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By

Suat-Cheng Peh

University of Leicester

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ABSTRACT

Previous studies of the pattern of lymphoma in multi-ethnic West Malaysian population have shown a high frequency of EBV association in childhood Hodgkin's lymphoma (HL) and adult NK/T-cell lymphomas, with a predilection of the former in Indians and the latter in Chinese. This thesis aims to expand knowledge of the EBV association pattern in childhood non-Hodgkin's lymphoma (NHL), Burkitt's lymphoma (BL), ethnic predilection and virus subtype pattern for Malaysian patients.

The childhood NHL pattern is similar to other parts of the world. BL, lymphoblastic lymphoma and diffuse large cell lymphoma (predominantly CD30-positive, ALK-positive anaplastic large cell type) form the 3 major groups of the disease. The difference in subtype composition results in different overall EBV association rates for T- and B-cell lymphomas when compared to the adults, being 3x higher in the B-cell lymphomas in children and the reverse for T-cell lymphomas. The frequency of BL in West Malaysian children is not higher and in the malaria endemic state of Sabah the pattern of lymphoma is similar to other Asian series. The low incidence of jaw presentation, more common abdominal and lymph node disease, and EBV association rate of 33% are features of sporadic BL.

Using a sensitive nested-PCR test on 38 lymphomas, 14 nasopharyngeal carcinomas, 12 reactive lymph nodes and tonsils, only EBV type A was identified. This was irrespective of the anatomical sites of the biopsy material, age group, sex and ethnicity of the patients. EBV was identified in sequential biopsies of EBV

associated lymphomas, and continued to be absent in non-EBV associated cases, supporting the probable pathogenetic role of the virus.

In conclusion, EBV type A is the prevalent subtype of virus present in Malaysian patients. East-West differences in lymphoma pattern are less distinct in children. BL is of the sporadic type and the predilection of EBV associated T-NHL to Chinese is again reflected in Malaysian children, supporting the notion that ethnic Chinese are at risk of developing EBV-associated cancers.

Chapter 1

Introduction and Objectives of Study

1.1 HISTORICAL BACKGROUND: Nomenclature and Lymphoma

Classification Scheme

Lymphoma, a term first introduced by Rudolph Virchow in 1858 (Virchow, 1858), is presently accepted to mean primary malignant neoplasm of the lymphoid tissue, which include Hodgkin's lymphomas (HL) and non Hodgkin lymphomas (NHL). However, the evolution of this concept and the nomenclature took more than a century. About 170 years ago in the year 1832, Thomas Hodgkin of Guy's Hospital, in his paper "On Some Morbid Appearance of the Absorbent Glands and Spleen" first gave a definitive vivid account on diseases originating in the lymphoreticular system. Earlier authors normally regarded changes that took place in the lymph glands as caused by diseases in the draining sites of the affected nodes. Classification schemes have traditionally been developed separately for Hodgkin's disease, and lymphomas other than Hodgkin's disease, more commonly referred to as non-Hodgkin's lymphoma. This was due to delineation of Hodgkin's disease as a separate entity from lymphosarcoma, a concept first initiated by Dreschfeld and Kundrat in the 19th century (Dreschfeld, 1893; Kundrat, 1893), which was favourably received and adhered to. This was partly because the neoplastic nature of Hodgkin's disease was not ascertained, nor was generally accepted for a very long time. It had been regarded as diverse a disease as an atypical form of tuberculosis, a specific infective granuloma of unknown origin, a tumour, or a transition form between a granuloma and a

tumour. Moreover, the uncertainty of the exact nature of the cell from which it is derived, if truly neoplastic, perpetuated this division.

The classifications of Hodgkin's disease were essentially histological. The earliest histological description, attributed to WS Greenfield (Greenfield, 1878), noted that the affected nodes were disrupted by chronic inflammation and fibrosis, with presence of multinucleated cells. The so called Sternberg-Reed cells, named after Carl Sternberg (1898) and Dorothy Reed (1902) constitutes an essential pre-requisite for the diagnosis of this disease. Rosenthal's report in 1936 documented the prognostic importance of histology in Hodgkin's disease (Rosenthal, 1936), in particular the density of lymphocytes. He divided the histology into 3 groups, predominance, subordination and absence of lymphocytes and nodules, and eight years later, Jackson and Parker (1944) also divided Hodgkin's disease into 3 groups, paraganuloma, granuloma and sarcoma. Based on the study of biopsy material of 377 cases from the US Armed Forces in World War 2, Lukes was able to distinguish 6 histological types (Lukes, 1963). During the Rye conference held in 1965, Luke's original classification was simplified to 4 groups, better known as the Rye classification (Lukes and Butler, 1966). In 1979, Poppema, Kaiserling and Lennert reported that the nodular lymphocyte predominance subtype of Hodgkin's disease was an entity different from the other subtypes (Poppema *et al*, 1979). In 1980, Poppema demonstrated that the L&H cells of this subtype contain J-chain and therefore are of B cell origin (Poppema, 1980). More recent studies that employed phenotyping and molecular biological techniques convincingly demonstrated the lymphoid origin of the Sternberg-Reed cells in the other subtypes. A large majority of cases were shown to be B cells, with aberrant hypermutation of immunoglobulin gene, suggesting B-cell derivation with

