-dependent Ah receptor transcription factor and UV induces CYP1A1 expression in skin suggest a role for allelism at this locus in skin carcinogenesis (Gonzalez, 1995). The influence of CYP1A1 Ile/Ile on tumour site shown here is more difficult to interpret than the GSTT1 effect since we have shown that this genotype was associated with slower BCC accrual, though this effect was relatively weak. It is not known whether CYP1A1 is uniformly expressed in skin; UV is known to

induce expression suggesting a differential effect in chronically and intermittently exposed skin is possible.

Though interactions between GSTM1 and CYP1A1 have been identified in mediating risk of lung cancer (Antilla et al., 1994), we believe this is the first report of an interaction between GSTT1 and CYP1A1. Indeed, particularly after correction for age, gender and BCC number, the odds ratio for this effect (3.56) was relatively high. This study presents further evidence that patients with truncal tumours represent a high risk group and that factors other than UV exposure are important in the pathogenesis of these tumours.

5.7. MULTIPLE BCC AND INTERNAL MALIGNANCY

In this small pilot study we found no association of multiple BCC and internal malignancy although there was a link with haematological neoplasms. Such a link was seen previously in MM and SCC (Adami et al., 1995). The mechanisms underlying this association are unclear but UV radiation has been suggested to be a critical factor. Indeed, the increasing incidence of non-Hodgkin's lymphoma has been associated with UV exposure (Bentham, 1996). UVB is associated with inhibition of the development of contact hypersensitivity in human skin, thus causing a degree of immunosuppression which is felt to be a contributing factor to the development of cutaneous malignancy (Yoshikawa and Streilein, 1990). This immunosuppression has been related to tumour necrosis factor alpha polymorphisms in mice (Vincek et al., 1993). It could be hypothesised therefore, that an association may exist between BCC and malignancies associated with immune suppression. Haematological neoplasms, particularly lymphomas, are seen in immunosuppressed individuals (Hoover and Fraumeni, 1973). This may be the mechanism behind the association. Clearly, a larger study with longer follow up is needed to confirm our observation and investigate its aetiology further.

We found no association between multiple BCC and internal malignancy overall. This correlates with two other small studies finding no association (Sandstrom et al., 1984; Moller et al., 1975). This contrasts with a study of 1973 patients in Sweden who found men with BCC have an increased risk of cancer of the lung and thyroid gland, whilst women with BCC had an increased risk of cancer of the uterine cervix (Lindelof et al.,

1991). The reason for these differing results is unclear but local exposure to carcinogens may be important. Arsenic exposure is associated with increased risk of both internal malignancy and BCC (Yeh et al., 1968) and so an association may be seen. There is still a need for a large study in the U.K. addressing this issue. However, it seems likely that any association will be seen in certain specific malignancies, especially those associated with immune suppression or UV exposure. Establishing an association between BCC and haematological malignancy has implications for follow up and in understanding the pathogenesis of this malignancy. Our pilot study suggests a link between BCC and haematological malignancy.

5.8. A COMPARISON OF RISK FACTORS FOR MM, SCC AND BCC IN THE UNITED KINGDOM.

Having previously assessed risk factors for BCC in a case-control study in the U.K. (see chapter 4.1.), we set out in this study to investigate SCC and MM in a case control manner and then to directly compare risk factors between the three skin cancer groups. We have shown that red/blonde hair and social class 1 and 2 are significant risk factors for MM. Outdoor occupation and blue/green eyes approached significance. There is no absolute definition of social class and therefore, all methods of determining this parameter are estimates and are open to criticism. They are also more limited especially with regard to females. We based our assessment on occupation. The association of MM with eye colour, hair colour and social class have all been documented (Breslow and Day, 1980) reflecting the importance of pigmentary factors in MM. In general higher social classes are associated with more highly paid occupations and it has been postulated that the increased risk of MM seen in the higher social classes may be due to more frequent overseas travel with resultant increased intermittent sun exposure (Boyle et al., 1995). Furthermore, skin type 1 has also been shown to be a risk factor but was not significant in our study. The reason for these differences are unclear but emphasises the influence of individual risk factors may differ in different countries, thus indicating the need for evaluation of local data for use in follow up strategies. We also found red/blonde hair to be a risk factor for SCC with outdoor occupation, skin type 1 and blue/green eyes approaching significance. Far fewer studies exist in SCC when compared to MM, but our results are consistent with other studies and support the view that cumulative exposure to sunlight is associated with SCC risk (Marks, 1995). Tobacco use is

associated with SCC of the lip (Cooke et al., 1984; Keller, 1970) but was not significant in our sample of SCC.

Since MM, SCC and BCC are clinically so distinct it might have been expected that differences would exist between them in a direct comparison of risk factors. However, we found few major differences. This may partly reflect sample size. Alternatively, it may reflect the crude, non-specific nature of the risk factors we have assessed, which have a relatively modest effect in our population, suggesting other factors are more important in the pathogenesis of these cancers. The main difference seen between those with MM and those with BCC or SCC was in tumour site distribution: MM was more likely to be on the trunk with odds ratios of 4.89 and 7.14 respectively. This has been documented before. The reason for this is unclear but may reflect differences in the importance of timing, nature and duration of ultraviolet exposure. Thus, intermittent UV exposure of the trunk may be more important in the development of MM than SCC or BCC. Alternatively, an ultraviolet-independent mechanism may be more involved in MM pathogenesis. Larger differences were seen between MM, SCC and BCC in the presence of tumours on the limbs. Why BCC does not occur as commonly on the limbs as SCC and MM is unclear. It has been noted previously (Lindqvist,1979) and may reflect differences in importance of UV exposure. Alternatively, variation in skin tissue structure and composition at different sites may be relevant. These observations merit further investigation. Many studies have grouped SCC and BCC under the title non-melanoma skin cancer and not assessed them individually. The site differences we have shown suggest that these cancers may have different pathogenetic pathways and so in future studies it may be better to assess them separately. In conclusion, we have compared risk factors between MM, SCC and BCC and found few major differences, indicating the non-specific nature of

these parameters. Major, as yet unexplained, variations in tumour site exist between these cancers, suggesting they are discrete cancers which share common risk factors whose individual contribution to risk is small.

5.9. RISK FACTORS IN INDIVIDUALS WITH SKIN CANCERS OF DIFFERENT HISTOLOGICAL TYPES.

Patients with BCC are at increased risk of both SCC (Karagas, 1994) and MM. One study found a relative risk of 17 for the development of MM in patients with a prior BCC (Margoob et al., 1994). Other studies have found relative risks between 2.8 to 6.6 (Lindelof et al., 1991; Holman and Armstrong, 1984). Differences between these rates may be explained by recruitment bias, histological classification and increasing incidence. A relative risk of 6.8 for the development of SCC has been described (Lindelof et al., 1991). Follow-up therefore, places considerable demands on clinicians. As few European studies have focused on those with skin cancers of different histological types, we assessed risk factors in such a group, firstly against controls and secondly compared to those with BCC only (as all our multi-cancer group had a BCC). We found that when compared to controls, those in the multi-cancer group were more likely to be of social class 1 or 2, have blue/green eyes and red/blonde hair and be skin type 1. This is not surprising as we have previously shown all these characteristics to be risk factors for BCC (see chapter 4.1). The influence of eye and hair colour indicates the importance of pigmentary factors in the development of these cancers. There was a significant difference in age between cases and controls which may bias our results. However, many of the parameters we have assessed change little with increasing age (eg gender, eye/hair colour, skin type, tumour site at presentation) suggesting the magnitude of bias to be small. In any case, all analyses were age corrected.

Clearly, it would be useful for follow up strategies to identify those who will develop skin tumours of different histological types, especially life threatening SCC and MM.

We therefore compared risk factors between the multi-cancer group against those with a single BCC. We compared them with single BCC patients for 2 reasons: 95% of our multi-cancer group had a BCC and we have shown previously that differences exist between those with single and multiple BCC (see chapter 4.6). The multi-skin cancer group were older but this is not surprising as they have longer to develop the disease. However, they were more likely to be male (OR 1.64). The reason for this is not clear but may reflect differences in UV exposure or hormonal factors. Patients in the multi-cancer group were also more likely to have truncal BCC at presentation and be skin type 1 than those with a single BCC (OR 3.52 and 2.56 respectively). This suggests that the multi-cancer group burn more on exposure to UV which may be important in cutaneous carcinogenesis. The truncal observation is interesting and correlates with the previous observation that those with truncal BCC are more likely to have multiple lesions. The mechanisms underlying these observations remain to be elucidated. Our results suggest that those patients with male gender, skin type 1 and a truncal BCC are at increased risk of skin cancers of different histological types. This may be of use in follow up strategies.

5.10. THE INFLUENCE OF CYP2D6 AND CYP1A1 POLYMORPHISMS, GENDER, EYE AND HAIR COLOUR ON SUN-REACTIVE SKIN TYPE.

This is the first study to address the role of detoxicating enzyme genotypes in mediating SRST. We have shown, in a large study group, that particularly the cytochrome P450 enzymes CYP1A1 and CYP2D6 are associated with SRST. We have also confirmed the known associations between skin type 1 and blue/green eye colour and blonde/red hair colour, both showing a negative trend from SRST 1 to 4. However, these results must be viewed with a degree of caution. We may have over proportioned our sample with skin type 1 by recruiting patients with skin cancer. Furthermore, possible confounding may have occurred by including patients with skin cancer, as it is known that certain genotypes and SRST 1 are associated with BCC (Heagerty et al., 1996). However, the trends were in the same directions in the non BCC group (although not significant due to the reduction in numbers) suggesting the magnitude of confounding to be small. Also, it is difficult to be certain of the applicability of our results to the mediation of SRST in people without skin disease. Therefore, it would be very useful to confirm our observations in a large cohort of people with no evidence of skin disease, but such a cohort of healthy people would be difficult to recruit.

The interaction with eye and hair colour remains the strongest known association with SRST. The mechanism behind the association is not clear but presumably reflects pigmentary factors which are important influences on the skin's response to UV radiation. The genetics of eye colour are not fully elucidated. Brown eye colour may segregate as a Mendelian dominant against blue or grey (Davenport and Davenport, 1907). Major genes of eye and hair colour have been localised to a region of

chromosome nineteen (Eiberg and Mohr, 1987). It is a possibility that the association between eye/hair colour and SRST may be due to other co-segregating genes. Indeed, mutations in the MSH receptor have recently been described which correlate strongly with skin type 1 and hair colour (Valverde et al., 1996). Mutations identified in the MSH receptor are present in over 75% of individuals with skin type 1 but less than 5% of individuals with skin types 3 and 4. A less strong association was seen with red hair colour. The absence of an association with indoor or outdoor occupation provides further evidence that skin type is predominantly genetically rather than environmentally determined. Some authors have questioned the validity of SRST (Rampen et al., 1988) but we feel, as do others (Fitzpatrick, 1988; Amblard et al., 1982) that it is a simple tool which is an easily identifiable risk factor for cutaneous malignancy, particularly malignant melanoma, and is an important area to study. The association of SRST with gender has been previously described in patients with malignant melanoma (Beral et al., 1983). This observation is difficult to explain but may reflect hormonal effects. We tested whether this observation was due to associations with eye and hair colour, but women were no more likely than men to have blue/green eyes or blonde/red hair. Differences in reporting of burning could exist between men and women (with men being more stoical) but this is difficult to assess in our sample.

It was surprising to find that GSTM1 null had no significant effects on SRST given its influence in anti Ro positive lupus (Ollier et al., 1996). This suggests that different substrates are involved in mediating susceptibility to photosensitivity and SRST 1. CYP1A1 was associated with SRST; the m1m1 genotype being associated with a decreasing trend from SRST4 to SRST1. As discussed earlier, the mutant gene may have increased inducibility. CYP2D6 EM is associated with increased activity of the

enzyme (Gough et al., 1990) leading to higher levels of oxidised intermediates. It approached significance with a decreasing trend from SRST 1 to 4. It is noteworthy that CYP1A1 and CYP2D6 have different substrates. CYP2D6 is not known to be expressed in skin but is found in liver. This suggests metabolism in sites other than skin may be involved in determination of SRST or that this gene is in linkage with another gene which is mediating the effect. It would be of interest to assess the influence of detoxication in other photosensitive disorders such as polymorphic light eruption and also to correlate MED's with detoxifying genotypes of which there is some pilot data (Kerb et al., 1997). The influence of detoxication on SRST is smaller than eye or hair colour. Other genes may be important: mediators of acute inflammation such as tumour necrosis factorα and variants of the melanocyte stimulating hormone receptor are possible candidates.

5.11. Conclusion

There is no as yet identified common precursor to BCC making the study of accumulation of mutations/genetic susceptibility from precursor lesion to cancer unfeasible. However, as we have shown, BCC affords the study of other interesting and clinically useful disease outcome measures such as total BCC number, site and accrual (measured as tumours/year and time to next tumour presentation). Thus, we have illustrated that polymorphism in loci encoding detoxifying enzyme genes affects total BCC number, tumour accrual, tumour site and time to next tumour presentation. Our results indicate that an individual's ability to deal with the products of UV-induced oxidative stress is important in the pathogenesis of this cancer. The mechanism behind this effect is not known but presumably relates to protection from and modification of DNA damage by relevant carcinogens. These enzymes, though apparently not strongly predisposing to BCC in a case-control comparison, may affect the type and number of mutations acquired by relevant tumour suppressor genes, or be important in the development of field cancerisation changes and thus partly account for the variable phenotype seen in BCC patients. In addition, we may not have studied the most influential detoxifying enzyme genes. With the recent identification of a candidate tumour suppressor gene for BCC, *PTC*, these hypotheses will be testable. Furthermore, BCC represents a major burden to health care provision and our results may be of use in the development of follow-up strategies.

BCC is a multifactorial disease which is likely influenced by the interaction of many genes and environmental factors. Other relevant areas would include: 1.) UV exposure; 2.)Immune modulation which may be effected by DNA damage, cytokine

release and inflammation; 3.)Other superoxide scavengers such as the superoxide dismutases; 4.) The level of protection afforded by melanisation; 5.) The DNA repair capacity of the host; 6.) Identification and characterisation of relevant tumour suppressor genes. Further studies to inter-relate our findings with these areas would be of great interest in elucidating BCC pathogenesis. These approaches would also have relevance to the study of other more life-threatening cancers.

5.12. Acknowledgements.

I would like to acknowledge all those listed in the appendix for their help and co-operation during the completion of this thesis. In particular, I would like to thank Professor Dick Strange for his supervision throughout and Dr. Tony Fryer for his invaluable help in the completion of this thesis. Finally, I would like to thank my wife, Mary, for her continuing encouragement during the time it has taken me to complete this work.

CHAPTER 6

6.1. Appendix

Location of Work

Patient Recruitment

Patients and controls were recruited from 3 centres: North Staffordshire NHS Trust, Hartshill Rd., Stoke on Trent, ST4 7PA; Stafford District General Hospital, Stafford and Royal Cornwall Hospitals NHS Trust, Truro, Cornwall. Over 85% were recruited from Stoke on Trent.

Laboratory Work

All assays were performed in the laboratories of the Department of Clinical Biochemistry and Pathology, Central Pathology Laboratory, North Staffordshire NHS Trust, Hartshill Rd., Stoke on Trent, ST4 7PA.

Supervision

Professor R.C. Strange, Professor of Clinical Biochemistry, Keele University.

Collaborations

Patient Recruitment

Drs. Adrian Heagerty and Andrew Smith, Department of Dermatology, North Staffs NHS Trust, Stoke on Trent and Dr. Bill Bowers, Consultant Dermatologist, Royal Cornwall Hospitals NHS Trust, Truro, Cornwall. Over 80% were recruited form Stoke on Trent.

Laboratory Work

Genotype Determination

Julie Alldersea, Janice Gilford, Lilian Yengi and Dr. Tony Fryer, Department of Clinical Biochemistry, North Staffs NHS Trust, Hartshill Rd., Stoke on Trent, ST4 7PA.

GSTM3 Immunostaining

Phillip Hand, Department of Pathology, North Staffs NHS Trust, Hartshill Rd., Stoke on Trent, ST4 7PA.

Statistical Analysis

Professor Peter Jones, Professor of Mathematics, Keele University, Keele, ST5 5BG.

This work describes a collaborative project but a substantial part is original work performed by myself. Before my arrival in Stoke on Trent, a pilot study had been undertaken. I became involved at this stage when it was clear that more details on the previous patients was needed, as well as recruiting new patients and expanding the number of genes to be assessed. I therefore recruited and characterised in great detail a substantial proportion of the patients and controls with respect to individual characteristics and details on tumours. I spent on average 1 day a week in the laboratory in Stoke on Trent becoming familiar with and undertaking some of the molecular genetic assays (under the supervision of Professor Strange and Dr. Fryer), especially in those with multiple tumours. I was involved in preparation of databases for analysis with Dr. Fryer. The statistical strategies were devised by Professor PW Jones and analysis was undertaken together with Dr. Fryer and myself.

Ethical Approval

All samples were collected with informed consent. Ethical approval was granted by the ethics committee of the North Staffordshire NHS Trust, Stoke on Trent.

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Publications Arising From Work In This Thesis

1.**Lear JT**, Heagerty A, Smith A, Bowers B, Jones P, Gilford J, Alldersea J, Fryer A, Strange R. Polymorphism in detoxifying enzymes and susceptibility to skin cancer. **Photochem Photobiol** 1996: 63; 424-8.

2.Heagerty A, Smith A, English J, **Lear JT**, Perkins W, Bowers B, Jones P, Gilford J, Alldersea J, Fryer A, Strange RC. Susceptibility to multiple cutaneous basal cell carcinomas: significant interactions between glutathione-S-transferase GSTM1 genotypes, skin type and male gender. **Br J Cancer** 1996; 73: 44-8

3.**Lear JT**, Tan BB, Smith AG, Jones PW, Fryer AA, Strange RC. Non-Hodgkin's lymphoma and solar ultraviolet radiation (letter). **Br Med J** 1996; 313: 298-9

4.Yengi L, Inskip A, Gilford J, Bailey L, **Lear JT**, Smith A, Heagerty AH, Bowers B, Hand P, Hayes JD, Jones PW, Strange RC, Fryer AA. Polymorphism at the glutathione S-transferase, GSTM3 locus: Interactions with cytochrome P450 and glutathione S-transferase genotypes as risk factors for cutaneous basal cell carcinoma. **Cancer Research** 1996; 56: 1974-7

5.**Lear JT**, Heagerty AHM, Smith A, Bowers B, Rowland-Payne C, Smith CAD, Jones PW, Gilford J, Yengi L, Alldersea J, Fryer AA, Strange RC. Multiple cutaneous basal cell carcinomas: glutathione S-transferase (GSTM1,GSTT1) and cytochrome P450 (CYP2D6, CYP1A1) polymorphisms influence tumour numbers and accrual. **Carcinogenesis** 1996; 17: 1891-6

6.**Lear JT**, Strange RC, Fryer AA. Relationship between sunlight exposure and a key genetic alteration in basal cell carcinoma(letter). **J Natl Cancer Inst** 1997; 89: 454-5

7.**Lear JT**, Smith AG, Bowers B, Heagerty AHM, Jones PW, Gilford J, Alldersea J, Strange RC, Fryer AA. Truncal tumour site is associated with high risk of multiple

cutaneous basal cell carcinoma and is influenced by glutathione S-transferase, GSTT1 and cytochrome P450, CYP1A1 genotypes and their interactions. **J Invest Dermatol** 1997; 108: 519-22

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9.**Lear JT**, Smith AG. Classic diseases revisited - Basal cell carcinoma. **Post Grad Med J** 1997; 73: 538-42

10.**Lear JT**, Heagerty AHM, Smith A, Bowers B, Jones PW, Gilford J, Alldersea J, Strange RC, Fryer AA. Truncal site and detoxifying enzyme polymorphisms significantly reduce time to presentation of next cutaneous basal cell carcinoma. **Carcinogenesis** 1997; 18: 1499-503

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12. Strange RC, **Lear JT**, Fryer AA. Glutathione S-transferase polymorphisms and skin cancer risk. **Chem-Biol Interact** (in press)

13. **Lear JT**, Smith AG, Strange RC, Fryer AA. Patients with truncal basal cell carcinoma (BCC) represent a high risk group (correspondence vignette). **Arch Dermatol** (in press)

14. **Lear JT**, Tan BB, Smith AG, Bowers B, Heagerty AHM, Jones PW, Fryer AA, Strange RC. A comparison of risk factors and tumour site for malignant melanoma, squamous cell carcinoma and basal cell carcinoma in the UK. **Int J Clin Prac** (in press)

Published Abstracts.

1.**Lear JT**, Heagerty A, Smith AG, Perkins W, Bowers B, Jones P, Gilford J, Alldersea J, Fryer A, Strange RC. Detoxifying enzyme genotypes and susceptibility to skin cancer. **Photodermatology, Photoimmunology and Photomedicine** 1996; 12: 755

2.**Lear JT**, Smith AG, Bowers B, Jones PW, Gilford J, Alldersea J, Yengi L, Fryer AA, Strange RC. Cutaneous basal cell carcinoma (BCC): Influence of glutathione S-transferase (GSTM1,GSTT1) and cytochrome P450 (CYP2D6, CYP1A1) on number of primary tumours. **Br J Dermatol** 1996; 134: 572

3.**Lear JT**, Fryer AA, Smith AG, Bowers B, Jones PW, Alldersea J, Gilford J, Strange RC. Detoxifying enzyme genotypes and susceptibility to cutaneous malignancy. **Br J Dermatol** 1996; 135 (supp 47): 24

4.**Lear JT**, Fryer AA, Smith AG, Bowers B, Jones PW, Alldersea J, Yengi L, Gilford J, Strange RC. Cutaneous basal cell carcinoma: Influence of glutathione-S-transferase (GSTM1, GSTT1) and cytochrome P450 (CYP2D6, CYP1A1) on rate of accrual of primary tumours. **Br J Dermatol** 1996; 135 (supp 47): 35

5.**Lear JT**, Smith AG, Bowers W, Jones PW, Fryer AA and Strange RC. Glutathione S-transferase and cytochrome P450 gene polymorphisms, gender and skin type. **Br J Dermatol** 1997; 136: 452

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7.**Lear JT**, Tan BB, Smith AG, Bowers B, Heagerty AHM, Jones PW, Fryer AA, Strange RC. Risk factors for basal cell carcinoma in the UK: a matched case control study in 806 patients. **Br J Dermatol** 1997; 137(supp 50): 43

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10. **Lear JT**, Heagerty AHM, Smith A, Bowers B, Jones PW, Gilford J, Alldersea J, Strange RC, Fryer AA. Truncal site and detoxifying enzyme polymorphisms significantly reduce time to presentation of next cutaneous basal cell carcinoma. **J Invest Dermatol** 1997; 109: 435

11. **Lear JT,** Smith AG, Bowers B, Jones PW, Fryer AA, Strange RC. Polymorphism in detoxifying enzymes and susceptibility to basal cell carcinoma. **Cancer Gene Ther** (in press)

Prizes

Published work from this thesis enabled me to win **"The Wycombe Prize",** in April 1997. This prize is sponsored by the British Association Of Dermatologists and is awarded every two years for an outstanding contribution to dermatology published from a district general hospital in the U.K. The Wycombe prize is judged on the basis of the applicants published work.

Reagents

Lysis buffer

0.32M sucrose	108.5 g/l
10mM Tris-HCl, pH 7.5	1.21 g/l
5mM Magnesium Chloride	1 g/l hexahydrate or
	0.47 g/l anhydrous
1% {v/v} Triton-X100	10 ml/l

They were dissolved in approximately 750 ml distilled water, brought to pH 7.50 with concentrated Hydrochloric Acid and made up to 1 litres with water. The solution was autoclaved (to inactivate nucleases) and stored at 4^0C until use

SE Buffer

750mM Sodium Chloride	21.9g in 500ml
24mM Disodium EDTA (pH8.0)	24ml 0.5M EDTA,pH8.0 in 500ml

0.5M EDTA, pH 8.0

37.2g of Disodium Ethylene Diamine Tetraacetic Acid was dissolved in approx. 150ml of distilled water, adjusted to pH 8.0 by addition of 10M sodium hydroxide (to enable it to dissolve) and stored at room temperature.

SDS/Proteinase K

5% (w/v) SDS (sodium dodecyl sulphate)	12.5g in 250ml
2mg/ml proteinase K	500mg in 250 ml

This was stored at -30^0C in 4ml aliquots.

Chloroform : isoamyl alcohol (24:1 v/v)

chloroform	240ml
isoamyl alcohol	10ml

Sodium Acetate

3M Sodium Acetate, pH 5.2	81.6g trihydrate in 200ml
	49.2g anhydrous in 200ml

This was dissolved in 150ml distilled water, brought to pH 5.2 with glacial acetic acid, made up to 200 ml with distilled water and stored at -30^{0}C.