biological properties of follicular-centre cell type. For the first time, Hodgkin's disease was put alongside non-Hodgkin's lymphomas in one classification scheme in the R.E.A.L. proposed list of lymphoid neoplasms (Harris *et al*, 1994). Added to the new WHO classification (Jaffe *et al*, 1998) is lymphocyte rich subtype. The nodular paragranuloma (lymphocyte predominant, other) as described in 1979 by Poppema, Kaiserling and Lennert differs from the classical types (mixed cellularity, nodular sclerosis, lymphocyte depletion and lymphocyte rich) in the phenotype expression of the atypical cells. The tumour cells in the classical types often express CD15, CD30 and EBV associated DNA, RNA and proteins, whereas the former does not express CD15 and EBV latent genes. Hodgkin's disease is now proposed to be named Hodgkin's lymphoma in the WHO classification, since the lymphoid nature of the Sternberg-Reed cells has now been convincingly established (Jaffe *et al*, 1998).

Various attempts at classification of non-Hodgkin's lymphomas have also been made over the years, from Robb-Smith (1938) and Gall and Mallory (1942) to the 1966 classification of Rappaport. Then in the 1970s, several classifications emerged, Dorfman (1974) (Working Classification of non-Hodgkin's Lymphoma), Bennett *et al.*, (1974) (British National Lymphoma Investigation Classification), Gerard-Marchant *et al.*, (1974) (Kiel Classification), Lukes and Collins (1974; 1975) (Lukes-Collins Classification), and Mathe *et al.*, (1976) (WHO Classification of Malignant Lymphoma). Non-Hodgkin's lymphomas being a diverse group of diseases, each of these classification schemes could not completely encompass every entity. Therefore, some entities were described in one and not in the others. The problem was compounded by the use of similar terms in different sense e.g. well and poorly differentiated lymphomas, and

similar disease entities were given different names. This led to much confusion since simultaneously all over the world, different centres each adopted a scheme familiar to them. Hence, an international study sponsored by the National Cancer Institute was carried out in an attempt to resolve the difficulties. The analysis at the end of the study showed that no one classification was more superior over the other, and a “Working-Formulation of non-Hodgkin’s Lymphoma for Clinical Usage” was formulated to enable translation among all systems. It was better known as the Working Formulation, and was published in 1982 (National Cancer Institute, 1982). In the ensuing 10 years, further information about these lymphomas became available, and newer entities were recognised. An international group of hematopathologists, met to address this issue. It was deemed that the most practical approach was to list the well-defined entities based on current morphological, immunologic and genetic profiles, as proposed in the Revised European-American Classification of Lymphoid Neoplasms (Harris *et al*, 1994). This classification encompasses the known lymphoid malignancies: the T-cell and B-cell lymphomas and Hodgkin’s disease. The T-and B-cell neoplasms are divided into 2 main categories, the “precursors” and “peripheral” types. The former corresponds to lymphoblastic lymphoma and leukaemia, and “peripheral” comprises the remainder types. The names given to these entities were based on the putative normal counterparts, the morphologic features, or established usage in the past. Additional categories of unclassifiable cases are permitted. The newly published “WHO Classification of Tumours on Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues” (IARC press, Lyon 2001) is the product of the collaborative project of European Association of Haematopathology and the Society of Haematopathology which began in 1995.

This scheme of classification on lymphoid tumours is based on the principles defined in the R.E.A.L.