Loading Buffer

Bromophenol Blue (0.15% w/v)	= 30mg
Xylene Cyanole (0.15% w/v)	= 30mg
Glycerol (30% v/v)	= 6ml
TBE (5x)	= 8ml
Sterile Water	= 6ml

This was filtered through a 0.2um filter before use

5xTBE. The following were dissolved in approx. 800ml of distilled water:

54g	Tris base
27.5g	boric acid
20ml	0.5M EDTA, pH 8.0

This was made up to 1 litre with distilled water and autoclaved before use

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SPECIAL NOTE

THIS ITEM IS BOUND IN SUCH A MANNER AND WHILE EVERY EFFORT HAS BEEN MADE TO REPRODUCE THE CENTRES, FORCE WOULD RESULT IN DAMAGE

ORIGINAL ARTICLES

k factors for basal cell carcinoma in the UK: e-control study in 806 patients

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IARY

cell carcinoma (BCC) is the commonest malignant neoplasm in white people. We present a large UK case–l study in which conditional logistic regression analysis of age-matched and gender-matched data sets was o compare, first, cases with controls (*n*=403) and second, patients having multiple BCC with those having a BCC (*n*=278). Eye/hair colour, occupation, skin type, social class, tumour site at presentation and smoking were assessed.

al class 1/2, skin type 1, red/blonde hair and blue/green eyes were all related to BCC risk, social class most y (odds ratio 2.36, *P*=0.007). Truncal site at presentation was a risk factor for the development of multiple dds ratio 4.03, *P*=0.002).

e data support the view that genetically mediated differences in ultraviolet responsiveness are important in hough the scale of their effect is small. They may be exploitable in primary and secondary prevention as well ng insights into pathogenesis. In particular, the fact that patients presenting with a truncal tumour are at ed risk of further BCC suggests that intermittent exposure in genetically predisposed individuals may ute to a cancer susceptibility syndrome.

DUCTION

h basal cell carcinoma (BCC) is the commonest nt neoplasm in white people, with an incidence of r 100 000 reported in North American men[1], it has d remarkably little research. World wide its ce is increasing at an annual rate of over 10%[2]. gh mortality attributable to BCC is not high, the is responsible for considerable morbidity[3,4] and thus s a growing burden on health care services. Local tion of tissue can be large if not limited by early on and treatment. By studying risk factors for BCC y be able to identify individuals with high sus- lity, with consequent opportunity for primary and ary prevention. However, there have been few ase–control studies of risk factors in BCC. Indeed, investigators have grouped BCC and cutaneous squamous cell carcinoma together as 'non-melanoma skin cancers'.

The aetiology of BCC is still unclear although ultraviolet radiation (UV) seems to be a critical factor in pathogenesis[5]. The relation between UV exposure and risk is complex and may depend partly on genetic factors. Thus, one study in Canada identified outdoor occupation (particularly farming), freckling and Scottish or Irish descent as particular risk factors[6], and a large multicentre south European study identified blonde hair, pale eyes, recreational sun exposure and skin type 1[7,8]. Further, it has been suggested that exposure between ages 0 and 19 is more important than lifetime exposure[9]. The genetic factors mediating susceptibility are not well understood. Specific chromosomal loss has been described[10] and subnormal capacity to repair UV-damaged DNA[11] has been incriminated. Our group has shown that detoxification of the products of UV-induced oxidative stress may be important, with allelism at the glutathione S-transferase GSTM1 and GSTM3 loci influencing susceptibility[12–14]. It is noteworthy that a substantial proportion of patients with BCC develop multiple lesions. In a North American study the overall five year risk of a new tumour was 50%[15]. This risk was related to the number of tumours already present—those with one tumour having a risk of 27%; those with ten or more, a

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risk of 90%. Few investigators have sought to determine whether patients with a single lesion have a different risk factor profile from those with multiple lesions. To address these issues we have conducted a matched case–control study.

PATIENTS AND METHODS

Patients with histologically proven BCC (n=827) were recruited, with ethics committee approval, from general dermatology outpatient clinics in the English Midlands (North Staffordshire Hospital, Stafford District General Hospital) and south of England (Royal Cornwall Hospitals) and were a representative sample of this patient group. Cases were from a mix of urban and rural environments. All patients were carefully examined and interviewed by a trained dermatologist to obtain information on: age; gender; eye and hair colour at age 21; skin type; smoking history (allowing classification as never or ever smokers); age of onset of first BCC; number of BCCs; indoor or outdoor occupation; social class; and site of tumours. Skin type was assigned as in the Fitzpatrick classification[16] and social class (1–5) was determined on the basis of occupation as proposed by the Office of Population Censuses and Surveys, 1980[17]. Site at presentation was classified as head/neck, trunk, upper limbs or lower limbs. Recurrent tumours were excluded from the total number of primary BCC. After the initial interview the patients were seen about every six months. All case notes were examined by JTL and WB to ensure accuracy. Patients with the basal cell naevus syndrome or other malignant disease (internal and cutaneous of a different histological type) were excluded. None of those approached refused to participate. Patients were also questioned regarding ingestion of arsenic-containing tonics and use of drinking water from potentially contaminated wells. No exposed patients were identified.

The control group (n=503), consisting of patients referred to dermatology clinics, were recruited, carefully examined and followed up by the same investigators. Their diagnoses included seborrhoeic warts (n=353) and eczema (n=150). People with any form of malignant disease or a photosensitive dermatosis were excluded from the control group. Again case notes were carefully examined to ensure accuracy of the data. We chose dermatological based controls for two reasons: first, they had been through a similar referral procedure and processing through the clinics as the BCC cases. Secondly, and importantly, they had all been examined by trained dermatologists to exclude the presence of skin cancer. (Given the age and number of controls needed for our study and the high BCC incidence, the presence of undetected skin cancers may be a substantial problem with use of neighbourhood controls.) Cases were then matched for age and gender with controls. Because of differences in age and gender between cases and [illegible] we were able to match only 403 cases to cont[illegible] patients with multiple BCC (2–35 primary tumo[illegible] then age and gender matched to those with a sin[illegible]

Statistical analysis

Since analysis was performed only on the matche[illegible] data, conditional logistic regression was used to [illegible] differences between cases and controls and [illegible] comparison of single and multiple BCC cases. C[illegible] logistic regression was conducted with the Egret [illegible] package (Revision 4.00.950, Statistics and Epid[illegible] Research Association, Seattle, 1993).

RESULTS

BCC case–control study

403 matched pairs were analysed. Table 1 illus[illegible] individual characteristics of the study groups an[illegible] shows differences between them. Red/blonde [illegible] type 1, blue/green eyes and social class 1 an[illegible] significantly associated with BCC, social class h[illegible] greatest odds ratio (2.36). We analysed the social [illegible] for males and females separately and found [illegible] strength of effect between the two genders (odds [illegible] for men and 3.5 for women). Smoking history an[illegible] occupation were not significant. No two-factor in[illegible] were identified.

Table 1 **Characteristics of the basal cell carcinoma and co[illegible]**

	Cases (%)	Control[illegible]
Age	63.0 SD 12.8	63.0 SD[illegible]
Male	165 (59.4)	165 (59.[illegible]
Ever smoked	245 (62.5)	238 (59.[illegible]
Blue/green eyes	289 (74.3)	248 (63.[illegible]
Red/blonde hair	61 (24.3)	60 (16.[illegible]
Skin type 1	57 (14.5)	36 (9.3)
Social class 1 and 2	38 (21.7)	36 (9.9)
Outdoor occupation	24 (12.6)	38 (10.5)

Table 2 **Significant differences between basal cell carcino[illegible] and age and gender matched controls**

	P value	Odds ratio	95% confidence interval
Social class 1 and 2	0.007	2.36	1.26
Skin type 1	0.024	1.66	1.07
Red/blonde hair	0.037	1.61	1.03
Blue/green eyes	0.001	1.71	1.23

parison of patients with single BCC st those with multiple BCC

atients with multiple BCC were age and gender d to those with a single BCC. In the multiple group, dian number of BCCs was 3 (range 2–35). Patients ultiple lesions were more likely to have a truncal r at first presentation (P=0.002, odds ratio 4.03, confidence interval 1.64–9.89). No significant nces were seen between multiple and single BCC ns of smoking history, eye/hair colour, skin type, class or outdoor occupation.

USSION

w of its high and increasing incidence and its low ity rate BCC may be more prevalent than all other s combined[18]. The lifetime risk for a North American orn in 1994 is estimated to be 28–33%[19]. Further, s with a BCC often develop more than one tumour, rs may recur, and patients may be unaware of [20,21]. Follow-up therefore places considerable ds on clinicians. However, there is surprisingly little h into BCC and only one large case–control study l) has previously been conducted in the UK[22]. Ours uch bigger, and we used a matched case–control ch to eliminate the confounding effect of age and r on assessment of risk factors. Our data show that class 1 and 2, skin type 1, red/blonde hair and blue/ eyes are all risk factors for development of BCC. class had the strongest effect with an odds ratio of Other studies in smaller groups and/or non-UK tions have identified age[23], red hair[7,8,24] blue/green skin type 1[7,8,24], freckling[24] and outdoor occupa- as risk factors.

e observation that higher social classes are at greater BCC has not been reported in the UK before. There absolute definition of social class (we based our nent on occupation), and all classification methods are tes and open to criticism. They are more limited ally with regard to females, which is why we matched nder. It is noteworthy that the social class effect was ilar strength in males and females. In general, higher classes are associated with highly paid occupations e postulate that the increased risk of BCC may be due re frequent overseas travel with resultant increased ittent sun exposure. This hypothesis has also been ed to explain the association of malignant melanoma higher social class[25]. However, another possible esis is that higher social class is associated with seeking behaviour which leads in turn to increased ting of BCC. This hypothesis is supported by the g that low socio-economic status and infrequent ian visits are associated with very large lesions[26].

The relations of skin type 1, red hair and blue/green eyes with BCC have been observed previously although not consistently. Reasons for this may be ethnic differences between studies. In our study outdoor occupation was not a factor; thus, intermittent recreational exposure may be more important than chronic ultraviolet exposure. Recent research supports this notion[7,8,27].

Identification of risk factors allows identification of susceptible individuals and provides insights into pathogenesis. It also helps us target preventive measures to those most at risk. In BCC, susceptibility is complex and a substantial proportion of patients acquire further primary lesions. This has major implications for follow-up and we need to identify the groups at risk. A study in the USA indicated that age over 60 years and more than one tumour at presentation were associated with greater risk of further BCC development[15]. Our data show that patients with a truncal tumour at first presentation are at increased risk of having more than one lesion, with an odds ratio of 4.03. Clearly, in the UK, the trunk is least likely to be chronically exposed to UV and it could be hypothesized that, in a UV-mediated disease, those with a BCC on the trunk are most susceptible and therefore likely to develop multiple lesions. These data also support the view that intermittent exposure is more important than chronic exposure in determining susceptibility. Alternatively, UV-independent mechanisms predisposing to truncal and multiple lesions may be involved. Systemic arsenic ingestion increases risk of BCC[28], so we enquired about possible arsenic exposure (some patients, of course, may have been exposed without their knowledge). It is noteworthy that the proportion of patients in our study with truncal BCC (10.2%) is similar to that reported in other studies on sporadic BCC. Our data suggest that those with truncal BCC are at increased risk of further tumours and patients should be informed of this. If close follow-up yielded earlier detection of tumours, patients with truncal lesions would benefit since, in general, smaller lesions are easier to treat, with better cosmetic outcome.

The associations identified in this report suggest that BCC results from an interaction between environmental exposure, including UV, and genetic factors (skin type, eye/hair colour). These observations are consistent with our previous studies identifying the importance of genetic variation in the ability to detoxify UV-induced reactive oxygen species, in mediating susceptibility to BCC[12] and in development of multiple lesions[13,14,29].

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Susceptibility to multiple cutaneous basal cell carcinomas: significant interactions between glutathione *S*-transferase GSTM1 genotypes, skin type and male gender

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Summary The factors that determine development of single and multiple primary cutaneous basal cell carcinomas (BCCs) are unclear. We describe a case-control study firstly, to examine the influence of allelism at the glutathione *S*-transferase GSTM1 and GSTT1 and cytochrome P450 CYP2D6 loci on susceptibility to these tumours and, secondly, to identify interactions between genotypes and relevant individual characteristics, such as skin type and gender. Frequency distributions for GSTM1 genotypes in cases and controls were not different, although the frequency of GSTM1 A/B was significantly lower ($P = 0.048$) in the multiple BCCs than in controls. We found no significant differences in the frequencies of GSTT1 and CYP2D6 genotypes in cases and controls. Interactions between genotypes were studied by comparing multinomial frequency distributions in mutually exclusive groups. These identified no differences between cases and controls for combinations of the putatively high risk GSTM1 null, GSTT1 null, CYP2D6 EM genotypes. Interactions between GSTM1 A/B and the CYP2D6 PM and GSTT1-positive genotypes were also not different. Frequency distributions of GSTM1 A/B with CYP2D6 EM in controls and multiple BCCs were significantly different ($P = 0.033$). The proportion of males in the multiple BCC group (61.3%) was greater than in controls (47.0%) and single BCC (52.2%), and the frequency of the combination GSTM1 null/male gender was significantly greater in patients with multiple tumours ($P = 0.002$). Frequency distributions of GSTM1 null/skin type 1 were also significantly different ($P = 0.029$) and the proportion of subjects who were GSTM1 null with skin type 1 was greater ($P = 0.009$) in the multiple BCC group. We examined the data for interactions between GSTM1 null/skin type 1/male gender by comparing frequency distributions of these factors in the single and multiple BCC groups. The distributions were almost significantly different (exact $P = 0.051$). No significant interactions between GSTT1 null or CYP2D6 EM and skin type 1 were identified. Comparisons of frequency distributions of smoking with the GSTM1 null, GSTT1 null and CYP2D6 EM genotypes identified no differences between patients with single and multiple tumours.

Keywords: basal cell carcinoma; allelism; GSTM1; GSTT1; CYP2D6; susceptibility

Basal cell carcinoma of skin (BCC) is the commonest malignancy in Caucasians and its incidence is increasing worldwide. Indeed, increases of over 10% per year are reported (Kricker *et al.*, 1993). Ultraviolet radiation (UV) is a critical causative factor, although the relationship between disease risk and exposure is complex. Thus, comparison of the distributions of BCCs and squamous cell cancers (SCCs) shows that BCCs are more prevalent on the trunk, a site usually only intermittently exposed, while SCCs are most common on the more exposed head and neck (Weinstock, 1994; Karagas and Greenberg, 1995).

Patients with a BCC are at high risk of suffering a further primary lesion, with studies in American patients showing that the crude 5 year risk of a new tumour is 50% (Karagas *et al.*, 1994). Importantly, this figure depends on the number of tumours already present; in subjects with one tumour the 5 year risk is 27%; in those with ten or more tumours the risk is 90%. These findings suggest that some subjects are at an inherently higher risk of this malignancy (Karagas *et al.*, 1994). However, while inherited factors are important in Gorlin's syndrome (Farndon *et al.*, 1992), the role of predisposing or protective genes in the genesis of sporadic, multiple BCC is less clear. Karagas *et al.* (1994) showed that the risk of further lesions increases with age, male gender and intermittent high exposures to UV. Susceptibility is also related to individual response to sunlight. Thus, subjects who readily burn and do not tan (skin type I; Fitzpatrick *et al.*, 1988) are at greater risk than those who tan easily and never burn (skin type IV) (Karagas *et al.*, 1994). Other factors that influence susceptibility to multiple tumours include the effectiveness of repair of damaged DNA. Thus, Wei *et al.* (1994) showed that a reduced capacity to repair a UV-damaged reporter gene is associated with an increased risk of multiple BCC. We have shown that allelism at the glutathione *S*-transferase GSTM1 locus also influences susceptibility to multiple BCC (Heagerty *et al.*, 1994). The mechanism for this effect is unclear as these enzymes metabolise a variety of potential carcinogens, including lipid and DNA products of UV-induced oxidative stress (e.g. DNA hydroperoxide). Indeed, their ability to catalyse the detoxification of 5-hydroxymethyluracil suggests a role in the repair of DNA damaged by oxidative stress (Ketterer *et al.*, 1993). GSTM1 enzymes also catalyse the metabolism of epoxides formed from pollutants such as polycyclic aromatic hydrocarbons (Ketterer *et al.*, 1993). GSTM1 genotypes result from combinations of *GSTM1*0*, *GSTM1*A* and *GSTM1*B*. *GSTM1*0* is deleted, suggesting that homozygotes will be more susceptible to inflammatory and/or malignant pathologies. There is evidence from studies in multiple skin cancers of different histological types and BCCs (Heagerty *et al.*, 1994) as well as other cancers such as lung, to support this view (Seidegard *et al.*, 1988; Nakachi *et al.*, 1993; Strange, 1993).

The polymorphic, theta class, glutathione *S*-transferase gene, GSTT1, also catalyses the metabolism of oxidised lipid and DNA as well as epoxides (Ketterer *et al.*, 1993). Homozygotes for deleted *GSTT1*0* constitute 17% of Caucasians and, while the consequences are unclear (Warwick *et al.*, 1994), comparison with GSTM1 null suggests

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t the genotype will influence susceptibility to ROS-uced damage. In particular, individuals null at both loci y be especially susceptible to oxidative or chemical stress. art from UV, skin is exposed to chemical carcinogens ose metabolism depends on the cytochrome P450 super-e family (CYP) (Jugert *et al.*, 1994). The products of ny CYP-catalysed reactions are substrates for GSTM1 l GSTT1, indicating the need for coordinated expression these genes to prevent accumulation of carcinogenic reac-oxygenated intermediates. While epidemiological surveys e not identified a link between BCCs and hydrocarbon osure, CYP genes may be relevant in mediating suscep-lity to skin cancers, as Wolf *et al.* (1992) found increased quency of CYP2D6 mutant alleles in patients with malig-t melanoma.

We describe a case–control study to determine the vance of GSTM1, GSTT1 and CYP2D6 genotypes in diating susceptibility to single and multiple BCC. As the cts of genotypes may be influenced by sex, skin type, eye our and smoking, interactions between genotypes and se factors have been studied.

terials and methods

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total of 737 unrelated Caucasian patients with his-gically proven BCC were recruited between November l and December 1994 from dermatology out-patient ics in the Midlands and South of England; Staffordshire rth Staffordshire Hospital, Stafford General Hospital), rnwall (Royal Cornwall Hospitals) and Hampshire (Royal th Hants Hospital). A total of 481 patients (52.2% males, an age 67 years) suffered a single tumour and 256 patients 3% males, mean age 70 years) more than one tumour tween 2 and 50 tumours per patient). Original hair colour, colour and skin type (types 1–5) (Fitzpatrick *et al.*, 1988) e recorded at sample collection. A smoking history was obtained allowing subjects to be classified as current okers, ex-smokers or never smokers. Four hundred and ty-five of these patients constitute the BCC case group cribed by Heagerty *et al.* (1994). Data on GSTM1 otype frequencies in these subjects were included in the sent study. Genotype frequencies in the original case up and the further 302 subjects recruited between October 3 and December 1994 were not different. A control group nprising 563 British Caucasians (47.0% males, mean age years) from these centres, who were without clinical or ological evidence of any malignancy, was also recruited. se hospital in- and out-patients suffered a variety of n-malignant diseases including varicose veins, hernias, morrhoids, mild iron deficiency anaemia, mild hyper-daemia, benign ovarian cysts (about 30% in total), ten-n headaches (~25%), benign skin papillomas (~20%), ign breast lumps (~5%) and cerebrovascular accidents 20%). Patients suffering inflammatory pathologies such as rative colitis, diabetes or asthma or receiving blood trans-ions within 3 months of blood sampling were excluded. ta on hair colour, eye colour, skin type and smoking ory was not available from all controls. Blood (5 ml) was en, with appropriate ethics committee approval, into TA and stored at −50°C.

ntification of GSTT1, CYP2D6 and GSTM1 genotypes in ocyte DNA

GSTM1 null, A, B and A/B genotypes were identified ng an amplification refractory mutation system (ARMS)-ed polymerase chain reaction (PCR) approach with ner sets to intron 6/exon 7 and exon 4/exon 5. The assay ntifies *GSTM1*0* homozygotes and *GSTM1*A/GSTM1*B* erozygotes and subjects with the GSTM1 A and GSTM1 B notypes. It does not distinguish the *GSTM1*0/GSTM1*A* l *GSTM1*A/GSTM1*A* genotypes or the equivalent GSTM1 B genotypes (Fryer *et al.*, 1993). GSTT1 null and expressing subjects were identified by PCR using the primer set and reaction conditions described by Pemble *et al.* (1994) and Warwick *et al.* (1994). The two mutant CYP2D6 alleles (G→A transition at intron 3/exon 4 and base pair deletion in exon 5) were identified (Gough *et al.*, 1990; Wolf *et al.*, 1992). Together these assays are about 90% predictive of phenotype (Wolf *et al.*, 1992).

Statistical analysis

χ^2 tests were used to examine for homogeneity between cases and controls. Since some genotype frequencies were small, the StatXact-Turbo statistical package was used to obtain exact *P*-values. As various factors (CYP2D6 EM, GSTT1 null, GSTM1 null, skin type, gender etc.) were studied, the influence on susceptibility of each (alone and in combination in pairs and triplets) was studied by comparing frequency distributions over the resulting mutually exclusive categories. The advantage of this approach is that it allows identification of those factors (alone and in combination) that contribute most to observed differences between cases and controls. *P*-values for the main comparisons (GSTM1, skin type 1, gender) were not adjusted for multiple comparisons as they were sufficiently small to remain significant if adjusted using the Bonferroni correction.

Results

Genotype frequencies in cases and controls

Table I shows the frequencies of GSTM1 genotypes in controls, the total BCC group and, patients with single and multiple BCC. The frequencies of the null, A and B genotypes were not different though the frequency of GSTM1 A/B was significantly lower in the multiple BCC than in the controls (odds ratio 0.29, 95% CI 0.055–0.098) confirming previous results in 435 of these patients (Heagerty *et al.*, 1994).

We found no differences in the frequencies of GSTT1 genotypes in controls and the BCC case groups (Table I). The frequencies of the CYP2D6 EM and HET genotypes in controls and case groups were also not different though the difference between the frequency of the PM genotype in controls and single BCC cases approached significance (Table I).

Interactions between GSTM1, GSTT1 and CYP2D6 genotypes

Interactions between genotypes were studied by comparing multinomial frequency distributions in mutually exclusive groups. Comparison of the frequency distributions for combinations of the putatively high risk GSTM1 null, GSTT1 null, CYP2D6 EM genotypes (i.e. GSTT1 null/GSTM1 null/CYP2D6 EM and GSTT1 null/GSTM1 null) showed no significant differences between the controls, patients in the total, single and multiple BCC groups (data not shown).

Corresponding interactions between GSTM1 A/B and the putatively protective CYP2D6 PM and GSTT1 positive genotypes were also examined. Thus, multinomial frequency distributions for combinations of GSTT1 expressers/GSTM1 A/B and, CYP2D6 PM/GSTM1 A/B in patients with single and multiple BCC and, controls and patients with multiple tumours were not significantly different (data not shown). The differences between frequency distributions of the three genotypes combined (CYP2D6 PM/GSTT1 expressers/GSTM1 A/B) in the multiple BCC and single BCC cases and, multiple BCC and controls approached significance ($\chi^2_6 = 11.24$, exact $P = 0.055$ and $\chi^2_5 = 10.06$, exact $P = 0.067$ respectively). These differences largely resulted from differences in the proportion of subjects with the combination GSTT1 positive/GSTM1 A/B; thus, the frequency of this combination was significantly lower ($\chi^2_1 = 6.83$, exact $P = 0.011$) in the multiple BCC group than in controls (data not

Table I CYP2D6, GSTM1 and GSTT1 genotype frequencies in patients with single and multiple basal cell carcinomas of skin

GSTM1 genotypes				
	GSTM1 null (%)	GSTM1 A (%)	GSTM1 B (%)	GSTM1 A/B (%)
Total BCC ($n = 699$)	376 (53.8)	197 (28.2)	108 (15.5)	18 (2.6)
Single BCC ($n = 454$)	236 (52.0)	128 (28.2)	75 (16.5)	15 (3.3)
Multiple BCC ($n = 245$)	140 (57.1)	69 (28.2)	33 (13.5)	3 (1.2)
Controls ($n = 561$)	306 (54.5)	158 (28.2)	74 (13.2)	23 (4.1)[a]
GSTT1 genotypes				
	GSTT1 null (%)	GSTT1 positive (%)		
Total BCC ($n = 584$)	97 (16.6)	487 (83.4)		
Single BCC ($n = 384$)	57 (14.8)	327 (85.2)		
Multiple BCC ($n = 200$)	40 (20.0)	160 (80.0)		
Controls ($n = 484$)	90 (18.6)	394 (81.4)		
CYP2D6 genotypes				
	EM (%)	HET (%)	PM (%)	
Total BCC ($n = 599$)	375 (62.6)	181 (30.2)	43 (7.2)	
Single BCC ($n = 396$)	243 (61.4)	121 (30.6)	32 (8.1)	
Multiple BCC ($n = 203$)	132 (65.0)	60 (29.6)	11 (5.4)	
Controls ($n = 310$)	194 (62.6)	99 (31.9)	17 (5.5)[b]	

[a] Frequency GSTM1 A/B in controls and multiple BCC $\chi^2_1 = 4.52$; exact $P = 0.048$. [b] Frequency CYP2D6 PM in controls and single BCC $\chi^2_1 = 3.57$, Yates corrected $P = 0.059$.

Table II Multinomial frequency distributions of GSTM1 A/B and CYP2D6 EM

	Controls (%)	*Single BCC (%)*	*Multiple* BCC (%)*
GSTM1 A/B + CYP2D6 EM	13 (3.4)	8 (2.0)	1 (0.5)**
GSTM1 A/B only	7 (1.8)	5 (1.3)	0 (0)
CYP2D6 EM only	235 (61.7)	235 (59.5)	131 (64.5)
Neither	126 (33.1)	147 (37.2)	71 (35.0)
Total	381 (100)	395 (100)	203 (100)

*Frequency distributions in controls and multiple BCC; $\chi^2_3 = 8.75$, $P = 0.033$. **Frequency GSTM1 A/B + CYP2D6 EM in controls and multiple BCC; $\chi^2_1 = 4.82$, exact $P = 0.042$.

Table III Interactions between male gender and GSTM1 null

	Controls (%)	*Single BCC (%)*	*Multiple* BCC (%)*	*Total BCC (%)*
GSTM1 null + male	79 (31.7)	115 (25.9)	90 (37.5)**	205 (30.0)
Male only	51 (20.5)	117 (26.4)	57 (23.8)	174 (25.4)
GSTM1 null only	62 (24.9)	114 (25.7)	47 (19.6)	161 (23.5)
Neither	57 (22.9)	98 (22.1)	46 (19.1)	144 (21.1)
Total	249 (100)	444 (100)	240 (100)	684 (100)

*Frequency distributions in single and multiple BCC; $\chi^2_3 = 10.49$, $P = 0.015$. **Frequency GSTM1 null + ♂ in single and multiple BCC; $\chi^2_1 = 9.44$, Yates corrected $P = 0.002$.

shown). Frequency distributions of GSTM1 A/B/CYP2D6 EM in controls and multiple BCC were significantly different (Table II). This difference largely resulted from the reduced frequency of subjects with CYP2D6 EM/GSTM1 A/B in the multiple BCC group compared with controls (Table II).

Interactions between gender and GSTM1, GSTT1 and CYP2D6 genotypes

The proportion of males in the multiple BCC group (61.3%) was significantly greater than in controls (47.0%) ($\chi^2_1 = 11.85$, Yates corrected $P = 0.0006$) and single BCC (52.2%) ($\chi^2_1 = 5.29$, Yates corrected $P = 0.0214$, odds ratio 1.45, 95% CI 1.05–2.00). Interactions between GSTM1 null and male gender were examined by comparing multinomial frequency distributions (Table III); distributions in single and multiple BCC were significantly different and, the frequency of the combination GSTM1 null/male gender was significantly greater in patients with multiple tumours (odds ratio 1.72, 95% CI 1.21–2.44).

Interactions between patient characteristics and genotypes

The proportions of patients in the single and multiple BCC groups with brown, blue or green eyes were not significantly different (data not shown).

Frequency distributions of skin types 1–5 in the single and multiple BCC cases were also not significantly different (Table IV-VI). Considering skin type in terms of no pigmentation (type 1) and variable protection to UV (types 2–5), we compared multinomial frequency distributions of GSTM1 null with skin type 1 in the patients with single and multiple BCC. The proportion of subjects with these factors was significantly greater in the multiple BCC group than in those with a single BCC (Table IV-VI). Thus, the frequency distributions of GSTM1 null/skin type 1 were significantly different and, the proportion of subjects who were GSTM1 null with skin type 1 was significantly greater (Table IV, odds ratio 3.25, 95% CI 1.30–8.27) in the multiple BCC group. We examined the data for interactions between GSTM1 null/skin type 1/male gender by comparing multinomial frequency distributions of these factors in the single and multiple BCC groups. The distributions were also significantly different (Table IV-VI). No significant interactions between GSTT1 null or CYP2D6 EM and skin type were identified (data not shown).

Interactions between smoking and genotypes

The proportion of cases who were current smokers or ex-smokers was not significantly different in the single and multiple BCC groups. Comparisons of multinomial frequ

Table IV Skin type frequencies in single and multiple BCC

Skin type	*1*	*2*	*3*	*4*	*5*
Single BCC ($n = 302$)	34 (11.3%)	94 (31.1%)	98 (32.5%)	59 (19.5%)	17 (5.6%)
Multiple BCC ($n = 162$)	26 (16.0%)	50 (30.9%)	54 (33.3%)	27 (16.7%)	5 (3.1%)

Table V Frequency distributions of GSTM1 null and skin type 1

	Controls (%)	*Single BCC[a] (%)*	*Multiple BCC (%)*
GSTM1 null + type 1	2 (4.4)	9 (3.3)	15 (10.0)[b]
Type 1 only	0 (0)	20 (7.4)	7 (4.7)
GSTM1 null only	24 (53.0)	131 (48.2)	66 (44.0)
Neither	19 (42.2)	112 (41.2)	62 (41.3)
Total	45 (100)	272 (100)	150 (100)

[a]Frequency distributions in single and multiple BCC; $\chi^2_3 = 9.06$; $P = 0.0285$.
[b] Frequency GSTM1 null + skin type 1 in single *v* multiple BCC; $\chi^2_1 = 6.87$; $P = 0.009$.

Table VI Interactions between GSTM1 null, skin type 1 and male gender

	Single BCC[a] (%)	*Multiple BCC (%)*
GSTM1 null + type 1 + male	5 (1.9)	7 (4.8)
GSTM1 null + type 1 only	5 (1.9)	7 (4.8)
Type 1 + male only	10 (3.7)	3 (2.0)
GSTM1 null + male only	60 (22.2)	44 (29.9)
Male only	63 (23.3)	34 (23.1)
Skin type 1 only	10 (3.7)	4 (2.7)
GSTM1 null only	69 (25.6)	22 (15.0)
None	48 (17.8)	26 (17.7)

[a] Frequency distributions in single and multiple BCC; $\chi^2_7 = 13.88$, exact $P = 0.051$.

tributions of smoking with each of the GSTM1 null, TT1 null and CYP2D6 EM genotypes identified no diff-nces between patients with single and multiple tumours ta not shown).

cussion

e role of factors other than UV in the pathogenesis of C is evident from work using a variety of experimental proaches (Heagerty *et al.*, 1994; Karagas *et al.*, 1994; Wei *al.*, 1994; McHenry *et al.*, 1995). We have described fur-r studies on the influence of allelism at loci encoding ase I and II detoxifying enzymes on susceptibility to this re-our. Genotype frequencies in controls have been com-ted with those in the total BCC group and patients with gle and multiple carcinomas. Interactions with other vant factors such as skin type, gender and smoking have tip been studied.

The present study confirms, in a substantially larger nt group, previous work from this laboratory showing dis heterozygote GSTM1 A/B genotype is associated with a duced risk of multiple BCC (Heagerty *et al.*, 1994). The chanism for this protective effect against multiple BCC is -V lear but is presumably related to the ability of these C ymes to catalyse the metabolism of a variety of products ve oxidative stress formed after exposure to UV and/or cons-ulents of cigarette smoke and other environmental poll-ngnts (Ketterer *et al.*, 1993). The finding that protection is no ciated with GSTM1 A/B but not GSTM1 A or GSTM1 B rat gely *GSTM1*0* heterozygotes) suggests a gene dosage pe ct that is specific to multiple BCC but not other skin lignancies such as squamous cell cancer or malignant lanoma. No protective effect for GSTT1 was identified, hough the genotyping assay used cannot differentiate *TT1*A/GSTT1*A* homozygotes and *GSTT1*0/GSTT1*A* ev erozygotes. It is possible the minority of subjects (about an %) with two expressed alleles are protected but this effect nc diluted by the larger number of *GSTT1*0* heterozygotes.

We also found no differences in frequency distributions of CYP2D6 genotypes in the cases and controls though the frequency of CYP2D6 PM was greater in patients with single BCC than in controls.

Recent studies showing the interactive effects of GSTM1 and CYP1A1 genotypes suggest that the influence of detoxifying enzymes in mediating cancer risk will depend on allelism at other relevant loci (Nakachi *et al.*, 1993; Warwick *et al.*, 1994). We identified no significant interactions between the putatively poor detoxification genotypes, GSTM1 null, GSTT1 null and CYP2D6 EM but did find significant differences between controls and patients with multiple BCC in the frequency of the combinations GSTM1 A/B with CYP2D6 EM and, GSTM1 A/B with GSTT1 expressers.

The importance of GSTM1 was emphasised by the finding that the frequency of the combination GSTM1 null/skin type I was significantly increased in patients with multiple BCC compared with those with a single tumour. Skin type is an arbitrary and subjective classification of individual response to UV. The classification of skin type 1 defines an extreme sensitivity to UV, which results in an inflammatory response but no pigmentary response (Fitzpatrick *et al.*, 1988). Our results show GSTM1 null alone is not a significant determinant of development of multiple BCC but the influence of skin type 1 is synergistic, such that in combination they are a significant predisposing factor to multiple BCC, possibly because these individuals are relatively less able to cope with the chemical products of UV or those of the resulting inflammation.

Significant interactions between GSTM1 null and male gender were also identified. The incidence of non-melanoma skin cancer is higher in men than women and Karagas *et al.* (1994) showed that in males with a prior tumour, the risk of a further BCC is 50% greater than in women. We also found a greater proportion of men in the multiple tumour group than in the single BCC or control groups. The mechanism for the observed interactions between GSTM1 null and gender and skin type 1 is unclear. Females may be relatively protected because oestrogens appear to stimulate melanin production both *in vivo* and *in vitro* (McLeod *et al.*, 1994).

Previous studies have failed to demonstrate an association between smoking and BCC or, smoking and risk of further tumours (Hunter *et al.*, 1990; Karagas *et al.*, 1994). As the number of controls from whom a reliable smoking history could be obtained was limited, we did not compare the proportions of ever/never smokers in the case groups with those in controls. However, it is noteworthy that the proportion of smokers in our BCC case group was significantly greater ($P<0.0002$) than that found by the Health Promotion Service of the North Staffordshire Hospital during a survey of 1957 unmatched, local adults (465, 23.9%) questioned during 1993. In agreement with previous findings our data showed that smoking alone did not increase the risk of multiple tumours (Karagas *et al.*, 1994). We have now shown that smoking does not influence risk of multiple tumours even in combination with putatively poor detoxification genotypes.

A better understanding of factors that predispose to single and multiple BCC will help devise preventative strategies for what is an increasing public health problem. While we identified few factors that influence the development of a single BCC, factors that mediate susceptibility to multiple tumours were found. The importance of GSTM1 has been emphasised, both the protective effect of GSTM1 A/B and the increased risk associated with the combination of skin type 1 and male gender with GSTM1 null. The influence of GSTT1 and CYP2D6 appeared to be less significant except in combination with GSTM1 A/B. We believe that our results are compatible with the view that development of multiple tumours is not merely determined by time but rather, certain patients have a genetically mediated increased susceptibility (Karagas *et al.*, 1994). We also presume that our data have underestimated the differences between patients with single and multiple tumours as some patients with single BCC are likely to eventually develop further tumours. There are no data from British patients, although local clinical experience suggests that the frequency of multiple tumours is lower than that found in American studies. The significant interaction between GSTM1 and skin type 1 indicates that other polymorphic genes that influence this phenotype, such as those determining melanin production and the immune response, are promising candidates.

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ultiple cutaneous basal cell carcinomas: glutathione transferase (GSTM1, GSTT1) and cytochrome P450 (CYP2D6, YP1A1) polymorphisms influence tumour numbers and accrual

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t genetic factors that mediate the pathogenesis of ltiple primary cutaneous basal cell carcinomas (BCC) largely unclear. Thus, some patients suffer many BCC 0) and/or rapid accrual (number of tumours/year from t presentation) of further lesions. We have studied, in English Caucasians, the influence of polymorphism carcinogen-metabolizing enzymes on susceptibility to cancer. Accordingly, we describe, first, a cross-sectional lysis of the influence of GSTM1, GSTT1, CYP2D6 and P1A1 genotypes on tumour numbers, and secondly, a gitudinal analysis, in 169 of these cases, of the effect of e genes on tumour accrual. We have confirmed the ected importance of age and number of lesions at sentation, and male gender and skin type as risk ors. Furthermore, the cross-sectional analysis showed P1A1 m_1m_1 ($P = 0.004$; rate ratio 1.242) and CYP2D6 l ($P < 0.001$, rate ratio 1.266) are associated with reased numbers of BCC. The longitudinal study showed, r adjustment for age and tumour number at presenta- , that GSTT1 null ($P < 0.001$, rate ratio 2.677) and P2D6 EM ($P < 0.001$, rate ratio 2.154) were significant rminants of accrual while CYP1A1 Ile/Ile was asso- ed with slower accrual than the Ile/Val and Val/Val otypes ($P = 0.008$, rate ratio 0.690). We believe these the first genetic factors to be associated with tumour rual. No significant interactions between genotypes were ntified, though the combinations GSTM1 null/skin type $P < 0.001$, rate ratio 2.702), CYP2D6 EM/male gender = 0.049, rate ratio 1.279) and CYP2D6 EM/blue+green s ($P = 0.046$, rate ratio 1.388) influenced tumour nbers. Previous studies indicate the importance of ctive repair of UV-damaged DNA in the pathogenesis multiple BCC; indeed the influence of GSTM1 may lt from its ability to utilize 5′-hydroxymethyluracil. wever, the finding that CYP2D6 and CYP1A1 influence our numbers and accrual indicates detoxification of nown molecules is important and supports the view that factors other than UV are important in the pathogenesis of BCC.

breviations: BCC, basal cell carcinoma; UV, ultraviolet radiation; GST, thione S-transferase; CYP, cytochrome P450; CI confidence interval; Ah, hydrocarbon hydroxylase.

Introduction

Patients with a basal cell carcinoma of skin (BCC*) are at high risk of suffering further primary BCC (1,2). Importantly, this risk depends on the number of lesions present. Thus, in subjects with one lesion the 5-year risk is 27%, in those with 10 or more tumours the risk is 90% (1,2) suggesting accrual of lesions is not just dependent on time, but rather some subjects have an increased susceptibility (2). Ultraviolet radiation (UV) is a critical factor in the pathogenesis of BCC though the relationship between exposure and risk of multiple tumours is complex; risk increases with age, male gender, exposure to UV and response to sunlight with subjects who readily burn and do not tan (skin type I) being at greatest risk (2). UV is an important carcinogen that has pleiotrophic effects in the skin including initiation of neoplastic change and impairment of immunosurveillance (3,4). Exposure to UVA constitutes an oxidative stress that leads to DNA strand breaks, peroxidation of membrane lipids and photolysis of amino acids (5). It is likely, therefore, individual differences in response to oxidative stress will mediate susceptibility. Thus, the effectiveness of repair of UV-damaged DNA is associated with risk of multiple BCC (6). Furthermore, comparison of genotype frequencies in patients with one BCC with those in patients with multiple lesions (2 to 35 tumours) showed allelism at the glutathione S-transferase GSTM1 locus also influences susceptibility (7,8). Thus, *GSTM1*A/GSTM1*B* heterozygosity confers protection against multiple BCC while homozygosity for *GSTM1*0*, in combination with skin type 1 confers increased risk. While both GSTM1 and theta class GSTT1 enzymes utilize lipid and DNA products of oxidative stress, the influence of GSTT1 on susceptibility to multiple tumours was less pronounced (8) showing the importance of specific detoxification reactions. Furthermore, allelism and, interactions between allelic variants at other loci may also influence risk of multiple BCC. However, while studies in Japanese with lung cancer showed interactive effects between GSTM1 and CYP1A1 (odds ratio 9.1) (9), it is unclear which combinations of genotypes mediate risk of particular cancers.

Several members of the cytochrome P450 family including CYP1A1 and CYP2D6 are candidates for cancer susceptibility (10–12). Both CYP1A1 and its ligand-dependent transcription factor, the Ah receptor, are widely expressed in extra-hepatic tissues including skin (10) and though the gene has largely been studied in the context of environmental pollutants (e.g. polycyclic aromatic hydrocarbons), there is evidence its products utilize endogenous ligands and participate in defence against oxidative stress (11,12). Thus, aryl hydrocarbon hydroxylase activity in rodent skin is increased by UV presumably because of photoproducts that induce expression. Indeed, *in vitro* studies show UV irradiation of amino acids such as

tryptophan, that are present in skin, results in oxidized products that bind to the Ah receptor with high affinity (13). CYP1A1 is polymorphic (9–15) with a mutation in the 3′-non-coding region giving a restriction site in the m_2 allele. The effects of this mutation are unclear though it is reported to segregate with a high CYP1A1 inducibility phenotype (9,14,15) and in Japanese, but not Caucasian smokers, is associated with increased risk of lung cancer (9,10,14). A mutation in exon 7 causes an Ile>val change in the haem binding region and may influence activity towards benzo[*a*]pyrene (14).

Skin is exposed to carcinogens that mimic the effects of endogenous ligands on activities such as proliferation and apoptosis (12). Effective metabolism of the products of such exposure is likely to mediate risk of multiple BCC. We now describe the influence of allelism at CYP1A1, CYP2D6, GSTM1, GSTT1 and physical characteristics (gender, skin type, hair colour, eye colour) on susceptibility assessed in terms of tumour numbers and their accrual (number of tumours/year from first presentation). The influence of allelism on numbers of BCC has been studied in a cross-sectional study as previously, we only compared genotypes in patients with one tumour with those in patients with more than 1 lesion giving a dichotomous outcome (7,8). We now describe associations with tumour numbers used without transformation, as the outcome. We also present a longitudinal study of the influence of genotypes, characteristics, patient age and number of lesions at first presentation on accrual of BCC.

Materials and methods

Patients

Cross-sectional study. The influence of genotypes and physical characteristics on BCC numbers was studied in 827 unrelated English Caucasians with histologically proven BCC. They were recruited between November 1991 and July 1995 from Dermatology out-patient clinics in the Midlands (North Staffordshire Hospital, Stafford District General Hospital) and South of England (Royal Cornwall Hospitals, William Harvey Hospital, Kent), and represented cases from a mix of urban and rural environments. Four-hundred-and-thirty-five patients comprised the BCC case group described by Heagerty *et al.* (7). Data on GSTM1 genotype frequencies in these subjects was included in the present study. Genotype frequencies in the original case group and the further 392 subjects recruited between October 1993–July 1995 were not different. 565 patients suffered one tumour, and 262 patients between 2 and 35 tumours. All patients were examined and interviewed by a trained Dermatologist (JTL, AHMH, AS, BB, CRP) to obtain information on hair and eye colour at 21 years of age, skin type (types 1–5) (8), smoking history allowing subjects to be classified as ever or never smokers. All case notes were carefully reviewed during August 1995 (JTL, BB) to determine sites and numbers of tumours, tumour recurrence, accrual and the presence of other malignancies (both skin and internal). Recurrences were excluded from the total number of primary BCC. Cases with basal cell naevus syndrome or BCC and another malignancy were excluded. The cases composed over 60% of those in the participating centres and were a typical sample of this patient group. The remaining 40% patients were lost to follow-up. None of those approached refused to participate.

Longitudinal study. The influence of genotypes and characteristics on tumour accrual was studied in 169 of the patients described above selected because accurate data on the time between first presentation and August 1995, and the number of primary lesions could be recovered by JTL and BB.

Identification of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes in leucocyte DNA

Blood (5 ml) was taken with Ethics Committee approval, into EDTA and stored at −50°C. GSTM1 null, A, B and A/B were identified using a PCR approach that identifies *GSTM1*0* homozygotes and *GSTM1*A/GSTM1*B* heterozygotes and the GSTM1 A and GSTM1 B phenotypes. It does not distinguish *GSTM1*0/GSTM1*A* and *GSTM1*A/GSTM1*A* or equivalent *GSTM1 B* genotypes (7,8). GSTT1 null and expressers were also identified by PCR (8). Two mutant *CYP2D6* alleles (G>A transition at intron 3/exon 4, base pair deletion in exon 5) were identified (8,11). Together, these assays are 90% predictive of phenotype in British Caucasians (11). Two mutant CYP1A1 alleles (exon 7 Ile>Val and 3′ flanking region) were detected usi PCR with restriction digestion. The exon 7 mutation was identified usi primers to intron 6 and exon 7 with the reverse primer modified at t penultimate base to generate a second NcoI site on amplification of the wil type, but not mutant allele (16). Both wild-type and mutant alleles amplifi a 322 bp fragment with a common NcoI site that acted as internal control ensure digestion. Homozygous wild-type DNA gave 232, 70, 20 bp fragmen homozygous mutant DNA gave fragments of 252 and 70 bp. Mutations at t 3′-end were identified as described (14).

Table I. Patient demographics

(i) Cross-sectional analysis: influence of genotypes and physical characteristics on numbers of BCC	
Number of BCC	1–35 tumours (n = 827) >5 tumours (n = 62) >10 tumours (n= 23)
Mean age (years)	67.3 ± 12.4 (SD) (n = 823)
Males/females	55.8%/44.2% (n = 813)
Blue and green eyes	72.8% (n = 418)
Brown eyes	27.2% (n = 156)
Skin type 1	14.2% (n = 78)
Skin type 2–6	85.8% (n = 473)
Ever smoker	62.5% (n = 367)
Never smoker	37.5% (n = 220)
Brown and black hair	74.9% (n = 242)
Blonde and red hair	25.1% (n = 81
(ii) Longitudinal analysis: influence of genotypes and physical characteristics on accrual of BCC	
Number of patients	169
Median number of BCC	3 (range 1–30)
Median number of new tumours/year	0.31 (range 0–18.7)
Median follow up (years)	4.54 (range 0–28.5)
Median number of new tumours during follow-up	1 (range 0–29)

Statistical analysis

Cross-sectional analysis. Poisson regression was used to model count da using the EGRET statistical package (SERC, Seattle, 1993). The mo assumes the Poisson rate parameter (mean number BCC) may be expres as a function of a set of covariates, i.e. age, skin type 1, eye colour, h colour, gender, genotypes. A rate ratio, defined as the multiplicative effect a change of a covariate by 1 was calculated (for these data usually being change from 0 to 1). Thus, the rate ratio for males [1] (1.43) against fema [0] is mean number BCC in males (2.38)/mean number BCC in femal (1.66), when gender alone (i.e. not in the presence of other covariates) considered. In the Poisson regression, this will change in the presence other covariates. The Poisson regression approach has the advantage that allows study of the ratio of the mean number of BCC corrected for oth covariates such as age and number of tumours at presentation.

Longitudinal analysis. Poisson regression with a rate multiplier was used adjust for the number of person years at risk since the increase in the numb of BCC is likely to change depending on the number of years between fi presentation and August 1995. The rate multiplier therefore, corrected for t different numbers of person years at risk in different patients, this is n present in the cross-sectional study. Cases were omitted when appropria data were missing for that analysis. Only results significant at the 5% lev or approaching this significance are described.