1.2 CHANGING TREND AND GEOGRAPHICAL VARIATION:

Message and Implication

Recent reports indicate changing trends in both Hodgkin's and non-Hodgkin's lymphomas (Vineis, 1996). The mortality rate from Hodgkin's lymphoma decreased world wide, partly attributed to effective therapeutic regimes and management plans (Levi *et al*, 1995; Levi *et al*, 1996). In addition, the application of immunophenotyping and immunogenetic characterisation of lymphomas may have also led to a shift in the categorisation of the worse prognostic type of Hodgkin's lymphomas to non-Hodgkin's lymphomas (Hartge *et al*, 1994; Levi *et al*, 1995; Glaser and Swartz, 1990). However, time trend reports on the true incidence of Hodgkin's lymphoma either remains unchanged (Devesa and Fears, 1992; Martissan *et al*, 1992), or has declined in the last 4 decades (Hartge *et al*, 1994; Cancer Facts and Figures, 1996). The diminishing incidence was observed in both sexes at about equal rate (Cancer Facts and Figures, 1996; Liu *et al*, 2000), and is more substantial in the older age group, whereas remains high in the young and developing countries, such as Puerto Rico and Bombay, India. There are also variations in the incidence of disease subtypes, mixed cellularity declined but nodular sclerosis increased over time. This interesting trend suggests a possible shift in the effect or influence of aetiological factors. EBV being the agent most convincingly associated to the development of the disease, one wonders if this trend is related to the changing exposure pattern to this virus. Age at first exposure to EBV infection has certainly changed in the

industrialised countries, shifting the primary infection to adolescence and young adults. How this could have changed the pattern is not immediately evident, though it may explain the rise in incidence in the young adults, but would not be able to explain the decline in the mixed cellularity subtype which is more closely associated with Hodgkin's lymphoma. In a more recent review on infant feeding and children cancer risk, the absence or short-term breast feeding is implicated to increase risk for Hodgkin's lymphoma but not non-Hodgkin's lymphomas (Davis, 1998).

Hodgkin lymphoma is relatively uncommon in Orientals when compared to the Western populations. It makes up only between 5% to 15% of all cases of lymphomas in Asia, such as Japan, Hong Kong, Taiwan and China (Ho *et al*, 1984; Ji and Li, 1992; Tomita *et al*, 1996; Lee *et al*, 1991), whereas, it is about 20 to 25% in Caucasian populations. Population studies report incidence rate of Hodgkin's lymphoma as approximately 3 per 100,000 person-year in North America, and only 0.5 in parts of Asia (Hartge *et al*, 1994). An exception was observed in India, where a higher frequency (30%) was reported (Nair and Krishnaswami, 1990). Hodgkin's lymphoma is also found to be common amongst children in Costa Rica, where its incidence is recorded to be similar to acute lymphoblastic leukaemia, and ranks among the highest recorded incidence rates in the world (Sierra *et al*, 1989). Investigations in the past decades implicate pathogenetic role of EBV in Hodgkin's lymphoma. Hence, it remains unsolved why Hodgkin's lymphoma is not more commonly seen in Asian countries, where other EBV-associated neoplasms are encountered more frequently than in the West. However, there is sufficient indication that the immune status of the host at the time of EBV infection is relevant to the development of EBV-associated

Hodgkin's lymphoma, as it is present in higher frequency in the elderly and young patients (Jarret *et al*, 1991; Khan *et al*, 1993). Moreover, there is strong association between EBV and childhood Hodgkin's lymphoma of 100%, even in widely different populations such as the Peruvian (Chang *et al*, 1993), Honduran (Ambinder *et al*, 1993), Chinese (Zhou *et al*, 1993; Chan *et al*, 1995b) and the Kenyan (Weinreb *et al*, 1996). These observations, together with the previous finding of a clonal episomal virus pattern strongly suggest that EBV play an important aetiological role in childhood Hodgkin's lymphoma in developing countries, regardless of the ethnicity of the populations. This notion is supported by a recent study of 277 childhood Hodgkin's lymphoma from 10 countries, which reported that the prevalence of EBV association varies from country to country, ranging from 50% in a developed country such as the UK, to 100% in Kenya (Weinreb *et al*, 1996).