Results

Cross-sectional study: influence of genotypes and individu characteristics on number of primary BCC

Patient characteristics. Table I shows the characteristics the study group and numbers of BCC in these patients: subjects had five or more tumours, and 23, 10 or more lesion As expected, the proportion of males was greater than in age matched controls (47%) from the study centres (8). Table I shows the frequencies of GSTM1, GSTT1, CYP2D6 a CYP1A1 genotypes in these patients. These were not signifi antly different from those in the 561 controls describ previously (8). Comparison of the frequencies of homo-

e II. Frequency of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes in patients with single and multiple basal cell carcinoma

M1	Null	A	B	A/B	*n*
	392	213	114	22	741
	(52.9%)	(28.7%)	(15.4%)	(3.0%)	
T1	Null	Expresser			*n*
	122	579			701
	(17.4%)	(82.6%)			
2D6	EM	HET	PM		*n*
	429	205	43		677
	(63.4%)	(30.3%)	(6.4%)		
1A1	Ile/Ile	Ile/val	val/val		*n*
7 mutation	572	94	7		673
	(85.0%)	(14.0%)	(1.0%)		
	m_1m_1	m_1m_2	m_2m_2		*n*
3′-flanking region	561	119	4		684
	(82.0%)	(17.4%)	(0.6%)		

e III. Age-corrected factors and interactions influencing number of BCC

	P	rate ratio	95% CI
ndividual factors[a]			
Male gender (n = 755)	<0.001	1.487	1.342–1.648
Skin type 1 (n = 529)	<0.001	1.393	1.205–1.609
Blue+green eyes (n= 522)	0.001	1.361	1.187–1.559
CYP2D6 EM (n = 623)	<0.001	1.266	1.125–1.425
CYP1A1 m_1m_1 (n = 635)	0.004	1.242	1.071–1.441
nteractions between factors[b]			
GSTM1 null.skin type 1 (n = 463)	<0.001	2.702	1.964–3.717
CYP1A1 m_1m_1.blue+green eyes (n = 446)	0.002	1.917	1.279–2.873
CYP2D6 EM.blue+green eyes (n = 429)	0.046	1.388	1.005–1.915
CYP2D6 EM.male gender (n = 614)	0.049	1.279	1.001–1.633

base-line is the complement (i.e. female gender, not skin type 1, etc).
ults shown are for individual factors alone (i.e. not in the presence of other factors).
ults for interactions are corrected for age in the presence of the main effects.

rozygotic combinations of the exon 7 and 3′-mutations in P1A1 showed, as reported (14,17), they were in linkage quilibrium (χ^2_4 = 56.12; $P < 0.0005$).

tors influencing the numbers of BCC. Table III shows genotypes and individual characteristics associated with reasing numbers of BCC. The data are adjusted for age e, as expected, this was a risk factor for multiple tumours = 0.013, rate ratio 1.005, 95% CI 1.001–1.009, n = 769). Neither GSTM1 null, GSTT1 null nor smoking were ificant risk factors. CYP1A1 Ile/Ile did not influence our numbers though both the CYP1A1 m_1m_1 and the P2D6 EM genotypes were associated with increased num- evs of primary tumours (Table III). Male gender, skin type 1, blue and green eyes were also significantly associated h increased numbers of BCC.

ractions between enzyme genotypes, male gender and skin ue 1. Table III shows significant, age-corrected interactions which the interaction term was more significant than either the main effects. Thus, the rate ratio for the interaction ween GSTM1 null and skin type 1 (2.702) was the highest ntified. Significant interactions between CYP2D6 EM and gender, and this genotype, and blue and green eyes, as as between CYP1A1 m_1m_1 and, blue and green eyes were identified. Significant interactions between male gender GSTM1 null (P = 0.045), gender and skin type 1 ($P <$ 01) and, skin type 1 and CYP1A1 m_1m_1 (P = 0.012) were ntified, but appeared to result from the strength of the main effects. No significant interactions between genotypes were identified though the interaction between CYP2D6 EM and CYP1A1 m_1m_1 approached significance (P = 0.095; rate ratio 1.326).

Longitudinal study: influence of genotypes and individual characteristics on accrual of BCC

Patient characteristics. Table I shows the details of the 169 patients in whom accurate information on the time between first presentation and August 1995, and the number of BCC was obtained. The median follow-up time (i.e. time between first presentation and August 1995) and median number of new lesions are recorded.

Factors influencing accrual of new BCC. Table IV shows the factors that influenced the rate of appearance of new BCC. As expected male gender, the number of lesions and age at first presentation were significant factors. Male gender appeared a particularly influential factor (rate ratio 2.260) though both CYP2D6 EM and GSTT1 null were significantly associated with the rate at which new tumours appeared. Current and ex-smoking (ever smokers) was also significantly associated with more rapid appearance of further tumours. The influence of GSTM1 null and CYP1A1 Ile/Ile on the rate of accrual of tumours also approached significance. Table IV also shows that GSTT1 null, CYP2D6 EM, male gender and skin type 1 were associated with faster appearance of new tumours after adjustment for the effect of age and the numbers of BCC at presentation. Comparison of the rate ratios indicated GSTT1

Table IV. Factors influencing accrual of BCC

	P	Rate ratio	95% CI
(a) Unadjusted[a]			
(i) Male gender (n = 143)	<0.001	2.260	1.793–2.848
(ii) BCC number at presentation (n = 146)	<0.001	1.213	1.167–1.260
(iii) Age at presentation (n = 140)	0.002	1.014	1.005–1.022
(iv) CYP2D6 EM (n = 128)	<0.001	2.398	1.880–3.059
(v) GSTT1 null (n = 126)	<0.001	2.321	1.821–2.959
(vi) Ever smoking (n = 110)	0.048	1.282	1.002–1.639
(vii) GSTM1 null (n = 143)	0.067	0.828	
(viii) CYP1A1 Ile/Ile (n = 121)	0.079	0.785	
(b) Adjusted for number of BCC and age at presentation[a]			
GSTT1 null (n = 121)	<0.001	2.677	2.075–3.452
Male gender (n = 135)	<0.001	2.174	1.720–2.749
CYP2D6 EM (n = 122)	<0.001	2.154	1.653–2.807
Skin type 1 (n = 94)	0.035	1.386	1.024–1.877
CYP1A1 Ile/Ile (n = 115)	0.008	0.690	0.524–0.909
(c) Interactions adjusted for number of BCC and age at presentation[b]			
Male gender.CYP2D6 EM (n = 121)	<0.001	2.633	1.510–4.593

The base-line is the complement (i.e. female gender, not skin type 1, etc.)
[a]Results shown are for individual factors alone (i.e. not in the presence of other factors).
[b]Results for interactions are corrected for number of BCC and age at presentation in the presence of the main effects.

null was the most influential. The CYP1A1 Ile/Ile genotype was also found to be a significant factor. The rate ratio (0.690) indicated it was associated with slower accrual of new tumours, and that it was relatively less influential than GSTT1 and CYP2D6 EM. No significant interactions between genes were identified. A significant interaction between CYP2D6 EM and male gender was observed, though significant interactions between male gender and GSTM1 null (P = 0.035) and skin type 1 (P = 0.009) appeared to result from the strength of the main effect.

Discussion

Previous studies from our laboratory have used a case-control approach to show that polymorphism in loci encoding carcinogen-metabolising enzymes influences susceptibility to BCC (7,8). We have now described further studies designed to determine, within the case group, the influence of these polymorphisms on tumour numbers and accrual. These studies comprised a cross-sectional analysis of the influence of *GST* and *CYP* genotypes on BCC numbers and a longitudinal analysis of the effect of these genotypes on tumour accrual. The first study allowed recruitment of large numbers of cases though, because follow-up was variable, the influence of genotypes may be underestimated as further lesions develop in some patients. The expected influence of gender, age and skin type was found indicating the value of this approach. The second study includes the variables shown to influence BCC numbers (1,2); however, since data on the time of accrual (or lack of accrual) was not always available the number of cases was smaller. The biochemical processes that determine BCC numbers and accrual are largely unclear because the relationship between each lesion and exposure to UV, a tumour initiator and promoter, is unknown. These parameters must reflect complex interactions between an initiating event(s) (UV and/or other factors), the extent of damage to host targets, their repair, immune surveillance and time. Thus, while the genetically mediated response to and detoxification of products of UV-induced oxidative stress will be important in determining numbers and accrual of lesions, some common and some different biochemical activities and polymorphisms may mediate these endpoints. We have confirmed the expected importance of male gender, skin type and, age and number of lesions at presentation (1,2). Furthermore, the cross-sectional analysis showed CYP1A1 m_1m_1 and CYP2D6 EM are associated with increased numbers of BCC and the longitudinal study, after adjustment for age and tumour number at presentation, that GSTT1 null, CYP2D6 EM and CYP1A1 Ile/Ile were significant.

While there is no apparent association between exposure to environmental pollutants and disease risk, we found allelism in the carcinogen-metabolizing enzyme CYP2D6 was associated with both increased numbers and accrual of BCC. The role of this enzyme is unclear (18). Its expression is mainly hepatic and, though found in brain and intestine, it has not been detected in skin (10). However, the association between CYP2D6 PM and susceptibility to Parkinson's disease suggests *in vivo* substrates include endogenous neurotoxins and possibly molecules containing amine- or guanidino- groups (11,19). Furthermore, as systemic agents such as arsenic predispose to multiple BCC (20), CYP2D6-mediated hepatic detoxification of photosensitizing agents may be important. Data showing UV-oxidized tryptophan binds to the Ah receptor and UV effects increased enzyme expression in skin suggest a role for CYP1A1 in skin carcinogenesis (10,13,21,22) though studies in Caucasians have generally failed to demonstrate an association between allelism at this locus and disease susceptibility (16,17). We found CYP1A1 genotypes were associated with altered risks of both BCC numbers, and accrual though m_1m_1 influenced numbers and the Ile/Ile genotype was associated with slower accrual. While these results appear contradictory and difficult to explain given uncertainty regarding the consequences of these mutations, it is noteworthy that the extent of altered risk associated with CYP1A1 genotypes is only moderate compared with other genes. If the 3′-mutation is associated with greater expression, m_1m_1 may confer less effective detoxification of an unknown carcinogen though it is suggested the Val/Val genotype is associated with greater activity towards benzo[*a*]pyrene. However, as argued

(23), the influence of a genotype must depend on ve substrates and it is possible the two CYP1A1 ons effect different responses on expression of CYP1A1 ner phase 2, Ah-dependent genes (12).

M1 and GSTT1 had different effects on numbers and of lesions. GSTT1 null did not influence the numbers ons possibly because its effect is diluted in a group that s subjects who accrue tumours quickly, slowly or not We speculate the influence of GSTM1 results from its to metabolize the products of UV-induced oxidative in skin, a view supported by the finding that GSTM1 also associated with increased production of anti-Ro t anti-La) antibodies in patients with systemic lupus matosus (24). Importantly, production of anti-Ro anti- in the absence of anti-La antibodies is associated with ensitivity. Available data are less clear regarding the n of GSTT1. Thus, GSTT1 null, but not GSTM1 associated with increased risk of astrocytoma and gioma (25), pathologies not associated with inflamma- s well as total ulcerative colitis (26). These data size current uncertainty of the role of GSTT1 and, re, the significance of the polymorphism. This appears end on substrate; while GSTT1 utilizes monohalome- and expressers are protected against the sister chromatid ge induced by these compounds (23), GST5-5, the TT1 homologue, has dehalogenase activity against omethane giving rise to formaldehyde. Thus, expression T5-5 in the Ames test leads to enhanced mutagenicity loromethane (23).

study has identified significant interactions between pes and individual characteristics associated with risk. pected, skin type 1 and male gender were associated creased numbers of BCC (1,2). These characteristics lated as women have higher skin reflectances and, ens stimulate melanin production (27). Interactions n these characteristics, and GSTM1 null and CYP2D6 re identified though the rate ratio for the CYP2D6 EM/ ender interaction mediating tumour numbers, but not was relatively small. While blue eyes have been linked creased risk of cutaneous malignancy (28), it has been blue and green eyes are not independently related to f BCC (1,2). We found eye colour was associated creased numbers of lesions presumably because it is ted with skin complexion. Interactions between eye and CYP2D6 EM and CYP1A1 m_1m_1 were also ed. Exposure to sunlight, whilst an important parameter, riously difficult to assess and studies showing that tibility to UVB-induced inhibition of contact hypersens- appears a better indicator of non-melanoma skin cancer an cumulative, life-time sun exposure indicate the ance of host genetic factors (29).

have identified genetic traits associated with suscepti- o increased numbers of BCC and, for the first time, that ce tumour accrual. Previous studies have shown the ance of DNA repair, indeed the influence of GSTM1 sult from its ability to utilize 5′-hydroxymethyluracil lowever, the finding that CYP1A1 and, particularly, D6 influence tumour numbers and accrual, while not able, is in keeping with the view these genes are tibility markers for many cancers. The mechanism of ffect is unclear, though may involve detoxification dentified molecules possibly containing amino- or ino-groups, or indole or steroid ring structures. Indeed, our finding that ever-smoking is associated with faster accrual of tumours also suggests the importance of carcinogen-metabolising enzymes such as members of the CYP 1 and 2 families. Our data, therefore, provide further support for the view that factors other than UV influence the pathogenesis of this cancer (29). Host genetic factors appear important in this process (30).

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morphism at the Glutathione *S*-Transferase Locus *GSTM3*: Interactions with ochrome P450 and Glutathione *S*-Transferase Genotypes as Risk Factors Multiple Cutaneous Basal Cell Carcinoma[1]

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influence of polymorphism in the glutathione *S*-transferase, *GSTM3* e susceptibility to cutaneous basal cell carcinoma (BCC) has been gated. We have reported previously two *GSTM3* alleles, *GSTM3*A* and 3*B*, distinguished by a recognition motif for the YY1 transcription in *GSTM3*B*. In this study, immunohistochemistry was used to identify 3 expression in the epidermis of skin samples from 11 controls and 9 s with BCC. A PCR method was used to identify *GSTM3*A* and 3*B* and thereby the GSTM3 AA, GSTM3 AB, and GSTM3 BB pes in 300 controls and 286 Caucasians with 1–35 primary BCCs. pes at GSTM1, GSTT1, and the cytochrome P450 *CYP1A1* and 6 loci were also determined. Frequencies of GSTM3, GSTM1, GSTT1, 6, and CYP1A1 genotypes in the cases and controls were not different. g the BCC cases into groups of 92 patients with 1 lesion and 194 s with 2–35 lesions showed that the frequencies of GSTM3 BB (2.6%) STM1 A/B (1.3%) in the group with 2–35 tumors were almost signif- lower than in the group with 1 lesion (7.6%, exact $P = 0.0601$, 3390; 6.5%, exact $P = 0.055$, $\chi^2_1 = 4.946$, respectively). Within the with 2–35 tumors, a Poisson regression model was used to identify pes, characteristics such as skin type, and interactions between geno- nd characteristics associated with increasing numbers of tumors. This d, after correction for male gender and age, that GSTM3 AA was not ted with risk of increased numbers of tumors, although in combination tin type 1, GSTM1 null, and CYP1A1 m1m1, the genotype did confer ed risk ($P < 0.001$, rate ratio, 2.058; $P < 0.001$, rate ratio, 1.606; 001, rate ratio, 1.470, respectively). The data suggest that, like other GST, GSTM3 influences cancer risk. As GSTM3 AA was associated creased tumor numbers, it appears that YY1 acts as an activator of the tion motif in *GSTM3*B*.

duction

ymorphism at the GST,[3] *GSTM1* locus has attracted much interest, e homozygosity for *GSTM1*0* confers altered risk for several s (1–3). However, although GSTM1 null may be a susceptibility r, data from some studies, including in lung cancer, are conflicting The reason for these discrepancies is unclear but may reflect the ce on detoxification of interactions between GSTM1 and other loci encoding detoxifying enzymes. Thus, a model based only on the hypothesis that GSTM1 null confers increased risk may be too simplistic (1). Furthermore, there is evidence *GSTM1*A* and *GSTM1*B* have different effects on susceptibility to some pathologies, including bladder cancer and Crohn's disease (1), and multiple cutaneous BCCs, in which GSTM1 A/B, but not GSTM1 A or GSTM1 B, is protective (4). The mechanism for these effects is unclear as the products of *GSTM1*A* and *GSTM1*B* demonstrate similar *in vitro* catalytic properties (3, 5). *GSTM1* is one of five μ genes (M1–M5; Refs. 3 and 6) the products of which demonstrate overlap in their substrate specificities (3, 5), implying that the protein products of one μ-class locus will compensate functionally for the absence of other family members. Indeed, studies in the lung suggest coordinated expression of some μ-class genes; thus, *GSTM1*0* homozygotes express less GSTM3 than do subjects with other GSTM1 genotypes (7). The mechanism for this observation is unknown but may be related to the finding that GSTM3 is also polymorphic, with two alleles, *GSTM3*A* and *GSTM3*B*, identified (8). Importantly, *GSTM3*B* is in linkage disequilibrium with *GSTM1*A* and contains in intron 6 a recognition motif for the YY1 transcription factor, which regulates gene expression from intragenic sites (8). The widely expressed YY1 factor influences the expression of many genes (9), suggesting that *GSTM3*A* and *GSTM3*B* are expressed at different levels and GSTM3 genotypes will confer different efficiencies in the metabolism of carcinogens. We propose, therefore, that assessment of the contribution of GSTM1 genotypes as susceptibility markers for cancer risk needs to take account of interactions with GSTM3. The nonfamilial skin cancers offer an interesting opportunity to test the hypothesis that allelism at GSTM3 influences risk, inasmuch as we have recently shown that GSTM1 null in combination with skin type 1 is associated with increased susceptibility to multiple BCCs (4, 10). Allelism at GSTT1 and the cytochrome P450 (CYP) *CYP2D6* and *CYP1A1* loci also influences the pathogenesis of multiple lesions (10, 11). Accordingly, we describe, first, immunohistochemical identification of GSTM3 in skin and, second, a case-control approach to compare GSTM3 genotype frequencies in controls and patients with BCC. Third, the case group was divided into patients with 1 tumor and those with 2–35 lesions. The patients with multiple lesions can be considered to have an increased susceptibility to BCC, and a cross-sectional study was performed to determine the influence of GSTM3 on susceptibility to multiple BCC and identify interactions with CYP and GST genotypes and characteristics such as gender and skin type.

Materials and Methods

Patient Samples. Genotypes were determined in leukocyte DNA from 286 English Caucasians attending the North Staffordshire and Royal Cornwall Hospitals suffering BCC [1–35 primary tumors per patient; median number of

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whom requests for reprints should be addressed. Phone: (01782) 716676; Fax: 716646.
e abbreviations used are: GST, glutathione *S*-transferase; BCC, basal cell ma.

tumors, 2; mean age, 68.3 ± 12.2 (SD) years; 58.4% males] during the period 1993–1995. These cases were divided into two groups: first, 92 patients who suffered one BCC (mean follow-up, 1.8 years; range, 8 months–11.5 years). These cases constituted a random sample of patients with one lesion from the participating centers from whom DNA was available. The second group comprised 194 patients with 2–35 tumors (mean follow-up, 7.5 years; range, 1.1–28.9 years). It was composed of 179 patients with 2–10 tumors and 15 patients with 11–35 tumors and represented about 60% of the cases in the two participating hospitals; the remaining patients were lost to follow-up. All patients were examined by a trained dermatologist (J. T. L., A. H. H., A. S., or B. B.) to obtain data on eye color (blue, green, or brown) and skin type (types 1–6; Ref. 10) and were reviewed in August 1995 by J. T. L. and B. B. to check tumor numbers and the presence of other malignancies. Recurrences were excluded from the number of BCCs, as were cases with basal cell nevus syndrome or BCC and other cancers. Diagnoses were confirmed histologically. Caucasian controls (300) from North Staffordshire and Cornwall [mean age, 62.0 ± 17.7 (SD) years; 41.1% males] without evidence of any malignancy were also recruited. These in- and outpatients suffered varicose veins, hernias, hemorrhoids, mild iron deficiency, mild hyperlipidemia, benign ovarian cysts, tension headaches, benign skin papillomas, benign breast lumps, and cerebrovascular accidents. Patient samples were collected with the approval of the Ethical Committees of the North Staffordshire and Royal Cornwall Hospitals and informed consent.

Immunohistochemical Studies. Normal skin from 11 patients was provided at operation for breast reduction. Skin from 9 patients suffering BCC was obtained from archive material in the Department of Histopathology, North Staffordshire Hospital, Stoke-on-Trent. All tissues were fixed in 10% phosphate-buffered formalin and processed through graded alcohols and xylene to paraffin wax (12). Serial sections (5 μm) were cut, treated with hyd peroxide, and covered with swine serum (diluted 1:3; Ref. 12). Sections incubated (1 h, 20°C) with a rabbit polyclonal antiserum specific to G (diluted 1:100; Refs. 3 and 5). The immunogen was purified from human (5). A section not treated with primary antiserum served as negative cont positive control was provided by sections of human kidney. A biotin swine antirabbit secondary antibody (Amersham, United Kingdom) was with an avidin-biotin-peroxidase complex (Dakopatts, Denmark). Pero activity was developed with diaminobenzidine tetrahydrochloride sub (Sigma Chemical Co., Poole, United Kingdom), counterstained with hem ylin, dehydrated, mounted, and assessed (12).

Identification of Genotypes. GSTM3 genotypes were identified primers to exon 6/7 (8). *GSTM3*B* was differentiated from *GSTM3*A*, is 3 bp larger, by digestion with MnlI. DNA from *GSTM3*A* homozy containing the additional MnlI site, gave fragments of 11, 51, 86, and 1 whereas *GSTM3*B* homozygotes gave fragments of 11, 125, and 13 GSTM1 genotypes were identified using ARMS-based PCR with prim intron 6/exon 7 (4). GSTT1 null and expressing subjects were also ide using PCR (10). Two mutant *CYP2D6* alleles (G→A transition at 3/exon 4 and bp deletion in exon 5) were identified (2, 10). Together, assays are 90% predictive of phenotype in British Caucasians (2). T flanking region mutation (m1m1 wild-type homozygotes) and the e Ile-Val mutation (Ile/Ile wild-type homozygotes) in *CYP1A1* were det using PCR with restriction digestion (11, 13).

Statistical Analysis. χ^2 tests were used to examine for homogene tween cases and controls. As some genotype frequencies were sma StatXact-Turbo statistical package was used. The influence of genotype characteristics alone and in combination, was studied by logistic regre

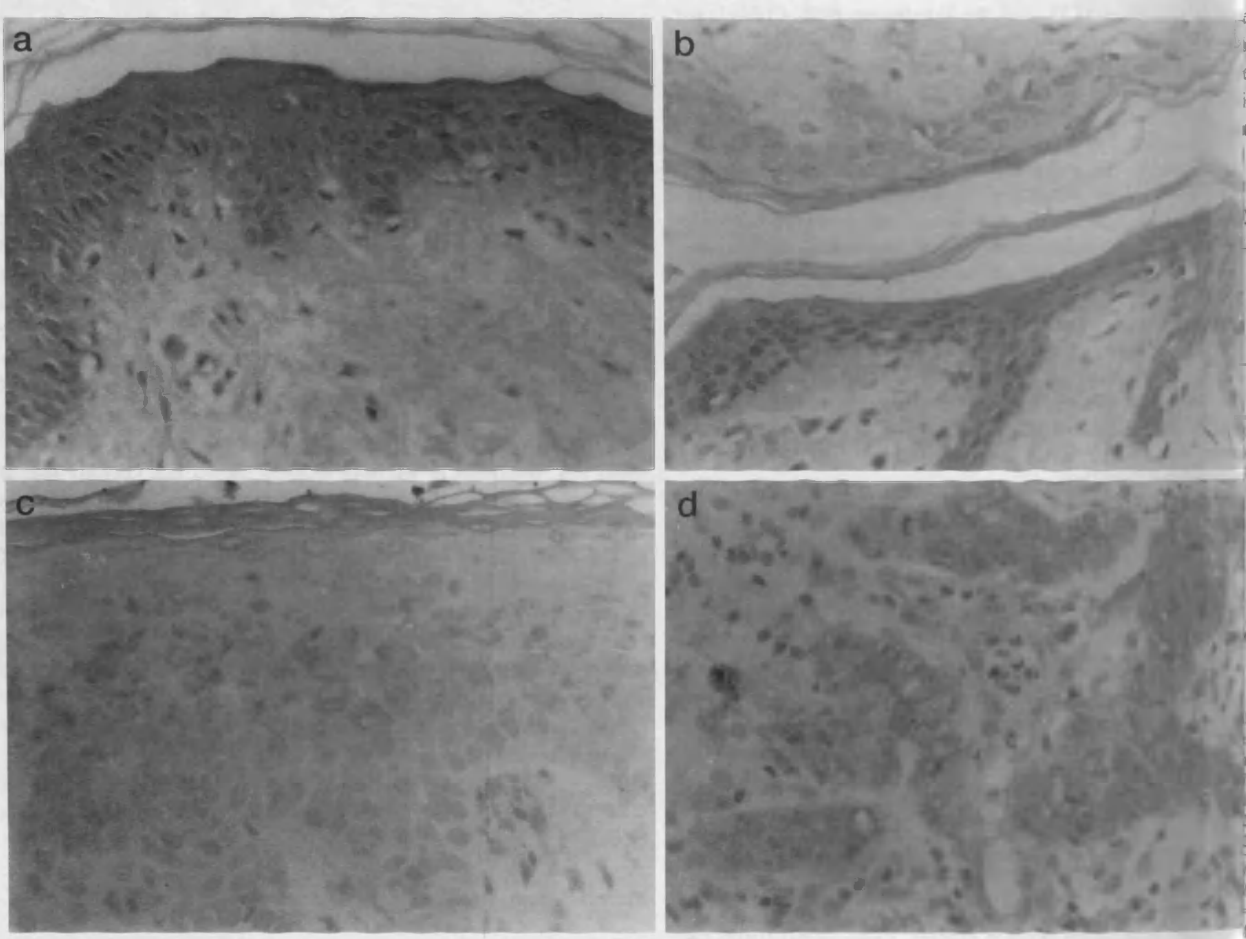

Fig. 1. Immunohistochemical identification of GSTM3 expression in skin samples from controls and patients with BCC. *a*, skin sample from control showing GSTM3 thro the epidermis; *b*, sample from control showing variable GSTM3 expression in skin fold; *c*, skin sample from a control demonstrating weak expression of GSTM3 in the stratum sp (×100); *d*, GSTM3 expression in tumor cells in a BCC (×400).

o he effect of genotypes on BCC numbers was studied by applying ression model (EGRET statistical package; SERC, 1993) to count s with 2–35 tumors. In the model, the Poisson rate parameter (mean BCCs) is expressed as a function of a set of covariates (*i.e.*, age, genotypes). The data were transformed to take account of all This analysis was exploratory and not intended to be predictive, our ing to identify covariates associated with the difference in trans- nts. A rate ratio, defined as the multiplicative effect of a change of by 1 was calculated (usually a change from 0 to 1). Thus, the rate males, 1, against females, 0, is mean number BCC in males/mean BCC in females when gender alone is considered. This will change in nce of other covariates.