The rise of non-Hodgkin's lymphoma on the other hand is world wide (Devesa and Fears, 1992; Martissan *et al*, 1992; Holdford *et al*, 1992; Pearce and Bethwaite, 1992; Seow *et al*, 1996; Morgan *et al*, 1997; Sukpanichnant *et al*, 1998). This steady up-ward trend in the last 4 decades has resulted in non-Hodgkin's lymphoma being placed as the eighth most common cancer in the UK (Office for National Statistics, 1996) and the sixth most common in the US (Wingo *et al*, 1999). Diagnostic improvements and emergence of acquired immunodeficiency syndrome-related NHL (AIDS-NHL) can contribute to the increment (Hartge *et al*, 1994; Gaidano *et al*, 1998). However, a Danish study on non-Hodgkin's lymphoma during 2 periods, 1943-1977 and 1978-1989, the pre-AIDS and AIDS era respectively, the increasing trend was observed in both periods, in all age groups and in both the sexes (Hjalgrim *et al*, 1996). Moreover,

in a French study over a period of 10 years from 1980 to 1989, on 380 cases of non-Hodgkin's lymphoma, only one case was proven to be associated with HIV infection. Hence, the conclusion that the increased incidence is independent of AIDS. (Carli *et al*, 1994). This observation is further supported by another French study (Rolland-Portal *et al*, 1997). Some reports indicated increment in some specific tumour subtypes, such as nodal peripheral T-cell lymphomas (Morgan *et al*, 1997) and extra-nodal B-cell lymphoma, in particular, the primary CNS lymphomas (Eby *et al*, 1988). Although AIDS contribute more to the overall increase in the incidence of CNS lymphomas (Cote *et al*, 1996), there is evidence that the increase of non-Hodgkin's lymphoma and CNS lymphomas antedates the AIDS epidemic (Carli *et al*, 1994, Eby *et al*, 1988). Some preliminary investigations suggest that the increase in lymphopietic neoplasms is related to agricultural practices, possibly the results of exposure to occupational chemicals such as pesticides and herbicides (La Vecchia *et al*, 1989; Morrison *et al*, 1994; Waterhouse *et al*, 1996). The compounds suspected include the dichlorophenoxyacetic acids in the herbicides (Zahm, 1997; Fontana *et al*, 1998) and the organochlorines and organophosphates in the pesticides (Brown *et al*, 1990). However, sunlight had also been implicated in a report from a study in Sweden (Adami *et al*, 1999). A recent review (O'Connor *et al*, 1999) implicates benzene, a class I carcinogen, proven to be highly effective in causing lymphoma and zymbal gland carcinoma in rats and mice experimentally (Farris *et al*, 1993; Huff *et al*, 1989). Reports that support the risk of benzene exposure in human subjects include increased incidences of lymphoma in children swimming in pools contaminated by petroleum products in The Netherlands (Mulder *et al*, 1994), living near oil refineries and petrochemical plants in the UK (Knox, 1994), and in

communities living in the vicinity of industrial plants in the US (Linos *et al*, 1991). Moreover, the rise in non-Hodgkin's lymphoma appears to take place after the Second World War. Almost all the countries where a tumour registry is kept, the increase parallels the rise in environmental levels of benzene introduced into the atmosphere from motor vehicles since the 1950s (O'Connor *et al*, 1999).

However, worthy of address is the probable role of virus in the variation of frequency of subtypes of non-Hodgkin's lymphoma in different geographical areas and populations. Published data show different malignant lymphoma patterns existing in Asian and Western populations (Harrington *et al*, 1987; Shih and Liang, 1991; Ho *et al*, 1984; Ji and Li, 1992). Substantial differences in the frequencies of subtypes of non-Hodgkin's lymphoma are also evident across geographical regions (Anderson *et al*, 1998). T-cell non-Hodgkin's lymphoma is more common in the Far East (Shih and Liang, 1991; Ji and Li, 1992; Anderson *et al*, 1998), and NK/T-cell lymphomas of the nasal and nasal-type are more common in the East Asians (Anderson *et al*, 1998; Jaffe *et al*, 1996; Aozasa *et al*, 1989; Ho *et al*, 1990; Chan *et al*, 1987; Harabuchi *et al*, 1990; Chiang *et al*, 1996) and Peru (Arber *et al*, 1993) than in the United States and Europe (Jaffe *et al*, 1996; Weiss *et al*, 1992b). In the case of ATL, the disease is clearly related to the aetiological factor, the HTLV-1 virus infection endemic in the South-Western part of Japan even though the pathogenetic mechanism is yet to be elucidated (Tajima, 1990). The sino-nasal NK/T-cell lymphoma disease is apparently related to EBV as the causative factor. However, EBV being ubiquitous, it is probably not the most important factor responsible for the varying geographical distribution pattern of some lymphoma subtypes. In Asia, EBV is also known to be closely associated with nasopharyngeal carcinoma, which is particularly prevalent in the ethnic

Chinese in Southern China, Taiwan, Hong Kong, Malaysia and Singapore. In the same ethnic population, there is also a higher frequency of upper aerodigestive tract T-cell/NK cell lymphomas with a similarly strong association with EBV (Peh *et al*, 1995; Chan *et al*, 1994). Hence, host related factors are probably more important in determining the pattern of occurrence of EBV associate NK/T-cell lymphoma.