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ience of GST Genotypes on Susceptibility. Table 1 shows 3 genotype frequencies in controls and cases. Frequency dis- ns and genotype frequencies in these groups were not different. y, frequency distributions and genotype frequencies for . GSTT1, CYP2D6, and CYP1A1 were not significantly dif-

GSTM3, GSTM1, GSTT1, CYP2D6, and CYP1A1 genotype frequencies in controls and patients with BCC

	Single BCC (%)[a]	Multiple BCCs (%)[b]	Total BCCs (%)[c]	Controls (%)[d]
3 AA	68 (73.9)	140 (72.2)	208 (72.7)	221 (73.7)
3 AB	17 (18.5)	49 (25.3)	66 (23.1)	64 (21.3)
3 BB	7 (7.6)	5 (2.6)	12 (4.2)	15 (5.0)
1 null	52 (56.5)	88 (57.5)	140 (57.1)	121 (57.3)
1 A	21 (22.8)	42 (27.5)	63 (25.7)	56 (26.5)
1 B	13 (14.1)	21 (13.7)	34 (13.9)	25 (11.8)
1 A,B	6 (6.5)	2 (1.3)	8 (3.3)	9 (4.3)
null	18 (19.6)	35 (21.0)	53 (20.5)	56 (19.7)
A	74 (80.4)	132 (79.0)	206 (79.5)	228 (80.3)
D6				
	53 (60.2)	104 (66.7)	157 (64.3)	129 (64.2)
	31 (35.2)	46 (29.5)	77 (31.6)	67 (33.3)
	4 (4.5)	6 (3.8)	10 (4.1)	5 (2.5)
1				
le	76 (83.5)	137 (82.0)	213 (82.6)	77 (91.7)
Val	15 (16.5)	27 (16.2)	42 (16.3)	6 (7.1)
Val	0 (0.0)	3 (1.8)	3 (1.2)	1 (1.2)
1	76 (83.5)	135 (81.3)	211 (82.1)	71 (86.6)
2	13 (14.3)	31 (18.7)	44 (17.1)	11 (13.4)
2	2 (2.2)	0 (0.0)	2 (0.8)	0 (0.0)

M3, GSTM1, and GSTT1, n = 92; CYP2D6, n = 88; CYP1A1, n = 91.
M3, n = 194; GSTM1, n = 153; GSTT1, n = 167; CYP2D6, n = 156; CYP1A1 = 167; CYP1A1 m1m1, n = 166.
M3, n = 286; GSTM1, n = 245; GSTT1, n = 259; CYP2D6, n = 244; CYP1A1 = 258; CYP1A1 m1m1, n = 257.
M3, n = 300; GSTM1, n = 211; GSTT1, n = 284; CYP2D6, n = 201; CYP1A1 = 84; CYP1A1 m1m1, n = 82.

Table 2 *Age- and gender-corrected factors and interactions influencing number of BCCs*

	P	Rate ratio	95% CI[a]
Individual factors			
Skin type 1 (n = 141)	<0.001	2.019	1.616–2.523
CYP1A1 m_1m_1 (n = 156)	<0.001	1.978	1.424–2.747
CYP2D6 EM (n = 146)	<0.001	1.608	1.246–2.074
GSTM1 null (n = 143)	0.002	1.436	1.144–1.802
Interactions between factors			
GSTM3 AA.skin type 1 (n = 141)	<0.001	2.058	1.628–2.601
GSTM3 AA.GSTM1 null (n = 143)	<0.001	1.606	1.287–2.004
GSTM3 AA.CYP1A1 m_1m_1 (n = 156)	<0.001	1.470	1.184–1.827

[a] CI, confidence interval.

ferent from those published for North Staffordshire BCC cases and controls (10, 11). The proportion of males and the mean age of the BCC group were significantly greater than in controls ($P < 0.001$ in both cases). Logistic regression showed that the age- and gender-corrected proportions of GSTM3 AA in the cases and controls were not different. Similarly, the age- and gender-corrected proportions of GSTM3 AA in combination with GSTM1 A, GSTM1 null, GSTT1 null, CYP2D6 EM, or CYP1A1 m1m1 in cases and controls were not significantly different.

To determine whether GSTM3 genotypes were associated with risk of multiple tumors, the BCC cases were divided into patients with 1 lesion and those with 2–35 tumors (Table 1). The frequencies of GSTM3 BB and GSTM1 A/B in the group with 2–35 tumors were almost significantly lower than in the group with 1 lesion (exact $P = 0.0601$; $\chi^2_1 = 3.390$; exact $P = 0.055$; $\chi^2_1 = 4.946$, respectively). Within the cases with 2–35 tumors only, Poisson regression was used to identify genotypes, individual characteristics and interactions between genotypes, and characteristics associated with increasing numbers of primary tumors. Thus, male gender and increased age were associated with a significant increase in the number of BCCs ($P < 0.001$). Neither GSTM3 AA nor GSTM1 A, GSTT1 null, or CYP1A1 Ile/Ile, alone or after correction for age and gender, were significantly associated with an increased number of BCC. However, after correction for age and gender, skin type 1, CYP1A1 m1m1, CYP2D6 EM, and GSTM1 null were significantly associated with increased numbers of tumors (Table 2). Significant age- and gender-corrected interactions in which the interaction term was more significant than either of the main effects were also identified; these interactions were between GSTM3 AA/skin type 1, GSTM3 AA/GSTM1 null, and GSTM3 AA/CYP1A1 m1m1 (Table 2).

Discussion

We have studied the influence of the newly described polymorphism at GSTM3 on risk of multiple BCC. This is the first report of allelism in a μ-class gene other than *GSTM1* as a candidate for susceptibility. Our immunohistochemical experiments showed specific expression of the gene in skin from cases and controls. The influence of GSTM3 on susceptibility was studied, first, by comparing genotype frequencies in controls with those in the BCC cases. Second, the frequency of genotypes in patients with 1 tumor was compared with that in patients with 2–35 lesions, a group that appears at increased risk of BCC. Third, a Poisson regression model was used in the cases with 2–35 tumors to identify genotypes and characteristics that influence tumor numbers.

Patients with a BCC are at high risk of suffering further lesions (14, 15). Importantly, this risk depends on the number of tumors present. Thus, in subjects with 1 lesion, the 5-year risk is 27%, and in those with 10 or more tumors, the risk is 90% (14, 15), indicating that accrual is not just dependent on time but rather that some subjects

the effect of genotypes on BCC numbers was studied by applying egression model (EGRET statistical package; SERC, 1993) to count s with 2–35 tumors. In the model, the Poisson rate parameter (mean BCCs) is expressed as a function of a set of covariates (*i.e.*, age, d genotypes). The data were transformed to take account of all This analysis was exploratory and not intended to be predictive, our ing to identify covariates associated with the difference in trans- nts. A rate ratio, defined as the multiplicative effect of a change of by 1 was calculated (usually a change from 0 to 1). Thus, the rate ales, 1, against females, 0, is mean number BCC in males/mean C in females when gender alone is considered. This will change in e of other covariates.

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ce of GST Genotypes on Susceptibility. Table 1 shows enotype frequencies in controls and cases. Frequency dis- nd genotype frequencies in these groups were not different. frequency distributions and genotype frequencies for STT1, CYP2D6, and CYP1A1 were not significantly dif-

STM3, GSTM1, GSTT1, CYP2D6, and CYP1A1 genotype frequencies in controls and patients with BCC

	Single BCC (%)[a]	Multiple BCCs (%)[b]	Total BCCs (%)[c]	Controls (%)[d]
A	68 (73.9)	140 (72.2)	208 (72.7)	221 (73.7)
B	17 (18.5)	49 (25.3)	66 (23.1)	64 (21.3)
B	7 (7.6)	5 (2.6)	12 (4.2)	15 (5.0)
ll	52 (56.5)	88 (57.5)	140 (57.1)	121 (57.3)
	21 (22.8)	42 (27.5)	63 (25.7)	56 (26.5)
	13 (14.1)	21 (13.7)	34 (13.9)	25 (11.8)
,B	6 (6.5)	2 (1.3)	8 (3.3)	9 (4.3)
ll	18 (19.6)	35 (21.0)	53 (20.5)	56 (19.7)
	74 (80.4)	132 (79.0)	206 (79.5)	228 (80.3)
	53 (60.2)	104 (66.7)	157 (64.3)	129 (64.2)
	31 (35.2)	46 (29.5)	77 (31.6)	67 (33.3)
	4 (4.5)	6 (3.8)	10 (4.1)	5 (2.5)
	76 (83.5)	137 (82.0)	213 (82.6)	77 (91.7)
	15 (16.5)	27 (16.2)	42 (16.3)	6 (7.1)
	0 (0.0)	3 (1.8)	3 (1.2)	1 (1.2)
	76 (83.5)	135 (81.3)	211 (82.1)	71 (86.6)
	13 (14.3)	31 (18.7)	44 (17.1)	11 (13.4)
	2 (2.2)	0 (0.0)	2 (0.8)	0 (0.0)

, GSTM1, and GSTT1, $n = 92$; CYP2D6, $n = 88$; CYP1A1, $n = 91$.
, $n = 194$; GSTM1, $n = 153$; GSTT1, $n = 167$; CYP2D6, $n = 156$; CYP1A1 167; CYP1A1 m1m1, $n = 166$.
, $n = 286$; GSTM1, $n = 245$; GSTT1, $n = 259$; CYP2D6, $n = 244$; CYP1A1 258; CYP1A1 m1m1, $n = 257$.
, $n = 300$; GSTM1, $n = 211$; GSTT1, $n = 284$; CYP2D6, $n = 201$; CYP1A1 84; CYP1A1 m1m1, $n = 82$.

Table 2 *Age- and gender-corrected factors and interactions influencing number of BCCs*

	P	Rate ratio	95% CI[a]
Individual factors			
Skin type 1 ($n = 141$)	<0.001	2.019	1.616–2.523
CYP1A1 m_1m_1 ($n = 156$)	<0.001	1.978	1.424–2.747
CYP2D6 EM ($n = 146$)	<0.001	1.608	1.246–2.074
GSTM1 null ($n = 143$)	0.002	1.436	1.144–1.802
Interactions between factors			
GSTM3 AA.skin type 1 ($n = 141$)	<0.001	2.058	1.628–2.601
GSTM3 AA.GSTM1 null ($n = 143$)	<0.001	1.606	1.287–2.004
GSTM3 AA.CYP1A1 m_1m_1 ($n = 156$)	<0.001	1.470	1.184–1.827

[a] CI, confidence interval.

ferent from those published for North Staffordshire BCC cases and controls (10, 11). The proportion of males and the mean age of the BCC group were significantly greater than in controls ($P < 0.001$ in both cases). Logistic regression showed that the age- and gender-corrected proportions of GSTM3 AA in the cases and controls were not different. Similarly, the age- and gender-corrected proportions of GSTM3 AA in combination with GSTM1 A, GSTM1 null, GSTT1 null, CYP2D6 EM, or CYP1A1 m1m1 in cases and controls were not significantly different.

To determine whether GSTM3 genotypes were associated with risk of multiple tumors, the BCC cases were divided into patients with 1 lesion and those with 2–35 tumors (Table 1). The frequencies of GSTM3 BB and GSTM1 A/B in the group with 2–35 tumors were almost significantly lower than in the group with 1 lesion (exact $P = 0.0601$; $\chi^2_1 = 3.390$; exact $P = 0.055$; $\chi^2_1 = 4.946$, respectively). Within the cases with 2–35 tumors only, Poisson regression was used to identify genotypes, individual characteristics and interactions between genotypes, and characteristics associated with increasing numbers of primary tumors. Thus, male gender and increased age were associated with a significant increase in the number of BCCs ($P < 0.001$). Neither GSTM3 AA nor GSTM1 A, GSTT1 null, or CYP1A1 Ile/Ile, alone or after correction for age and gender, were significantly associated with an increased number of BCC. However, after correction for age and gender, skin type 1, CYP1A1 m1m1, CYP2D6 EM, and GSTM1 null were significantly associated with increased numbers of tumors (Table 2). Significant age- and gender-corrected interactions in which the interaction term was more significant than either of the main effects were also identified; these interactions were between GSTM3 AA/skin type 1, GSTM3 AA/GSTM1 null, and GSTM3 AA/CYP1A1 m1m1 (Table 2).

Discussion

We have studied the influence of the newly described polymorphism at GSTM3 on risk of multiple BCC. This is the first report of allelism in a μ-class gene other than *GSTM1* as a candidate for susceptibility. Our immunohistochemical experiments showed specific expression of the gene in skin from cases and controls. The influence of GSTM3 on susceptibility was studied, first, by comparing genotype frequencies in controls with those in the BCC cases. Second, the frequency of genotypes in patients with 1 tumor was compared with that in patients with 2–35 lesions, a group that appears at increased risk of BCC. Third, a Poisson regression model was used in the cases with 2–35 tumors to identify genotypes and characteristics that influence tumor numbers.

Patients with a BCC are at high risk of suffering further lesions (14, 15). Importantly, this risk depends on the number of tumors present. Thus, in subjects with 1 lesion, the 5-year risk is 27%, and in those with 10 or more tumors, the risk is 90% (14, 15), indicating that accrual is not just dependent on time but rather that some subjects

have an increased susceptibility (15). Risk increases with age, male gender, exposure to UV, and skin types associated with burning without tanning (skin type I; Ref. 15). Exposure to UV constitutes an oxidative stress, and it is likely that individual differences in response to this stress will mediate risk. μ-class GSTs are attractive candidates for susceptibility to skin cancers, including BCC, as recent studies from our laboratory suggest that GSTM1 contributes to detoxification of the products of UV-induced oxidative stress. For example, GSTM1 null is associated with anti-Ro (but not anti-La) antibodies in patients with systemic lupus erythematosus (16). Significantly, production of anti-Ro antibodies in the absence of anti-La is associated with photosensitivity. Furthermore, GSTM1 null in combination with skin type 1 confers increased susceptibility to multiple BCCs (10, 11). If the influence of GSTM1 results from its metabolism of products of UV-induced oxidative stress, GSTM3 is also a candidate, as it utilizes hydrogen peroxide and cumene hydroperoxide (3). Our finding that GSTM3 is expressed in the epidermis and basal layer is compatible with the view that this enzyme is part of local antioxidant defenses. Thus, the basal layer includes the stem cells believed to be the targets for UV-induced damage from which BCCs arise.

Our analysis did not identify differences in the frequencies of GSTM3 genotypes in the total BCC group and controls or between cases with 1 tumor and those with 2–35 lesions, although it is noteworthy that the difference in the frequency of GSTM3 BB in these two case groups approached significance, suggesting that this relatively uncommon genotype is protective and worthy of further study. Within the cases with 2–35 tumors, a Poisson regression model was used to study the association between genotypes and number of lesions. This analysis showed that GSTM3 AA alone was not associated with an increased number of tumors. However, combinations of GSTM3 AA with skin type 1, GSTM1 null, or CYP1A1 m1m1 were significantly associated with increased tumor numbers. Indeed, the rate ratio for the interaction between GSTM3 AA and skin type 1 (2.058) was the highest identified. The classification of skin type 1 defines extreme sensitivity to UV that results in an inflammatory response but no tanning. Presumably, individuals with this skin type and GSTM3 AA or GSTM1 null are less able to metabolize the products of the oxidative stress associated with UV exposure. The finding of a significant interaction between GSTM3 AA and GSTM1 null complements studies showing lower levels of immunohistochemical positivity for GSTM3 in the lungs of subjects with GSTM1 null (7) and suggests a similar effect in skin. These data may reflect the linkage of *GSTM3*B* with *GSTM1*A* (8) and the consequent association of *GSTM3*A* with the other *GSTM1* alleles, the majority of which will be *GSTM1*0*. Our finding of a significant interaction between CYP1A1 m1m1 and GSTM3 AA provides further support for the view that polymorphism in *CYP* genes influences susceptibility to multiple BCC (10). Thus, CYP1A1 is expressed in skin and is induced by UV exposure (17). The consequences of the 3′-downstream mutation are unclear but may confer increased inducibility, suggesting that CYP1A1 m1m1 provides less effective detoxification on exposure to carcinogens (17, 18). Our finding of an interaction between CYP1A1 and a μ-class GST is compatible with data showing that GSTM1 null is associated with high inducibility of CYP1A1 transcription (19). The mechanism for the increased susceptibility to BCC conferred by CYP2D6 EM is also unclear, although recent studies indicate the importance of this gene in skin and brain tumors (10, 11, 20).

Our data suggest that GSTM3 AA confers increased risk of multiple BCC. Although the mechanism is unclear, we speculate that the YY1 transcription factor acts as a GSTM3 inducer in skin via 5′-AAGATA-3′ motif in *GSTM3*B*. YY1 expression is altered by many molecules, including growth factors (9), suggesting that *GSTM3*B* homozygotes are better able to induce expression of the gene after UV exposure. Our results provide further evidence for the importance of effective metabolism of potential carcinogens in skin and support the view that other factors in addition to UV exposure are determinants of disease risk.

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ıncal Tumor Site Is Associated with High Risk of ıltiple Basal Cell Carcinoma and Is Influenced by ıtathione *S*-Transferase, GSTT1, and Cytochrome ɔ0, CYP1A1 Genotypes, and Their Interaction

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l cell carcinoma (BCC) places increasing burdens linicians; incidence is rising and patients may lop multiple primary tumors. Although UV exre is critical, many patients develop tumors at exposed sites, such as the trunk, suggesting a tic predisposition. We previously showed that morphism in loci encoding the detoxifying enes, glutathione *S*-transferase (GSTM1, GSTM3, T1) and cytochrome P450 (CYP2D6, CYP1A1) ences susceptibility to BCC. We now describe a control approach in 345 patients with BCC that ines the role of these polymorphisms and pat characteristics (age, gender, skin type, hair r, eye color, smoking, occupation) in determinusceptibility to truncal tumors. GST and CYP types were identified using polymerase chain tion-based methods. Patients with one or more cal tumors were significantly younger (p = 0.0170) than those with no truncal tumors. Male gender also appeared more common in the truncal tumor group, although this did not achieve significance (p = 0.0925). Patients whose first tumor was truncal had significantly more tumors (p = 0.0297). GSTT1 null (p = 0.0245, odds ratio 2.24) and CYP1A1 Ile/Ile (p = 0.0386, odds ratio 2.86) were associated with truncal site after correction for age and gender. The combination, GSTT1 null and CYP1A1 Ile/Ile, was particularly significant (p = 0.0059, odds ratio = 2.95). These effects were present after correction for tumor numbers. These data show first, patients with truncal tumors constitute a high-risk group for BCC, second, a significant genetic influence on BCC site, and third, a significant interaction between GSTT1 and CYP1A1 genotypes. *Key words: genetic predisposition/detoxifying enzymes/polymorphism. J Invest Dermatol 108:519–522, 1997*

Basal cell carcinoma of skin (BCC) is the commonest cancer in Caucasians, accounting in 1994 for about 35% of all newly diagnosed neoplasms in the United States (Boring *et al*, 1993, 1994; Miller and Weinstock, 1994). A remarkable feature of this pathology is isk suffered by patients of developing further tumors at nt sites. Importantly, this risk depends on the number of s already present; 27% of patients with one tumor will suffer her tumor within 5 y compared with 90% in those with 10 or lesions (Kricker *et al*, 1993). Exposure to ultraviolet radiation is recognized as a critical factor in the pathogenesis of BCC ker *et al*, 1993; Kripke, 1994; Karagas and Greenberg, 1995), ugh the relationship between amount, timing, and nature of exposure and risk is complex and poorly understood. Indeed, compared with cutaneous squamous cell cancer, BCC are more common on generally less-exposed sites, especially the trunk (Kricker *et al*, 1993; Karagas and Greenberg, 1995), with lesions infrequently found on the forearms or backs of the hands. Also, whereas the incidence of BCC is increasing, it is the proportion of tumors on the trunk that demonstrates the greatest increase (Kricker *et al*, 1993). Together, these data suggest that susceptibility to BCC is dependent not merely on UV exposure but also on host genetic factors. This view is supported by data showing susceptibility to UVB-induced inhibition of contact hypersensitivity appears to be a better indicator of non-melanoma skin cancer risk than cumulative UV exposure (Schmieder *et al*, 1992).

Because UV exposure is a critical factor in the pathogenesis of BCC (Kripke, 1994; Karagas and Greenberg, 1995), factors that mediate individual response to the pleiotrophic effects of radiation are candidates for susceptibility to BCC. Thus, skin type and male gender are recognized as risk factors (Kricker *et al*, 1993). The concept of susceptibility, however, is complex because genetic factors, as well as influencing BCC risk in individuals without tumors, may also influence tumor numbers, rate of appearance of

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onreviations: BCC, basal cell carcinoma; GST, glutathione *S*-transCYP, cytochrome P-450; CI, confidence interval.

rs (accrual), and their site. We have studied genetic predis-on to BCC using each of these risk parameters. Some ising candidates for BCC risk are identified, polymorphism in involved in repair of UV-damaged DNA and detoxification products of oxidative stress being significant (Heagerty *et al*, Wei *et al*, 1994; Heagerty *et al*, 1996; Lear *et al*, 1996). Thus, ve shown that polymorphism at detoxifying enzyme gene loci as members of the glutathione *S*-transferase (GSTM1 null, M3 AA, and GSTT1 null genotypes) and cytochrome P450 2D6 EM and CYP1A1 m1 m1 genotypes) supergene families, ell as patient characteristics such as skin type 1, mediate ptibility to increased numbers of primary tumors and their rate crual (Lear *et al*, 1996; Yengi *et al*, 1996). GSTM1, GSTM3, GSTT1 appear attractive candidates, because these enzymes e the products of oxidative stress-induced damage to DNA ipids. Both CYP1A1 and its ligand-dependent transcription r, the Ah receptor, are widely expressed in extrahepatic tissues ding skin (Raunio *et al*, 1995), and although the gene has ly been studied in the context of environmental pollutants , polycyclic aromatic hydrocarbons), there is evidence that its ucts utilize endogenous ligands and participate in defense st oxidative stress (Nebert, 1994; Smith *et al*, 1995). The role YP2D6 is unclear (Ingelman-Sundberg and Johansson, 1995). xpression is mainly hepatic and although found in brain and tine, it has not been detected in skin (Raunio *et al*, 1995). The iation between CYP2D6 PM and susceptibility to Parkinson's se, however, suggests *in vivo* substrates that include endoge- neurotoxins and molecules containing amine or guanidino ps (Guengerich, 1995; Smith *et al*, 1995). Further, because mic agents such as arsenic predispose to multiple BCC (Yeh *et* 968), CYP2D6-mediated hepatic detoxification of photosensi-g agents may be important.

ecause the trunk is less exposed, or at least intermittently sed, to UV, it could be hypothesized that patients with truncal ors represent a high-risk group because they are less able to lle the products of UV-induced damage. Little research has essed this issue or investigated possible differences in individ- with and without truncal tumors, although it is known that sure to arsenic-containing tonics predisposes to truncal tumors h *et al*, 1968), supporting the view that factors other than UV e may be important. We hypothesize that the presence of ical tumors is more strongly associated with genetic susceptibil- Accordingly, we now describe studies to investigate genetic rences and individual characteristics in patients with and with-truncal tumors.