1.3 AETIOLOGY OF LYMPHOMA: Role of Viruses

There are 2 families of viruses known to be aetiologically associated with human malignant lymphomas, the retrovirus and the human herpesvirus (Cohen, 1999). Human T-cell leukaemia virus-1 (HTLV-1), a T-cell (CD4) trophic retrovirus, is closely related to the adult T-cell lymphoma-leukaemia (ATL), first described in the 1977 in Japan (Uchiyama *et al*, 1977). This variety of lymphoma is most common in South-Western Japan, the Caribbean basin and the sub-Saharan Africa. The specific geographical distribution prompted the suspicion of an infective cause for this disease. In the early 1980s, a retrovirus named HTLV-1 was isolated from ATL patients (Poiesz *et al*, 1980). It is estimated that 1% of population in Japan is carrier of this virus, and 1% to 4% of these carriers develop ATL (Kondo *et al*, 1987; Kondo *et al*, 1989). Outside these geographical locations, HTLV-1 infection and associated lymphomas are relatively infrequent. More than 10 years of research showed that there are no identifiable oncogene or tumour suppressor genes in this virus, or specific cytogenetic abnormalities found associated with ATL (Lyons and Liebowitz, 1998). The site of viral DNA integration appears to be at random, and there is no activation, deregulation or loss of important cellular genes. However, there is increased expression of IL-2R-

α , IL-2 and granulocyte-macrophage stimulating factor (GM-CSF), and the cells showed diminished dependence on IL-2 for growth in vitro (Popovic *et al*, 1984). Research has been focused on the regulatory factor, Tax. The reason being that Tax protein is the first HTLV-1 virus protein to be produced, and is required for replication of the virus. It acts both as the antigen and mitogen, driving the cell division of infected CD4 positive and CD8 T-cells. Tax interacts with the 21-bp repeat regions of the long terminal repeats (LRT) termed TRE1 (Tax responsive element-1) and the upstream region named TRE2, via DNA binding proteins CREB and CREM (Kondo *et al*, 1987). However, in-vitro and transgenic mouse model studies on the pathways of Tax in transformation and tumorigenesis have so far not lead to conclusive results (Bangham, 2000). The knowledge on the exact pathogenetic mechanism of this virus is to date relatively limited.

Human Herpes Virus:

In the past 15 years, there has been massive accumulation of information and knowledge on human herpes virus and lymphomas with respect to the 2 important viruses, the Epstein-Barr virus (EBV) and human herpesvirus-8 (Gaidano *et al*, 2000, Caesarman *et al*, 1995).

a. Epstein-Barr virus

Following its discovery in the tumour cells of Burkitt's lymphoma in 1964 (Epstein *et al*, 1964), EBV has since been shown to be closely associated with nasopharyngeal carcinoma, T-cell and NK/T-cell lymphomas, immunodeficiency/AIDS-related lymphomas, post-transplant lymphoproliferative disorders and Hodgkin lymphoma. Numerous major reviews have been published in the recent past on the role of EBV in lymphomas (Lyons and Liebowitz, 1998; Joske and Knecht, 1993; Takada *et al*, 1995; Chang and Weiss, 1996; Meijer *et al*,

1996; Oudejans *et al*, 1997; Baumforth *et al*, 1999; Kawa, 2000). There is convincing evidence that this virus is not just an innocent bystander. In most instances the virus is shown to be clonal, based on the study of terminal repeats sequence of the viral episomal form by Southern blot analysis. Although the exact pathogenetic role(s) of EBV in each lymphoma type has yet to be elucidated, some hypotheses can be made based on scientific evidence and observations.