MATERIALS AND METHODS

lents The influence of genotypes and characteristics on tumor site was led in 345 unrelated Northern European Caucasians with histologically ed BCC. They were recruited from Dermatology clinics in the North fordshire Hospital, Stafford District General Hospital, and Royal Corn- Hospitals. Of first tumors, 80.1% were on the head/neck, 11.4% were ral, 6.5% were lower limbs, and the remaining 2.0% were on the upper k; 170 patients suffered one tumor and 175 patients between two and 30 ors. Recurrences were excluded from the total number of primary C. No patients with basal cell nevus syndrome, xeroderma pigmento- or BCC and another malignancy (cutaneous or internal) were ded. None of those approached refused to participate. All patients were ined and interviewed by a trained dermatologist (J.T.L., A.S., M.H.) to obtain information on hair (blonde/red and brown/black) eye color (blue/green and brown) at 21 y of age, skin type (types 1–4), pation (indoor/outdoor), smoking history (ever/never smokers) (Lear 1996), as well as the time between tumor appearance and presentation physician. Patients were also questioned regarding ingestion of arsenic-aining tonics and use of drinking water from potentially contaminated s. No arsenic-exposed patients were identified.

ntification of GSTM1, GSTM3, GSTT1, CYP2D6, and CYP1A1 notypes Blood (5 ml) was taken with Ethics Committee approval into lenediamine tetraacetic acid and stored at −50°C. GSTM1 null, A, B, A/B were identified using a polymerase chain reaction approach (Lear , 1996). GSTM3 genotypes were identified using primers to exon 6/7 ngi *et al*, 1996). *GSTM3*B* was differentiated from *GSTM3*A* by digestion with MnlI. GSTT1 null and expressers were also identified by polymerase chain reaction (Lear *et al*, 1996). Two mutant CYP2D6 alleles (G-A transition at intron 3/exon 4, base pair deletion in exon 5) were identified (Lear *et al*, 1996). Together these assays are about 90% predictive of phenotype in European Caucasians (Lear *et al*, 1996). Two mutant CYP1A1 alleles (exon 7 Ile-Val and 3′-flanking region *MspI* mutations) were detected using polymerase chain reaction (Lear *et al*, 1996; Yengi *et al*, 1996).

Table I. Patient Demographics

	Patients with No Truncal Tumors	Patients with at Least One Truncal Tumor
Mean age (y)	68.3 ± 12.2 (SD) (n = 263)	66.2 ± 11.9 (SD) (n = 74)
Males	54.1% (n = 268)	64.9% (n = 77)
Mean BCC no.	1.99 ± 1.81 (SD) (n = 270)	5.40 ± 6.03 (SD) (n = 75)
Blue and green eyes	70.1% (n = 148)	75.8% (n = 47)
Brown eyes	29.9% (n = 63)	24.2% (n = 15)
Skin type 1	16.4% (n = 34)	16.9% (n = 10)
Skin type 2–4	83.6% (n = 173)	83.1% (n = 49)
Ever smoker	65.1% (n = 142)	62.9% (n = 39)
Never smoker	34.9% (n = 76)	37.1% (n = 23)
Brown and black hair	74.8% (n = 101)	64.1% (n = 25)
Blonde and red hair	25.2% (n = 34)	35.9% (n = 14)
Outdoor occupation	16.7% (n = 13)	19.2% (n = 5)
Indoor occupation	83.3% (n = 65)	80.8% (n = 21)

Statistical Analysis T tests were used to assess differences in ages and number of BCCs between the two groups. χ^2-tests were used to examine for homogeneity between cases with and without at least one truncal tumor. As some genotype frequencies were small, the StatXact-Turbo statistical package was used to obtain exact p values. As various factors (GSTM1, GSTM3, GSTT1, CYP2D6, CYP1A1, gender, age, skin type, eye color, hair color, smoking status, occupation) were studied, the influence on tumor site of each alone and in combination was studied by logistic regression analysis. Combinations of genotypes and characteristics were studied in the presence of the main effects, and only those in which the interactive term was more significant than either of the main effects were included. For example, the combination GSTT1 null+CYP1A1 Ile/Ile was considered in the presence of GSTT1 null alone and CYP1A1 Ile/Ile alone. Because age and gender were significant confounding factors, analysis of the influence of genotypes and other patient characteristics were corrected for these factors. Since patients with at least one truncal tumor suffer more BCC, the associations with tumor site were further analyzed by correction for BCC number as well as age and gender.

RESULTS

Comparison of Patient Characteristics between Truncal and Nontruncal Groups **Table I** shows the characteristics of the study groups. The mean age at presentation of patients with at least one truncal tumor was lower than those without truncal tumors [p = 0.0170, χ^2_1 = 5.70, odds ratio = 0.975, 95% confidence interval (CI) = 0.955, 0.995], and the proportion of males was greater although this did not reach significance (p = 0.0925, χ^2_1 = 2.83, odds ratio = 1.571, 95% CI = 0.928, 2.659). The number of BCC in patients with at least one truncal tumor was greater than the nontruncal tumor group because the larger the number of tumors, the greater the probability that one will be truncal. The mean number of primary tumors in patients whose *first* tumor was truncal (n = 40; mean tumor number ± SD = 3.96 ± 4.74), however, was also significantly greater than those whose *first* tumor was not truncal (n = 312; mean tumor number ± SD = 2.58 ± 3.32; p = 0.0297, χ^2_1 = 4.73, odds ratio = 1.079, 95% CI = 1.008, 1.156). Other patient characteristics (skin type, eye color, smoking, occupation, hair color) were not significantly different between the two groups, although the presence of red or blonde

e II. Factors Demonstrating Significant Differences ween Patients with at Least One Truncal Tumor and Those with No Truncal Tumors[a]

	p Value	χ^2	Odds Ratio	95% CI
null	0.0245	5.06	2.24	1.11–4.53
1 Ile/Ile	0.0386	4.28	2.86	1.06–7.72
null + CYP1A1				
le	0.0059	18.70	2.95	1.37–6.39

are corrected for age and gender.

orrected for age and gender, approached significance (p =).

ic Factors Associated with the Presence of Truncal rs **Table II** shows the genotypes, corrected for imbalances ent age and gender, associated with tumor site. By logistic ion, the age-corrected proportion of GSTT1 null and A1 Ile/Ile genotypes was significantly greater in patients with one truncal tumor. The importance of GSTT1 and CYP1A1 pes is further emphasized by the increased significance of the tion (the combination of both GSTT1 null and CYP1A1). In order to further assess the importance of these genotypes rminants of tumor site, the associations with tumor site were rrected for BCC number as well as age and gender, because s with at least one truncal tumor suffer more BCC **(Table I)**. demonstrated that individually, both GSTT1 null and A1 Ile/Ile remained significant but with reduced odds ratios 0.0316, odds ratio = 1.44, 95% CI = 1.24, 1.66, and p =), odds ratio = 1.51, 95% CI = 1.29–1.77, respectively). The nation of these genotypes (both GSTT1 null and CYP1A1), however, remained highly significant, with a decreased p and increased odds ratio (p = 0.0035, odds ratio = 3.56, 95% 1.52–8.34) despite this rigorous correction. None of the other pes examined (GSTM1 null, GSTM3 AA, CYP2D6 EM, and A1 m1 m1) was associated with tumor site, either alone or in nation with other genotypes or patient characteristics.

DISCUSSION

ave studied the influence of detoxifying enzyme genotypes atient characteristics on the development of truncal BCC by aring these factors in patients with at least one truncal BCC ose with no truncal tumors. BCC are a major burden to care agencies, with an incidence in the United States as high per 100,000 people and reported annual increases of about (Karagas and Greenberg, 1995). In view of this high and sing incidence, and because mortality is low, it is predicted evalence of this tumor will be greater than that for all other s combined (Boring *et al*, 1994). Thus, the lifetime risk of for an American child born in 1994 is estimated to be 28–33% r and Weinstock, 1994). Further, although exposure to UV is gnized risk factor, our data show that about 20% of patients BCC develop at least one tumor at sites generally believed to relatively little exposure.

e mean age at first presentation of patients with at least one al tumor was lower than in those with no truncal tumors. rmore, patients whose first tumor is truncal are at increased further tumors, suggesting that patients with truncal tumors ent a high-risk group. The increased prevalence of males in uncal compared with the nontruncal tumor group supports cal clinical impression, although this failed by a small margin hieve statistical significance. This is consistent with the ation of both male gender and truncal tumor site with sed risk of multiple tumors (Lear *et al*, 1996). Outdoor ation was not a significant factor in our analysis, suggesting ales do not have increased chronic UV exposure compared females. Other explanations for the increased risk associated male gender, such as less effective melanization than females od *et al*, 1994), are worthy of investigation.

Since both age and gender were significant confounding factors, data on the influence of genotypes and patient characteristics were corrected for imbalances in these factors. Following correction, none of the patient characteristics studied were associated with tumor site. In particular, skin type 1 was not significantly different between the two groups, suggesting that patients with truncal tumors are no more likely to burn on UV exposure than patients with nontruncal tumors.

Analysis of the role of detoxifying enzymes in determining tumor site showed that GSTT1 and CYP1A1 are important, even after correction for BCC number as well as age at first presentation and gender. Although the level of significance of these factors was relatively low, the influence of the highly significant interactive term (both GSTT1 null and CYP1A1 Ile/Ile) suggests the effects are real. We have previously shown that GSTT1, whose enzyme substrates include the products of oxidative stress–induced damage to DNA, is significantly associated with rate of accrual of BCC in patients with multiple tumors (Lear *et al*, 1996). The data presented here support the view that GSTT1 null genotypes are associated with faster appearance of further tumors and the presence of truncal tumors. Thus, it appears that this genotype exerts its effect on the rate of BCC appearance, because it predisposes to tumors on both chronically and intermittently exposed sites. These data suggest that individuals deficient in the ability to repair UV-derived oxidative stress–induced damage to DNA and/or lipids are genetically predisposed to BCC and are more likely to develop subsequent tumors. GSTT1 null individuals may be more susceptible to UV-induced BCC following even relatively little UV exposure, resulting in an increased number of tumors at a younger age and the development of lesions on intermittently exposed sites such as the trunk. Unlike GSTT1 null, however, no GSTM1 null effect was identified, complementing data showing that the products of these loci have some differences in substrate specificities (Norppa *et al*, 1995).

Data showing that UV-oxidized tryptophan binds to the CYP1A1 ligand-dependent Ah receptor transcription factor and UV induces CYP1A1 expression in skin suggest a role for allelism at this locus in skin carcinogenesis (Gonzalez, 1995). The influence of CYP1A1 Ile/Ile on tumor site shown here is more difficult to interpret than the GSTT1 effect, because we have shown that this genotype was associated with slower BCC accrual, although this effect was relatively weak. It is not known whether CYP1A1 is uniformly expressed in skin; UV is known to induce expression, suggesting that differential effect in chronically and intermittently exposed skin is possible.

Although interactions between GSTM1 and CYP1A1 have been identified in mediating risk of lung cancer (Anttila *et al*, 1994), this report describes an interaction between GSTT1 and CYP1A1. Indeed, particularly after correction for age, gender, and BCC number, the odds ratio for this effect (3.56) was relatively high. This study presents further evidence that patients with truncal tumors represent a high-risk group and that factors other than UV exposure are important in the pathogenesis of these tumors.

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feron beta 1b has to be self administered
neously every other day. Patients run the
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ised the drug by the development of anti-
after three years.[3]
feron beta 1b has no significant effect on
velopment of disability in multiple
s.[3] Quite why it has been licensed is a
to many of us. Why is Schering allowed
rtise it in the *BMJ*?

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Hodgkin's lymphoma and ultraviolet radiation

re inconsistent

—Graham Bentham confirms known geo-
al patterns when he reports a positive
ion between the incidence of non-
in's lymphoma and ambient solar ultraviolet
n in England and Wales.[1,2] His results con-
th those from the United States, where a
e association was reported,[3] further fuelling
bate over whether exposure to solar
let radiation increases the risk of non-
in's lymphoma. If there is a positive
ion then those people working in outdoor
tions might be expected to be at increased
this malignancy.
examined the risk of non-Hodgkin's
oma among people judged to spend most
r working day outdoors, using one of the
population based cancer registration
s in the world. The data and methodology
een fully described elsewhere.[4] Briefly,
tional information was available on
3 men and 119 227 women registered as
cancer in England between 1981 and
Ten job groups were defined as outdoor
tions (see table 1); they accounted for a
f 17 636 registrations in men and 960 in
. Proportional registration ratios were
estimate the relative risk of specific can-
outdoor workers compared with workers
ccupations combined, with adjustment for
ve year age groups), social class (six
), and cancer registry of origin (13
es). All registrations for which the
tion was adequately described formed the
d for comparison.
e 1 shows the risks for all cancer sites in out-
orkers by sex. In men there was a significant
xcess of non-melanocytic skin cancer,
ing high exposure to solar ultraviolet
n[5] but no excess of non-Hodgkin's
oma. In women the reverse was found, with
ificant 56% excess of non-Hodgkin's
oma but no excess of non-melanocytic skin
. It is notable that 25 of the 27 women diag-
as having non-Hodgkin's lymphoma were
s and that similar observations among
ural employees have been attributed by
to exposure to pesticides.[6]
also examined the relation between the
tion specific risks for non-melanocytic
ancers and non-Hodgkin's lymphoma

Table 1—*Adjusted proportional registration ratios (95% confidence intervals) for outdoor workers* aged 20-74, England, 1981-7*

Cancer (ICD code, 9th revision)	Men: No Observed	Men: Adjusted proportional registration ratio	Women: No Observed	Women: Adjusted proportional registration ratio
Oral cavity (141, 143-145)	130	96 (81 to 115)	6	160 (59 to 349)
Salivary (142)	22	91 (57 to 138)	0	—
Pharynx (146-148)	79	78 (62 to 98)	2	70 (9 to 255)
Oesophagus (150)	452	103 (94 to 113)	14	111 (61 to 187)
Stomach (151)	1180	96 (91 to 102)	26	89 (59 to 131)
Small intestine (152)	37	124 (88 to 172)	2	161 (20 to 584)
Colon (153)	927	95 (90 to 102)	49	103 (77 to 137)
Rectum (154)	848	103 (97 to 111)	31	117 (80 to 167)
Liver (155)	116	99 (82 to 119)	1	29(1 to 167)
Gall bladder (156)	66	74 (58 to 95)	8	152 (66 to 300)
Pancreas (157)	591	104 (96 to 113)	21	101 (63 to 155)
Retroperitoneum (158.0)	10	78 (38 to 145)	0	—
Peritoneum (158.8-158.9)	10	80 (38 to 147)	1	234 (6 to 1307)
Nose and nasal sinuses (160)	40	111 (80 to 152)	1	70 (2 to 394)
Larynx (161)	233	95 (84 to 109)	0	—
Lung (162)	5354	98 (95 to 101)	115	101 (84 to 122)
Pleura (163)	67	69 (54 to 89)	1	61 (2 to 342)
Thymus and mediastinum (164)	22	138 (87 to 209)	2	309 (38 to 1117)
Bone (170)	32	103 (71 to 146)	2	123 (15 to 448)
Soft tissue (171)	65	93 (72 to 119)	7	207 (84 to 428)
Melanoma (172)	125	94 (79 to 113)	9	67 (31 to 128)
Non-melanocytic skin (173)	1564	119 (114 to 126)	49	88 (65 to 117)
Female breast (174)	NA	—	190	96 (84 to 112)
Male breast (175)	28	98 (65 to 142)	NA	—
Uterus (179, 181, 182)	NA	—	30	99 (67 to 143)
Cervix (180)	NA	—	37	88 (63 to 123)
In situ cervix (233.1)	NA	—	105	93 (76 to 113)
Ovary (183)	NA	—	44	99 (72 to 133)
Prostate (185)	1126	108 (102 to 115)	NA	—
Testis (186)	190	100 (86 to 115)	NA	—
Other male genital organs (187)	57	110 (84 to 144)	NA	—
Bladder (188, 189.1-189.9)	925	89 (84 to 95)	15	84 (48 to 140)
Kidney (except pelvis) (189.0)	268	88 (79 to 100)	8	98 (43 to 195)
Eye (190)	19	82 (49 to 128)	1	85 (2 to 479)
Brain and meninges (191, 192, 225, 237.5-9, 239.6)	489	104 (95 to 114)	24	113 (73 to 169)
Malignant brain (191)	373	107 (97 to 119)	11	87 (44 to 156)
Thyroid (193)	39	97 (69 to 133)	8	131 (57 to 259)
Suprarenal and other endocrine organs (194)	8	59 (26 to 118)	0	—
Ill defined and secondary (195-199)	894	94 (89 to 101)	50	112 (84 to 149)
Non-Hodgkin's lymphoma (200, 202)	401	95 (86 to 105)	27	156 (103 to 228)
Hodgkin's disease (201)	120	88 (73 to 105)	3	63 (13 to 187)
Myeloma (203)	211	112 (98 to 128)	3	42 (9 to 126)
Leukaemia (204-208)	395	105 (96 to 117)	12	85 (44 to 149)

ICD = *International Classification of Diseases*. NA = Not applicable.
*Outdoor workers were defined by using the Southampton occupational classification and included the following job groups (number of registrations given in parentheses): farmers (men, 8245; women, 838); foresters (men, 194; women, 6); fishing and related workers (men, 292; women, 3); scaffolders (men, 298; women, 4); bricklayers and tilesetters (men, 2957; women, 0); roofers and glaziers (men, 556; women, 16); builders etc (men, 3760; women, 79); rail track workers (men, 358; women, 2); road construction workers and paviours (men, 662; women, 9); mains and service layers (men, 314; women, 3).

because it is well known that exposure to solar ultraviolet radiation increases the risk of skin cancer.[5] No linear association was observed, for either men (correlation coefficient 0.07) or women (correlation coefficient –0.02).

The aetiology of non-Hodgkin's lymphoma is poorly understood, and many factors are likely to be important. Given the inconsistencies in the data presented here and elsewhere, more detailed research is clearly required before a causal role is ascribed to solar ultraviolet radiation.

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Basal cell carcinoma may be linked to haematological malignancy

EDITOR,—Graham Bentham shows that the incidence of non-Hodgkin's lymphoma is positively associated with exposure to solar ultraviolet radiation,[1] giving insights into the previous

1—*Comparison of internal malignancies in patients with multiple basal cell carcinomas and controls*

ancy	Cases	Controls
ological*	5	0†
	3	1
testinal tract	1	2
inary tract	4	4
	13	7

ients had non-Hodgkin's lymphoma and one had yeloid leukaemia.
25, McNemar's test (StatXact Turbo statistical

tion of a link between non-Hodgkin's ma, malignant melanoma, and squamous cancer.[2] He did not, however, assess any ion with basal cell carcinoma, the nest malignancy in white people.

ure to ultraviolet radiation is recognised as l factor in the pathogenesis of basal cell car- , presumably partly because of resulting : suppression.[3] It could therefore be sised that an association would exist basal cell carcinoma and malignancies d with immune suppression, such as logical neoplasms.[4] Importantly, many with basal cell carcinoma develop multiple and it might be presumed that these represent a group with high susceptibility is, one at greatest risk of internal ncy. We report findings from a case-control identify an association between multiple cell carcinoma and haematological ncy.

ther 141 white patients from northern (mean age 71; 62 women) with histologi- wed primary basal cell carcinoma (range nours per patient) were recruited over 18 from dermatological outpatient clinics and up for roughly three years. Patients with pes of skin cancer or Gorlin's syndrome luded. Controls matched for age and sex e to one control) who had benign skin ns (benign naevi, 67; eczema, 47; leg 0; rosacea, 7; others, 10) were recruited in clinics. The presence of any histologically nternal or haematological malignancy in controls was noted. Thirteen cases, d with seven controls, had an internal ncy (table 1). This difference was not nt, though the difference between the of cases (5) and controls (0) with a logical malignancy approached signifi- ur of the five cases developed the haema- malignancy after developing their first carcinoma.

ilot study suggests a link between basal noma and haematological malignancy. nk would have implications for follow up erstanding of the pathogenesis of this ncy.

knowledge the support of the Cancer Campaign in this study.

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Peanut and nut allergy

Creams and ointments containing peanut oil may lead to sensitisation

EDITOR,—Pamela W Ewan suggests that peanut allergy in children is due to the ingestion of peanut butter before the age of 1 year but acknowledges that some children react after their first known exposure.[1] She supposes that minute amounts of allergen might be present in breast milk or hidden in foods, but it is not widely appreciated that arachis oil (peanut oil) is present in many preparations that are applied topically. Breast feeding mothers often treat sore nipples with chamomile ointment, the main ingredient of which is arachis oil. Presumably some of this is ingested by the infant, which could lead to sensitisation.

Children might also become sensitised to peanut allergen through skin contact. Despite the name, zinc and castor oil ointment, which is often used to treat napkin dermatitis, is 30% peanut oil. Napkin eruptions are common in children with atopic dermatitis, and absorption of allergens is increased across broken or inflamed skin, so sensitisation to peanuts could occur in this way. Certainly, skin contact leading to sensitisation occurs with other allergens, and although no evidence exists of specific induction of peanut allergy by this route, I prefer not to recommend the use of creams or ointments that contain arachis oil.

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Baby massage oils could be a hazard

EDITOR,—The recent articles on peanut allergy do not mention the fact that baby massage is becoming popular and that the oils used in this might pose a hazard.[1,2] If tiny babies suck their hands after a hand massage with arachis (peanut) oil they may ingest large quantities of nut products. Special care baby units such as that at Queen Charlotte's and Chelsea Hospital recommend arachis oil for massages of premature babies. Perhaps the potential risk should be indicated on the labels of massage oils and in baby massage books and at classes. Alternative products could be used.

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Serious adverse reactions to adrenaline are becoming more likely

EDITOR,—Pamela W Ewan's findings with regard to peanut and nut allergy[1] agree with my personal experience of the problem: such allergy occurs in otherwise atopic subjects, it is acquired early (possibly in utero in some cases), and after the first adverse reaction sufferers are almost invariably aware of the problem—although in some this is at a subconscious level, and they become averse to all nuts without remembering the reason.

I doubt, however, Ewan's implication that the problem is becoming much more common. It is difficult to obtain meaningful figures of prevalence in the past, but I have estimated a probable prevalence of hypersensitivity to any nut of between 1% and 5% in the population from which my patients have been drawn over some 30 years, and without much variation. Previously, those affected knew that they could not eat nuts, avoided them assiduously, were generally free of symptoms, and did not consult a doctor, which explains the medical profession's lack of awareness of the problem in the past. Those affected were detected, if at all, when they attended allergy clinics for investigation of other manifestations of atopy. Now, on the other hand, as a consequence of publicity generated by such organisations as the Anaphylaxis Campaign and British Allergy Foundation,[2] many people who managed very well by themselves over many years have been informed that they must seek medical advice and be referred to clinics, where investigation confirms only what they know already.

A consequence of this is that we are seeing a true increase in serious adverse reactions to adrenaline injections, which are now being offered routinely to such patients and used for any symptoms, whether trivial or even unrelated to hypersensitivity. Parenteral adrenaline certainly plays a major part in the management of dangerous anaphylaxis and angio-oedema, but these are exceedingly rare (especially when one considers the high prevalence of sensitivity to nuts), and they probably usually involve additional, non-atopic factors. Avoidance remains the golden rule and is usually practised successfully because the patient's tongue and lips are aware of traces of the allergen in prepared foods to which the patient has been blinded. Better labelling of foods would help, but I fear that we may see more frequent dangerous episodes, including deaths, due to adrenaline than to anaphylaxis unless a more measured response to the problem is developed.

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2 Sampson HA. Managing peanut allergy. *BMJ* 1996;312:1050-1. (27 April.)

Study was not designed to measure prevalence

EDITOR,—Media coverage of Pamela W Ewan's study of peanut and nut allergy[1]—for example, in BBC Radio 4's *PM* programme—highlighted the conclusion in the abstract that "peanut and nut allergy is becoming common"; we note that in the key message this statement has become "peanut and nut allergy are becoming more common." The study, however, was not designed to measure the prevalence of such allergies and, indeed, was restricted to patients seen during one year at a particular allergy clinic; the only evidence given in support of the supposed

es, isomerization of urocanic acid and activation of a surising range of cytokine genes within keratinocytes. The NA photoproducts (18) and UVB-generated oxygen interediates (19) have been implicated as damaging to cutaneus immunity. The possibility exists that polymorphisms at ci that encode excision and repair genes or at loci regutting free radical quenching may exist and could have a ofound effect on cutaneous immunity. Similarly, polymorisms at cytokine gene loci may contribute to UVB-S.

Finally, UVB radiation produces multiple, mechanistically stinct immune aberrations. *Locally,* at the irradiated site, VB radiation (1) impairs induction of CH, (2) promotes pten-specific tolerance and (3) creates a sustained immusuppressive microenvironment (20). *Systemically,* UVB diation (1) impairs both delayed and CH to a variety of tigens (21) and (2) induces active, nonspecific unresponveness. Our studies have revealed genetics factors operatg *in only one* of these aberrations, but we suspect that, in ne, other polymorphic genetic factors will be identified at contribute to the damaging immune effects of UVB raation and thereby alter an individual's risk of developing nlight-induced skin cancer.

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Polymorphism in Detoxifying Enzymes and Susceptibility to Skin Cancer

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Apart from Gorlin's syndrome (1), the role of genetic factors in the pathogenesis of the nonfamilial skin cancers is generally unclear. Although excessive ultraviolet radiation (UV) appears the major causative agent, its effects are complex and other factors (soot, cigarette smoke, arsenic) are relevant, suggesting that the effectiveness of detoxification of the products of UV-induced oxidative stress as well as xenobiotics will partly mediate susceptibility. Many genes encoding detoxifying enzymes are polymorphic indicating particular genotypes identify detoxification-deficient and therefore, at-risk subjects (2). An understanding of the factors predisposing to skin cancers is important as their incidence is increasing (3). In particular, basal cell carcinoma

is the commonest malignancy in Caucasians. Ge-
ences appear to be important in this cancer as pa-
one lesion are at high risk of further primary BCC
risk depends on the number of lesions present;
with one lesion the 5 year risk is 27%, in those
more tumors the risk is 90% (3,4), suggesting
not just dependent on time but rather some sub-
increased risk (4). This risk increases with age,
r, exposure to UV and skin type 1 (*i.e.* subjects
s burn and never tan) (5).

xygen species and antioxidant enzymes

A has pleiotropic effects on skin cells including
)NA strand breaks and oxidation of membrane
mino acids in skin (6), suggesting differences in
this stress will mediate risk of skin cancer. A
rted by studies showing the effectiveness of re-
-damaged reporter genes is associated with risk
BCC (7). Because exposure to UV constitutes an
ress with the formation of reactive oxygen spe-
in skin (8), the detoxification of these species
oducts is also likely to be critical, thus implicat-
volved in these reactions as candidates. Because
itially deleterious effects of ROS, cells use var-
nisms to modulate their concentration including
expression of the antioxidant enzymes, CuZn-
eroxide dismutase, catalase and Se-glutathione
(9). Coordinated expression of these genes en-
tion of the levels of superoxide radical and H_2O_2
suppression of formation of the dangerously re-
xyl radical ($HO^{\bullet}$). While appropriate expression
zymes protects cells, many cellular molecules
evidence of oxidative damage even in health
g conditions. Much interest has focused on the
mage to membrane lipid and mitochondrial and
lA. Peroxidation of membrane lipid is continu-
in healthy tissues, levels of oxidized products
londialdehyde and alkenals are low indicating
letoxication of these dangerous compounds. In-
peroxidation has been found in various patholhile it may be a consequence rather than cause
is potentially deleterious as it alters membrane
permeability. Lipid peroxidation results in for-
eroxy radicals that form lipid hydroperoxides.
pose to a complex mixture that includes cyto-
les such as malondialdehyde, 2-alkenals and 4-
als. The DNA strand breaks and damage to
gars are a further early and significant result of
ss. They result from $HO^{\bullet}$-induced damage or
nucleases. The $HO^{\bullet}$-induced damage to bases
ied by products such as 5-hydroxymethyluracil,
mine and 8-hydroxyguanine (10).

S-transferase phenotype and BCC

xygen as an electron acceptor requires expres-
nes that decrease production of $HO^{\bullet}$ and/or al-

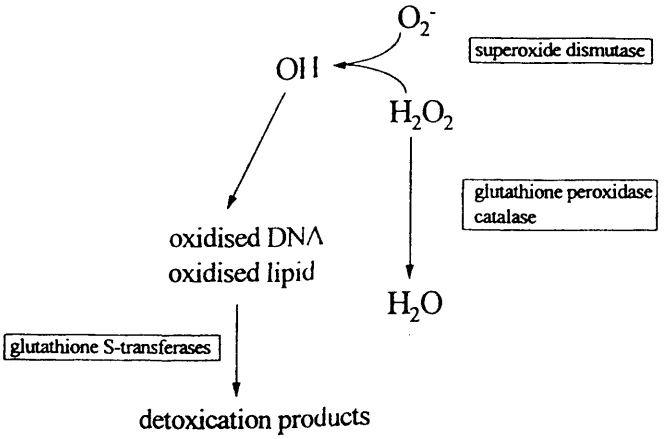

Figure 9. Relationship between glutathione *S*-transferase and other antioxidant enzymes.

low repair/replacement of damaged lipid and DNA. Although these processes remain poorly understood, peroxidase activity appears central in detoxication of lipid and DNA hydroperoxide. Thus, recent data suggest the enzymes of the glutathione *S*-transferase (GST) supergene family are critical in the detoxification of cytotoxic, lipid and DNA products of oxidative stress including DNA hydroperoxides (Fig. 9) (11). Four GST gene families are expressed in mammalian tissue cytosols; alpha, mu, theta and pi. Each of these GST families may include multiple genes, with active enzymes comprising homo- and heterodimeric combinations of monomers encoded by genes of the same family. Five mu class genes are arranged in tandem on chromosome 1. One of these, GSTM1 has been intensively studied. Indeed, the observation that GSTM1 can utilize 5′-hydroxymethyluracil led to the suggestion it is involved in repair of UV-damaged DNA (12). Two cytosolic theta class GST, T1–1 and T2–2, have been isolated from human liver. Importantly, the distribution of enzymes of different GST gene families expressed in cytosol can be different; thus, immunofluorescence studies in cultured glomerular cells show both mesangial and epithelial cells express alpha class Ya and mu class Yb1/Yb2 though their subcellular distributions differ. Ya was distributed in cytosol while Yb1/Yb2 was predominantly perinuclear. These data support reports of nuclear staining for GST and the view that mu GST catalyze the reduction of peroxidised DNA (11).

Several GST genes are polymorphic with common null alleles identified at GSTM1 and GSTT1 (12). Thus, GSTM1 demonstrates phenotypes that arise from combinations of the *GSTM1*0, GSTM1*A* and *GSTM1*B* alleles. *GSTM1*0* is entirely deleted and homozygotes (GSTM1 null) who comprise about 50% of many populations studied (13), express no protein. So far two alleles have been identified at GSTT1; *GSTT1*0* and *GSTT1*A* (12,14). Homozygosity for *GSTT1*0* is found in about 20% of control subjects (13). Both *GSTT1*0* and *GSTM1*0* homozygotes appear at greater cancer risk though the specificity (*i.e.* why some cancers but not others) and sensitivity (*i.e.* how much extra risk is conferred) remains unclear (2,13).

The establishment of polymerase chain reaction-based genotyping methods for many detoxifying enzymes allows for the use of genetic epidemiological approaches to exam-

ns: BCC, basal cell carcinoma; GST, glutathione *S*-
ROS, reactive oxygen species; SLE, systemic lupus
is.

Table 12. Factors influencing susceptibility to single and multiple BCC

GSTM1 A/B	(χ^2_1 = 4.52; P = 0.048; odds ratio = 0.29)
Male gender	(χ^2_1 = 5.29; P = 0.021; odds ratio = 1.45)
Male gender + GSTM1 null	(χ^2_1 = 9.44; P = 0.002; odds ratio = 1.72)
Skin type 1 + GSTM1 null	(χ^2_1 = 6.87; P = 0.009; odds ratio = 3.25)
GSTM1 null + GSTT1 null	No significant effect
GSTM1 null + CYP2D6 EM	No significant effect
Ever smoking	No significant effect
GSTM1 null + smoking	No significant effect
GSTT1 null + smoking	No significant effect

ne the influence of allelic variation on risk of single and nultiple BCC (15,16). Thus, the importance of putatively igh risk null genotypes can be assessed *in vivo*, in the presnce of other influencing factors. Accordingly, we have studed genotype frequencies in 737 English Caucasians with ingle and multiple BCC (between 2 and 50 tumors per paient) and compared them with those in 563 corresponding ontrols recruited from in- and out-patients in the same areas n the Midlands and south of England. As the effects of enotypes are likely to be influenced by characteristics (genler, skin type, eye color), interactions with these factors were studied. Frequencies of GSTM1 null, A and B were ot different though GSTM1 A/B was significantly lower in nultiple BCC (1.2%) than in controls (4.1%), confirming the protective effect of this genotype (Table 12) (15). We found no differences in GSTT1 genotype frequencies in cases and controls. Recent studies showing the interactive effects of GSTM1 and CYP1A1 genotypes suggest that the influence of detoxifying enzymes will depend on allelism at other relevant loci (17). Such interactions were studied by comparing multinomial frequency distributions in mutually exclusive groups. Comparison of the frequency distributions for combinations of GSTM1 null and GSTT1 null showed no significant differences between the controls and patients with single and multiple BCC. In separate studies, we also failed to identify interactions between these GST genotypes and polymorphic variants at the cytochrome P450 CYP2D6 and CYP1A1 loci.

The importance of gender in the pathogenesis of multiple BCC was shown by the significantly greater proportion of males in the multiple BCC group (61.3%) compared with controls (47.0%) and single BCC (52.2%) (Table 12). Further, the frequency of the combination GSTM1 null/male gender was significantly greater in patients with multiple tumors (P = 0.002) as was the proportion of subjects who were GSTM1 null with skin type 1 (P = 0.009) (Table 12). This latter combination gave an odds ratio greater than that for skin type 1 or male gender, indicating its importance. Frequency distributions of GSTM1 null/skin type 1/male gender in single and multiple BCC were almost significantly different (exact P = 0.051). No significant interactions between GSTT1 null, gender or skin type were identified.

These data emphasize the importance of GSTM1 in mediating susceptibility to multiple BCC. Protection is associated with GSTM1 A/B but not GSTM1 A or B, suggesting a gene dosage effect specific to multiple BCC but not other skin cancers (15). Homozygous deficiency of the gene, though not a factor alone, is significant in combination with skin type 1. Presumably such individuals are less able to cope with products of oxidative stress. The mechanism for interaction between GSTM1 null and gender is unclear though females may be protected as estrogens stimulate melanin production and their skin is more reflective to UV. A further insight into the role of GSTM1 and GSTT1 in handling oxidant stress in skin comes from studies in systemic lupus erythematosus (SLE) a disease characterized by multisystem inflammation and autoantibodies to intracellular antigens. While GSTM1 and GSTT1 null frequencies in patients antibody positive for dsDNA, La and Ro together and U1RNP were not different to those negative for these antibodies. All 12 cases with anti-Ro (in absence of La) were GSTM1 null, a significantly different frequency to that in 79 Ro-negative subjects (exact P = 0.0016). Further, the difference in the frequency of GSTT1 null in these groups approached significance (exact P = 0.0639). Multinomial frequency distributions of GSTM1 and GSTT1 null in anti-Ro positive and control subjects were significantly different (P = 0.0034), providing evidence for an association between GSTM1 and GSTT1 and production of anti-Ro in SLE. Importantly, this phenotype is strongly associated with photosensitivity. Photosensitivity appears to result from enhanced ROS modifying proteins to reveal autoantigenic, cryptic determinants and formation of surface microblebs that contain Ro-antigen. Thus, UV-induced ROS may both induce antigenicity of Ro and perpetuate its exposure to the immune system. If GSTM1 and GSTT1 enzymes limit ROS-associated effects, null genotypes would be associated with increased UV-induced damage and enhanced surface expression of Ro. Our data provide further evidence that these genes limit the effects of UV and present a mechanism for disease heterogeneity in SLE (13).

Conclusions and future directions

The importance of factors other than UV in the pathogenesis of BCC has been suspected from data from studies using a variety of experimental approaches (4,7,15,18). The use of case control studies can help identify interactions between genotypes and characteristics. Our data support the view that the development of multiple BCC is not merely determined by time but rather, some patients have a genetically mediated increased risk (4). As no environmental factor apart from UV has been identified, we propose GSTM1 is part of cellular antioxidant systems. Clearly further studies are needed to define the sensitivity and specificity of GSTM1 null and function of GSTT1 as well as other detoxifying enzymes, including

of the cytochrome P450 supergene family, many of
embers appear critical in the metabolism of endog-
micals involved in control of cell-specific functions
xpressed in skin (19,20). The studies described in
w are based on comparison of factors in controls,
with a single tumor and those with more than one
ing a dichotomous outcome. Presumably such stud-
nderestimate the influence of genetic and other fac-
me cases (and controls) will develop further tumors.
ore, the factors mediating susceptibility to two tu-
not be the same as those influencing development
more primary lesions. Clearly studies based on iden-
sociations between factors and tumor numbers with-
ormation are needed. Tumor accrual (number of new
r year) is a further parameter of susceptibility. Such
owever, require accurate data on the number and
lesions and often such information is not readily
. Such studies will help understand the biochemical
at determine tumor numbers and accrual, which are
ncertain because the relationship between exposure
d events in skin and the eventual appearance of a
unclear.

presently also studying the influence of GST and
es on susceptibility to squamous cell carcinoma of
malignant melanoma. Initial studies on GSTM1 ge-
identified no differences between cases and controls
ugh because these pathologies are considerably less
than BCC, it is harder to recruit the large case groups
study of interactions between genotypes and indi-
aracteristics. Interestingly, Wolf *et al.* (21) showed
sed frequency of mutant CYP2D6 alleles in patients
ignant melanoma compared with controls though the
failed to achieve significance. The biochemical ba-
is observation is unclear as this gene has not been
be expressed in skin. It may reflect the importance
ive hepatic detoxification of unidentified photosensi-
nts. Thus, arsenic has been shown to be associated
increased risk of Bowen's disease (22).

studies on the influence of GST on risk have com-
notypes in cases and controls. Because cases are
nous in terms of the events leading to malignancy,
associations between mutations in target genes such
d GST genotypes will help to assess the importance
genes. Thus, studies in lung cancer patients showed
frequencies of mutations in p53 and H-ras 1 was
n *GSTM1*0* homozygotes than those subjects who
d at least one functional allele (23). However, al-
hese data suggest GSTM1 protects p53 against mu-
, this effect is not universal as we found no rela-
between GSTM1 genotype and p53 overexpression
ations in ras and gsp in pituitary adenomas (24).

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Oncogenes and Tumor Suppressor Genes in Photocarcinogenesis

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Photocarcinogenesis of the skin is a multistep process that begins when the cellular DNA is damaged upon exposure to UV light. A cascade of events that occurs following the DNA damage include accumulation of p53 and overexpression of certain p53-regulated genes, cell cycle arrest and DNA repair. Occasional errors in DNA repair lead to the generation of mutations. Although the precise genetic alterations that occur during photocarcinogenesis are unknown, there is evidence to indicate that alterations in the *p53* tumor suppressor gene, and to a lesser extent, *ras* oncogenes, contribute to the development of skin cancer. Genetic alterations involving mutation, amplification or loss of heterozygosity (LOH)**** in *ras* and *p53* genes have been detected in human skin cancers and UV-induced mouse skin cancers.

Ras gene mutations in human and UV-induced mouse skin cancers

H-*ras*, K-*ras* and N-*ras* proto-oncogenes each encode protein products of 21 kDa (1,2), which are localized at the cell membrane. The Ras proteins display intrinsic GTPase activities and participate in the transduction of signals to the nucleus *via* a cascade of kinases (1,2). Several studies have shown that human skin cancers contain mutations in the *ras* oncogenes (3–6), although the frequency of such mutations is variable. This variation may reflect differences in the ethnic and environmental backgrounds of the patients or may be due to differences in the methods of mutational analysis.

Analogous to human skin cancers, UV-induced murine skin cancers also display *ras* mutations. Our studies have shown that 4 of 20 UV-induced mouse skin tumors contained either C → A or A → G base substitutions at codon 61 of the N-*ras* gene (7). Interestingly, *ras* gene mutations in UV-induced mouse skin tumors appear to be restricted to certain strains of mice. For example, UV-induced C3H mouse skin cancers display *ras* mutations, albeit at 20% frequency, whereas skin cancers induced by UV in SKH-1/hr strain of mice do not harbor *ras* mutations (8,9). The reasons for this observation is unknown but it is possible that differences in repair mechanisms or repair efficiency of UV-induced DNA damage between C3H and SKH-1/hr strains of mice may account for this phenomenon.

H-*ras* polymorphism and LOH in human skin cancers

The human c-H-*ras*-1 proto-oncogene located on the short arm of chromosome 11 (11:p15) exhibits a *Bam*HI restriction fragment length polymorphism (RFLP) (10). This polymorphism has been attributed to the presence of tandemly repeated 28 base-pair nucleotides at the 3′ end of the H-*ras* coding sequence. This structural feature results in the generation of two allelic restriction fragments following digestion with *Bam*HI. Several studies have shown that one of the c-H-*ras* alleles is deleted in several types of human cancers (11–13). The LOH of specific genes is thought to unmask recessive mutations. Our previous studies have shown that about 29% of human skin cancers displayed LOH at the H-*ras* locus (13).

Our recent studies on human skin cancers of the head and neck indicate that the H-*ras* proto-oncogene exhibits a new genetic polymorphism at codon 27. Amplification of exon 1 of H-*ras* by polymerase chain reaction (PCR) followed by single-strand conformation polymorphism (SSCP) analysis gave rise to two bands in some individuals and only one band in others. When the DNA from these two bands was extracted, reamplified and subjected to nucleotide sequence analysis, it was found that the slow-migrating band harbored the nucleotides CAT at codon 27 while the fast-migrating band harbored CAC at the same codon (14). Both codons encode the same amino acid, histidine, and this genetic polymorphism at codon 27 of the H-*ras* gene was detected in both normal tissues from skin cancer patients as well in healthy individuals with no history of cancer. Analysis of matching normal and tumor tissues revealed that 6 of 10 skin cancer patients were heterozygous, *i.e.* contained both CAT and CAC alleles in their normal tissue at codon 27 of H-*ras*. In addition, two of six (33%) heterozygous patients exhibited LOH in tumor DNA as compared to the corresponding adjacent nonmalignant skin or lymphocyte DNA. Interestingly, the two tumors in which LOH occurred at the H-*ras* locus also contained multiple *p53* mutations and were unusually aggressive (14).

Although it is known that activating mutations at codon 12, 13 or 61 of *ras* genes play a role in tumorigenesis, the involvement of LOH of H-*ras* gene in tumor developmnent is less clear. Loss of chromosomal sequences in human and mouse tumors has been regarded as evidence that the affected regions contain tumor suppressor genes. It is quite possible that the presence of 28 base-pair variable tandem repeats near the H-*ras* gene may cause genomic instability leading to allelic imbalance, which in turn may contribute to the development of cancer by unmasking recessive muta-

****Abbreviations:* AK, actinic keratosis; BCC, basal cell carcinoma; LOH, loss of heterozygosity; NMSC, nonmelanoma skin cancer; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SCC, squamous cell carcinoma; SSCP, single strand conformation polymorphism.

'ORRESPONDENCE

e: Relationship Between ınlight Exposure and a Key enetic Alteration in Basal ell Carcinoma

Cutaneous basal cell carcinoma CC) is the most common cancer oc- ring in Caucasians, with an incidence 175 per 100 000 reported in American n (*1*). The disease causes consider- e morbidity and is placing increasing rdens on health services because mor- ity is low and incidence is increasing 10% per annum (*2*).

While exposure to UVB radiation is ical, the relationship between this ex- ure and BCC risk is complex, and t factors including response to UV iation are implicated (*2,3*). Indeed, ent studies have provided molecular dence implicating host response. A didate gene (patched) for Gorlin's drome, a familial condition associ- l with multiple BCCs, maps to chro- some 9q22 (*4*), and loss of heterozy- ity (LOH) in this region has been orted to occur in 60%-70% of spo- ic BCCs (*3*). Recent studies, how- ever, found no association between 9q LOH and UV radiation exposure, as assessed by site or UV radiation-related mutations in p53.

Gailani et al. (*3*) concluded, "BCC incidence may reflect an etiologic contribution of additional environmental agents" acting synergistically with UVB radiation. Suggested agents included arsenicals, polycyclic aromatic hydrocarbons (PAHs), ionizing radiation, and/or UVA radiation. We have obtained molecular epidemiologic data that support this conclusion.

Ionizing radiation and arsenic are BCC risk factors (*5*), possibly via reactive oxygen species (ROS)-mediated mechanisms. While various enzymes detoxify ROS and their oxidation products, much data indicate the importance of the glutathione *S*-transferase (GST) supergene family (*6*). Various environmental, carcinogenic nitrosamines and PAHs are metabolized by enzymes of the cytochrome P450 (CYP) supergene family (*6*). We have examined the hypothesis that effectiveness in detoxifying the products of ROS action and environmental carcinogens is significant in determining susceptibility to various cancers, including BCC. Thus, we identified associations between GSTM1, GSTM3, GSTT1, and CYP2D6 polymorphisms and susceptibility to BCC, assessed by use of tumor numl ic- crual, and site as end points (*7* lo- mozygosity for the GSTM1*0 allele in combination with skin type 1 (individuals who always demonstrate an inflammatory response with burning but without tanning on exposure to UV radiation) predisposes to multiple tumors; these patients have been reported to develop an average of 4.6 tumors compared with 2.0 tumors in those without this combination (*8,9*). GSTT1 null is associated with faster tumor appearance (*8*). Similarly, the CYP2D6 EM (extensive metabolizer) genotype predisposes to multiple tumors and faster accrual. Furthermore, in individuals with a truncal tumor, an area generally not heavily exposed to UV radiation, these polymorphisms are associated with a reduced time to development of further BCCs; GSTM1-null subjects have a median time to next tumor presentation of 1.6 years compared with 5.0 years in patients with other site–genotype combinations (P = .0009; Fig. 1).

Our observations suggest the importance of host response to agents other than just UVB radiation. They indicate that the GSTM1 and GSTT1 enzymes detoxify products of oxidative damage and that polymorphisms in their genes influence the effectiveness of these reactions. The finding that CYP2D6 mediates susceptibility was less expected inasmuch as no link between BCC risk and exposure to environmental pollutants has been identified. Since CYP2D6 has not been detected in skin (*6*), we have suggested a role for hepatic detoxification of unknown carcinogens (*8*). The importance of UV radiation in less exposed areas such as the trunk is unclear, although, in genetically susceptible individuals, even intermittent exposure may be sufficient to promote tumorigenesis (*7*).

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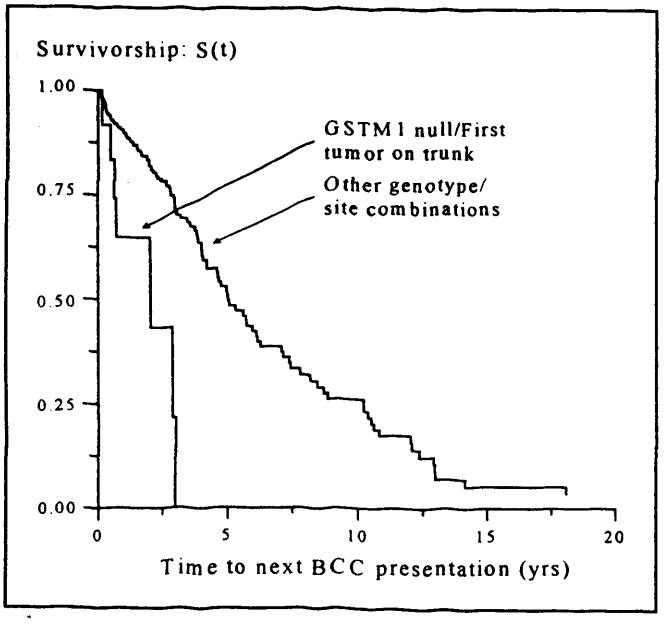

. 1. Survival analysis es for time to presen- n of subsequent new ary basal cell carci- as (BCC) in patients the GSTM1-null ge- pe and whose first tu- was truncal (n = 12) pared with all other nts with BCC (n = Cox's proportional rds regression model ected for number of at presentation), n 97, P = .0009, and rd ratio = 3.82 (95% fidence interval = 8.42). Uncorrected ian time to next BCC GSTM1-null plus cal tumor = 1.6 ; other genotype and combinations = 5.0 . Proportion tumor free at 3 years/5 years was 22%/0% for GSTM1-null plus truncal tumor and /52% for the other genotype plus site combinations.

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Paradoxical Effect of Cytotoxic Chemotherapy on Anemia in Cancer Patients With β-Thalassemia Minor

Thalassemia syndromes (e.g., α-, β-, δ-, δβ-thalassemia) are inherited anemias characterized by reduced or absent production of one or more globin chains (α, β, γ, δ). In β-thalassemia, the reduction in β-chain production causes an excess of α chains, which are unstable and precipitate in red blood cell (RBC) precursors leading to both ineffective erythropoiesis and reduced RBC survival. β-Thalassemia minor is characterized by mild or minimal anemia, which, however, in some circumstances, such as during pregnancy, may become more severe and require transfusions.

Cytotoxic chemotherapy, leading to myelosuppression, would be expected to worsen the anemia of patients with β-thalassemia minor, thus inducing the choice of less myelotoxic regimens or prompting early transfusion practice, mainly in patients with low baseline hemoglobin (Hb) values. We evaluated the effect of chemotherapy on the anemia in breast cancer patients with β-thalassemia; these patients were undergoing chemotherapy as adjuvant treatment.

From November 1992 through D cember 1995, 245 patients with stag breast cancer (*1*) were randomly assig to receive six cycles of CEF21 (i.e., c clophosphamide at a dose of 600 mg/m^2, epirubicin at 60 mg/m^2, and fluorouracil at 600 mg/m^2 intravenously on day 1) given every 21 days or the same treatment given every 14 days (CEF14) with the support of granulocyte colony-stimulating factor (*2*). Four of these 245 patients had a diagnosis of β-thalassemia (Hb A_2 level >2.5%). Two patients (#1 and #2) received CEF21, and two patients (#3 and #4) received CEF14. As a control group, a sample of 80 patients (40 treated with CEF21 and 40 treated with CEF14) was randomly extracted from the 241 patients without β-thalassemia.

The mean ± standard deviation of the Hb decrease (differences between the sixth and the first cycle) was significantly greater in control patients (−1.9 ± 1.09 g/dL) than in patients with β-thalassemia (−0.02 ± 1.13 g/dL) (P = .001, Student's t test) (Table 1). Two β-thalassemic patients (#1 and #2) even showed an increase in Hb values, but no patient in the control group showed such an increase. The hypothesis that the patterns of Hb values among the two groups were different was tested by the analysis of variance for repeated measures, after adjustment for baseline Hb values. The interaction between the two groups and Hb values during the chemotherapy was significant (P<.001), indicating that the patterns of modification in Hb levels throughout the five chemotherapy cycles are different between β-thalassemic patients and control patients. Moreover, two patients in the control group and none in the β-thalassemic group needed RBC transfusions.

Although surprising, the paradoxical effect of chemotherapy on Hb levels in patients with β-thalassemia is not unexplainable. Some cytotoxic drugs, e.g., hydroxyurea, can induce an increase in the γ chain and thus in the hemoglobin F (HbF) level in both normal individuals and patients with β-thalassemia (*3-5*). In

Table 1. Hemoglobin (Hb) values during chemotherapy in patients with β-thalassemia and in control patients

	Hb values, g/dL, during chemotherapy						
	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6	Diff.*
emic patients							
	8.9	9.3	9.6	9.4	10.4	9.4	0.5
	11.0	11.1	10.6	11.3	11.7	12.0	1.0
	10.8	10.4	11.0	11.2	11.0	10.8	0
	11.6	10.8	10.2	10.3	9.9	10.0	−1.6
	Mean values ± standard deviation						
emic patients	10.6 ± 1.2	10.4 ± 0.8	10.3 ± 0.6	10.5 ± 0.9	10.7 ± 0.8	10.5 ± 1.1	−0.02 ± 1.1
atients	12.9 ± 1.1	12.7 ± 1.1	12.2 ± 1.0	11.8 ± 1.2	11.4 ± 1.3	11.0 ± 1.3	−1.9 ± 1.1

ence in Hb values between the sixth and the first cycle of chemotherapy.

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runcal site and detoxifying enzyme polymorphisms significantly educe time to presentation of further primary cutaneous basal ell carcinoma

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asal cell carcinoma (BCC) is the commonest cancer in aucasians. Its incidence is rising and many patients evelop multiple primary tumours at separate sites. Factors etermining time between first primary tumour presentation and the next new primary lesion are unclear. We sed Cox's proportional hazards model to study, in 856 aucasians, the influence of tumour site, individual characteristics and polymorphism in glutathione *S*-transferase GSTM1, GSTT1) and cytochrome P450 (CYP2D6, CYP1A1) loci on time to next primary tumour presentation. More than one tumour at first presentation ($P < 0.0001$, hazard ratio 2.72) and GSTT1 null ($P = 0.028$, hazard ratio 1.74) were associated with decreased time to next primary tumour presentation. Significant two-factor interactions, corrected for number of tumours at presentation, were identified between a truncal tumour at first presentation and each of male gender, GSTM1 null and CYP2D6 EM ($P < 0.003$, hazard ratios 3.09–3.82). In each of these cases, all patients with the risk combination demonstrated further separate tumours within 5 years of first presentation. Thus, patients with a truncal tumour at first presentation, especially males and those presenting with more than one lesion have a significantly decreased time to presentation of further tumours and should receive more meticulous follow-up. Polymorphism in GSTM1 and CYP2D6 also influences the rate of new primary tumour accrual giving insights into the link between ultraviolet exposure and multiple tumour development.

Introduction

Basal cell carcinoma of skin (BCC*) is the commonest cancer in Caucasians, accounting in 1994, for ~35% of all newly diagnosed neoplasms in the US (1–3). A remarkable feature seen in patients who develop BCC is the risk of further, separate primary tumours (as distinct from recurrence of the original lesion) (4,5). Importantly, this depends on the number of lesions already present: 27% of patients with one tumour suffer a further tumour within 5 years compared with 90% of those with 10 or more lesions (5). These data suggest accrual is not merely dependent on time but rather, some subjects have a genetic susceptibility to faster accrual. Exposure to ultraviolet radiation (UV) is critical in the pathogenesis of BCC (4–6), though the relationship between exposure and risk of multiple tumours is complex and poorly understood. We have proposed that, as UV has pleiotrophic effects on skin (6), genes that mediate individual response to these effects, such as protection from oxidative stress, are putative candidates for susceptibility to multiple tumours. However, while data from case-control studies support this view, it is clear that the concept of susceptibility to multiple BCC is complex, as genetic factors appear to influence both tumour numbers and their rate of appearance (7–10). A further important aspect of susceptibility is the identification of genetic factors that influence tumour site. Thus, compared with cutaneous squamous cell cancer, BCC are more common on generally less exposed sites especially the trunk (4,5) with lesions infrequently found on the forearms or backs of the hands. Also, while the incidence of BCC is increasing, it is the proportion of tumours on the trunk that demonstrates the greatest increase. The factors that determine susceptibility to tumours on the trunk rather than the more sun-exposed head and neck are unclear, though it might be expected that truncal tumours are associated with different susceptibility genes.

Various individual characteristics are associated with risk of multiple BCC including skin type and male gender (4,5). Some promising candidate susceptibility genes are also identified with polymorphism in genes involved in DNA repair and detoxification of the products of oxidative stress being significant (7–11). Thus, the glutathione *S*-transferase (GST) GSTM1 null genotype in combination with skin type 1 confers increased risk of multiple tumours while GSTM1 A/B is protective (7,8). Allelism at GSTT1 also influences tumour accrual, a finding compatible with the view that these enzymes are part of the cellular defence against UV-induced oxidative stress (9). Polymorphism at the cytochrome P450 CYP2D6 and CYP1A1 loci is also associated with tumour numbers and accrual (8–9).

A better understanding of the role of genetic factors in the pathogenesis of BCC would be useful as few common cancers offer an opportunity to compare their influence on tumour numbers, accrual and site. Furthermore, a pilot study from our laboratory indicates an association between BCC and susceptibility to haematological malignancies suggesting shared genetic influences (12). We now describe, in a large cohort of cases ($n = 856$), the use of Cox's proportional hazards model to investigate the influence of genetic and individual characteristics on the time between first tumour presentation and presentation of the next new primary BCC. The influence of a variety of relevant individual characteristics (gender, age, number of tumours at first presentation, skin type, hair colour and eye colour at age 21, smoking status and occupation) is described though particular attention has been given to tumour site at first presentation to identify the

*Abbreviations: BCC, basal cell carcinoma; UV, ultraviolet radiation; GST, glutathione-*S*-transferase; CYP, cytochrome P-450; CI, confidence interval

significance of truncal lesions. This parameter has not been previously studied in the context of the role of polymorphism in mediating susceptibility. We believe the use of the Cox's regression model offers significant advantages over our previous studies on the influence of genotypes on the rate of tumour appearance (9); thus, previously, in a study of only 169 cases, we used tumour numbers per unit time as our susceptibility endpoint. Use of this parameter depends on the assumption that time between first and second, second and third presentations, and so on, is similar. It is not known if this is the case. We also suggest that the identification of genetic factors that influence the time interval between first and second primary tumour presentation will be useful clinically in the identification of individuals with a fast rate of BCC development (i.e. within 2 years of a previous tumour) who would benefit from more meticulous follow-up.

Patients and methods

Patients

The influence of genotypes and characteristics on time from first to next primary tumour presentation was studied in 856 unrelated Northern European Caucasians with histologically proven BCC. They were recruited between November 1991 and November 1995 from dermatology clinics in the North Staffordshire Hospital, Stafford District General Hospital and Royal Cornwall Hospitals. Of the total, 566 patients suffered one tumour and 290 patients between 2–35 tumours. Of this case group, 435 patients comprised the BCC case group described earlier (7) and data from these patients were included in the present study. Patient characteristics and genotype frequencies between the original 435 patients and the further 421 recruited between October 1993 and November 1995 were not different. Recurrences of original BCC lesions were excluded from the total number of primary BCC. No patients with basal cell naevus syndrome, xeroderma pigmentosum or BCC and another malignancy (either internal or cutaneous of a different histological type) were included. Cases comprised over 60% of those in the participating centres and were a typical sample of this patient group with patient characteristics comparable to other BCC populations (5). The remaining 40% were inadvertently missed in busy clinics. These patients did not comprise a particular subgroup and were not clinically different to the study group. None of those approached refused to participate. All patients were examined by a trained dermatologist (JTL, AGS, AHMH, BB) and information on hair (blonde/red and brown/black) and eye colour (blue/green and brown) at 21 years of age, skin type (types 1–4), occupation (indoor/outdoor) was obtained (8). A smoking history was also obtained allowing classification as 'ever' (had previously smoked at least 10 cigarettes/day for at least 5 years or current smokers) or 'never' smokers were obtained (8). Patients were also questioned regarding ingestion of arsenic-containing tonics or use of drinking water from potentially contaminated wells, though no exposed patients were identified. All case notes were reviewed during November 1995 (JTL,BB) to determine tumour site at presentation, tumour numbers and time between first BCC presentation and next presentation with a separate primary tumour.

Identification of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes

Blood (5 ml in EDTA) was taken with the Ethics Committee approval and stored at −50°C until analysis. GSTM1 (null, A, B, A/B) and GSTT1 (null and expressers) genotypes were identified using PCR approaches (9). Two variant CYP2D6 alleles (CYP2D6A, base pair deletion in exon 5; CYP2D6B, G-A transition at intron 3/exon 4) were also identified using PCR with diagnostic restriction enzyme digestion (9). These assays are ~90% predictive of phenotype in Europeans (13,14). Two variant CYP1A1 alleles (exon 7 Ile−Val and 3′ flanking region MspI mutations) were detected using PCR with restriction enzyme digestion (9). Comparison of the frequencies of homo- and heterozygotic combinations of these two alleles showed they were in linkage disequilibrium ($\chi^2_4 = 52.2$; $P < 0.0001$) (13,14).

Statistical analysis

Methods of survival analysis were used to study factors that influence time from first BCC presentation to presentation of a subsequent new primary tumour. Although in this study, death is not the endpoint, this approach can be applied to assess disease 'progression' using other endpoints (15). We used time from first presentation with BCC to presentation of a separate primary BCC (not recurrence of an origianl lesion) as our main outcome measure. In our clinical experience this is a more reliable endpoint than time to tumour appearance determined by the patient since the latter parameter relies on accurate memory in an often elderly population. Where patients presented with a single tumour, or with more than one tumour but did not suffer further BCC by November 1995, their times were considered to be censored. For these patients the time of appearance of the next tumour obviously exceeded the study period, or their event-free times must be at least the time to the cessation of follow-up. These censored times are incorporated into the calculation of the estimates of the survival probabilities (15). For all the multiple BCC patients, the time interval between first presentation of the new primary tumour (not necessarily the second lesion) was used in the analysis. Cox's proportional hazards regression was used to determine which factors, alone and in combination, significantly influenced the time from first presentation to presentation of a subsequent new tumour (15). Since number of tumours at presentation, categorized as single versus multiple, was highly significant alone, all subsequent calculations were carried out by correcting for this factor (i.e. including it in the regression model together with the parameter under study). The results are presented as the following summaries: corrected hazard ratios with 95% confidence intervals and significance levels and uncorrected median time to next BCC and proportions of patients with further tumours at 3 and 5 years.

Table I. Patient demographics

Number of BCC	1–35 tumours (n = 856) >4 tumours (n = 71) >9 tumours (n = 24)
Mean age (years)	67.5 ± 12.2 (SD) (n = 85
Males/females	56.2% to 43.8% (n = 843
Blue and green eyes	73.0% (n = 435)
Brown eyes	27.0% (n = 161)
Skin type 1	14.0% (n = 80)
Skin type 2-4	86.0% (n = 491)
Ever smoker	63.1% (n = 383)
Never smoker	36.9% (n = 224)
Brown and black hair	75.0% (n = 249)
Blonde and red hair	25.0% (n = 83)
First tumour on trunk	10.8% (n = 36)
First tumour on head/neck, upper limbs or lower limbs	89.2% (n = 297)
Median number BCC	1 (range 1–35)
Median number BCC at presentation	1 (range 1–10)
Outdoor occupation	11.7% (n = 25)
Indoor occupation	88.3% (n = 189)

Results

Patient characteristics

Table I shows the characteristics of the cases. The number of patients with 1–4, >4 and >9 separate primary lesions is shown to illustrate the spread of BCC numbers and is not used in subsequent analyses. The median event-free time for the total case group was 5.72 years and the proportion remaining tumour free at 3 and 5 years was 73% and 51%, respectively. The proportion of males was greater than in the 563 age-matched controls (47%) from the study centres described by Heagerty *et al.* (8). Frequencies of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes were not significantly different from those in these controls (data not shown) (8,9).

Single factors influencing time to presentation of next BCC

Table II shows the genotypes and characteristics associated with time from first presentation to next new primary BCC. Data were adjusted for number of tumours at first presentation since, as expected (4,5), the presence of more than one tumour at first presentation was significantly associated with a decreased time to next tumour ($P < 0.0001$, hazard ratio 2.72, 95% CI 1.84–4.03, median time to next new tumour single tumour at first presentation 5.75 years, multiple tumour at first presentation 3.58 years). The association of a truncal tumour at first presentation with a decreased time to ne

ible II. Factors influencing time from first presentation to next presentation (corrected for number of tumours at presentation)

	P-value	Hazard ratio (95%CI)	Median time (years)	% with further tumour at 3–5 years
irst tumour on trunk (n = 33)	0.096	1.58 (0.92,2.70)	3.00	41/54
on truncal first tumour (n = 282)			5.04	26/47
STT1 null (n = 80)[a]	0.0208	1.74 (1.09,2.79)	5.07	38/59
STT1 expresser (n = 359)[a]			6.12	16/35
teractions (corrected for tumour numbers at presentation)				
STM1 null/first tumour on trunk (n = 12)	0.0009	3.82 (1.73,8.42)	1.63	78/100
ther cases (n = 285)			5.03	27/48
YP2D6 EM/first tumour on trunk (n = 16)	0.0031	3.09 (1.46,6.50)	1.88	53/100
ther cases (n = 246)			5.14	27/46
ale gender/first tumour on trunk (n = 19)	0.0002	3.33 (1.76,6.30)	1.53	60/100
ther cases (n = 290)			5.04	25/47

e results are presented as the following summaries; corrected hazard ratios with 95% confidence intervals and significance levels for the model, and corrected median time to next BCC and proportions of patients with further tumours at 3 and 5 years. The hazard ratio is related to odds ratio in case-ntrol studies. It can be approximated, assuming no correction for confounding factors, from the ratio of median times to next new BCC presentation in the k group (e.g. GSTT1 null) divided by the non-risk group (e.g. GSTT1 expressers).
STT1 was assessed independent of tumour site (i.e. tumours at all sites are included).

sion approached significance (Table II). Male gender also appeared to be associated with a shorter time to presentation of further tumours, though the effect failed to achieve significance (P = 0.0815, hazard ratio 1.38). Skin type, hair colour, eye colour, smoking status and occupation were not significant. GSTT1 null was associated with a significantly reduced time to presentation of next tumour (Table II) whereas GSTM1 null, CYP2D6 EM, CYP1A1 Ile/Ile and CYP1A1 m1m1 were not significant.

Interactions between genotypes, characteristics and tumour site

Table II shows significant two-factor interactions, corrected for number of tumours at first presentation, in which the interaction term was more significant than either of the main effects. Only two-factor interactions were considered. Thus, highly significant two-factor interactions between the presence of the first tumour on the trunk and each of male gender, GSTM1 null genotype and CYP2D6 EM genotype were identified. Data on the interaction between truncal tumour at first presentaion and GSTM1 null genotype are illustrated in Figure 1. These interactions (truncal tumour + male gender, truncal tumour + GSTM1 null genotype, truncal tumour + CYP2D6 EM genotype) appeared particularly significant as all of the cases with these combinations demonstrated further tumours within 5 years, whereas only half of the cases without these combinations suffered a further lesion during this time (Table II). Indeed, 3 years after first presentation, 78% of the cases with GSTM1 null and a first truncal tumour had suffered a further BCC. No significant two-factor interactions between pairs of GSTM1, GSTT1, CYP2D6 and CYP1A1 genotypes were identified. Other interactions between genotypes and patient characteristics, including skin type 1 and male gender were not significant.

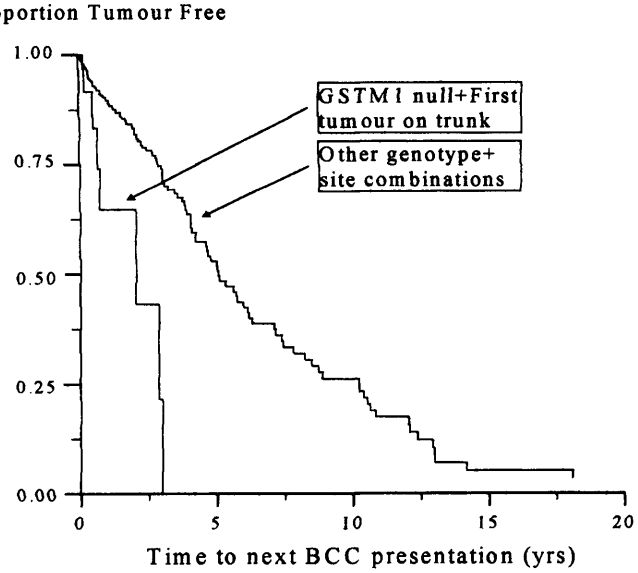

Fig. 1. Cumulative event-free probability curve for time to presentation of subsequent primary BCC in patients with the GSTM1 null genotype and whose first tumour was truncal compared with all other BCC patients.

Discussion

We have reported the use of a Cox's proportional hazards model approach to study the influence of GST and CYP genotypes, patient characteristics and tumour site on the time from first presentation with a BCC to next presentation of a separate primary BCC. These tumours are a major burden to health-care agencies with incidences in the US as high as 300 per 100 000 people and reported annual increases of ~10% (4). In view of this increasing incidence and the low mortality associated with BCC, it is predicted that the prevalence of this tumour will be greater than that for all other cancers combined (2). Further, patients with a BCC are often unaware of or, ignore the development of further lesions (16,17). The ability to identify, at first presentation, which patients will develop further lesions and at what rate would be useful if it allowed earlier detection of lesions. This is particularly true since smaller tumours are usually more easily removed with better cosmetic outcome (18).

We have identified in 856 patients, factors associated with a rapid time to presentation of next new primary tumour. Although there must be a time interval between appearance of the BCC and presentation to dermatologists, we have used the latter parameter (see statistical anlaysis section). It is our clinical impression that this is a more reliable endpoint than time to tumour appearance determined by the patient, which is dependent on accurate memory in an often elderly population. While it is possible that the time difference between these two parameters varies between patients, we detected no significant

differences in this interval between the subgroups shown in Table II. As expected, patients with more than one lesion at first presentation demonstrated a decreased median time to next tumour (4,5). We also report, for the first time, that truncal site at presentation in combination with other factors is associated with a reduced median time to next tumour and an increased proportion of patients suffering a further tumour within 3 and 5 years. Thus, male patients with a truncal tumour at first presentation demonstrate a decreased time to next tumour with all cases suffering a further tumour within 5 years. Since consumption of arsenic is a potential confounding factor for truncal tumours, it is noteworthy that the proportion of our cases with truncal tumours was similar to that in other studies of sporadic BCC (19).

Polymorphic variants associated with a decreased time to next tumour were also identified; thus GSTT1 null was a significant factor with about two-thirds of these cases suffering a further tumour within 5 years compared with only one-third of patients who expressed the gene. GSTM1 and CYP2D6 genotypes alone did not influence subsequent tumour presentation time, though significant interactions with truncal site were identified. Thus, cases with a truncal tumour at first presentation and GSTM1 null, demonstrated a markedly decreased time to next lesion such that all patients with this combination suffered a further tumour within 5 years. We speculate the influence of GSTM1 and GSTT1 results from their ability to metabolize the products of UV-induced oxidative stress in the skin, a view supported by the finding that GSTM1 null is associated with photosensitive systemic lupus erythematosus via increased production of anti-Ro (but not anti-La) antibodies (20). However, unlike GSTM1 null, no interaction between GSTT1 null and tumour site was found, a finding that complements data showing the products of these loci have some different substrate preferences.

While there is no known association between environmental pollutants and risk of BCC, CYP2D6 EM in combination with tumour site was associated with decreased time to presentation of subsequent BCC. The role of this enzyme is unclear and though expressed in liver, brain and intestine, it has not been detected in skin (13,21). As systemic agents such as arsenic predispose to multiple BCC, CYP2D6-mediated hepatic detoxification of unknown carcinogens may be important. Data showing UV-oxidized tryptophan binds to the CYP1A1 ligand-dependent Ah receptor transcription factor, and UV induces CYP1A1 expression in skin, suggest a role for allelism at this locus in skin carcinogenesis (13,21). However, while polymorphism at this locus influences tumour numbers (9), the present study shows that allelic variation in exon 7 and the 3′ region do not influence time to next tumour presentation.

Why patients with more than one lesion or a truncal tumour at first presentation, or are male, demonstrate a shorter time to next tumour is unclear. Indeed, factors that influence rate of presentation of a further tumour may differ from those that influence susceptibility. Thus, we found GSTT1 null influences accrual but not susceptibility to BCC while GSTM1 null in combination with skin type 1 influences susceptibility to multiple tumours but not tumour accrual (9). The susceptibility factors that determine tumour site are unclear, though patients who demonstrate tumours on less exposed sites such as the trunk, might be considered the most susceptible and consequently likely to suffer further tumours. The finding that males with truncal lesions demonstrated decreased time to next tumour presentation is compatible with data showing males are at increased risk of multiple BCC. Outdoor occupation was not a significant factor in our analysis suggesting cumulative UV-exposure alone is not the explanation. This view is compatible with data showing susceptibility to UVB induced inhibition of contact hypersensitivity appears a better indicator of non-melanoma skin cancer risk than cumulative UV exposure (22).

From a statistical viewpoint, the GSTT1 null effect is relatively weak and would require confirmation in subsequent studies. However, since the observations reported here are exploratory and not intended to be predictive, correction for multiple analyses is not appropriate. In the case of the interactive effects, although correction for multiple analyses in exploratory studies is unnecessary, the size of the *P* value is such that they are likely to remain significant even after correction. To reduce the risk of spurious association, Christensen (15) recommends that the number of variables tested is 5–10% of the number of cases studied. The number of variables used in this report is considerably less than this.

The biochemical processes that determine rate of appearance of further tumours in BCC are unclear. Our data indicate patients with a truncal tumour at presentation, especially males and those presenting with more than one lesion should receive meticulous and more frequent follow-up to expedite early diagnosis. Our data show that inter-individual differences in the efficiency of detoxification reactions also determine susceptibility. Thus, the highly significant influence of GSTM1 null or CYP2D6 EM on time to next tumour presentation suggests a possible use of genetic markers in a follow-up strategy.

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