At least 11 genes can be expressed during latency infections, EBNA-1, -2, -3A, -3B, -3C, -LP, LMP1, LMP2A, LMP2B, EBER1 and EBER2. Each of these possesses their own function(s). There are 3 patterns of latent gene expression in EBV-associated lymphomas:

Pattern	Gene expression	Tumour types
Latency I	EBNA-1, EBERs	Burkitt's lymphoma
Latency II	EBNA-1, LMP-1 EBERs	Hodgkin's lymphoma NK/T-cell lymphoma
Latency III	EBNAs-1,-2,-3A,-3B,-3C,LP LMPs, EBERs	AIDS-related lymphomas and post-transplant lymphoproliferative diseases

Several of these latent gene proteins are involved in the transformation process e.g. EBNA-2, EBNA-3A and 3C, LMP1 and LMP2 (Baumforth *et al*, 1999). LMP1 has been shown to transform rodent fibroblast cell lines (Wang *et al*, 1985), and induces contact inhibition in Rat-1 and BALB/3T3 cells (Baichwal and Sugden, 1988), and LMP1 expressing Rat-1 cell are tumorigenic in nude mice whereas LMP1-negative Rat-1 cells are not (Wang *et al*, 1985). At least 4 signalling pathways are involved in the function of LMP1, namely nuclear factor- κ B (NF- κ B), c-Jun N-terminal kinase (JNK)-AP-1, p38/MAPK (mitogen

activated protein kinase), and Janus kinase (JAK)-STAT (signal transducers and activators of transcription).

(i) *T- and NK/T-NHL*

Even though EBV is B-cell lymphotropic, it is found to be present in the T-cells in rare, fulminant cases of infectious mononucleosis and some T-cell lymphoproliferative diseases. Such as, the virus-associated haemophagocytic syndrome-associated T-cell lymphocytosis, nasal NK/T-cell lymphoma, some varieties of peripheral T-NHL and rare cases of post-transplant T-NHL (Kumar *et al*, 1993; Van Gorp *et al*, 1994). It is presently still unclear what host and viral factors contribute to the T-cell infection. It is postulated that EBV can infect T-cell as CD21, the C3d (EBV) receptor is expressed in low level in normal T-cells. Other proposed modes of entry of virus into the cells include endocytosis, or via cytotoxic T-cell killing of EBV infected cells through close proximity of the CTLs with released viral particles. The tumour cells express LMP1 gene protein and CD30. Studies on cases of nasal NK/T-cell lymphoma reveal almost 100% EBV association rate. Gene expression is detected in almost all the tumour cells and the virus is demonstrated to be monoclonal. The ligand for CD30 receptors has recently been cloned and studied. It was found to have pleiotropic biological activities. Hence, modulation of expression of CD30 by EBV, perhaps via LMP1, and CD30-CD30 ligand interactions may influence the growth of EBV-associated lymphomas (Meijer *et al*, 1996). On the other hand, the viral gene expression pattern is more variable in peripheral T-NHL of AILD-type. The majority of EBV is present in the reactive B lymphocyte and immunoblasts, and smaller numbers of neoplastic and non-neoplastic T-cells (Weiss *et al*, 1992a). It is deemed unlikely that EBV would play a central role in the pathogenesis of these lesions.

However, it may explain the occurrence of rare B-immunoblastic lymphomas arising in AILD (Takada *et al*, 1995).

(ii) *Burkitt's lymphoma,*

Assay on tumorigenicity of EBV-positive and negative Akata cell clones in nude mice (Joske and Knecht, 1993) showed tumour growth from EBV-positive and not EBV-negative clones. This finding is very convincing of EBV conferring growth advantage to the Burkitt's lymphoma cells. One hypothesis based on the available data for endemic Burkitt's lymphoma is as follows. Early in the evolution, after primary EBV infection, malnutrition and chronic malaria infections results in polyclonal B lymphocyte stimulation and T lymphocyte immunosuppression. The setting favours the proliferation of EBV-infected B-cells, resulting in prolonged survival and higher steady state number. This may therefore favour the occurrence of the characteristic cytogenetic alterations involving the Ig and MYC loci. Up-regulation of MYC allows these altered cells growth advantage over other cells in the expanded pool (Lombardi *et al*, 1987). Altered MYC expression may then replace the function of EBV, allowing cells to proliferate and survive. Hence, the EBNA and LMPs expression are down-regulated. Because EBNA and LMPs are targets for CTL response, decreased expression of these immune targets may lead to evasion of immunological surveillance, and hence increases the chances of survival.

(iii) *Post-transplant lymphoproliferative disease*

It was documented that patients with active EBV infection are at risk of developing post-transplant lymphoproliferative disease (PTLD). Also, patients who acquire primary EBV infection in the post-transplant period are at special high risk. PTLD can present in 3 broad groups, based on the morphology and

molecular pattern. First, plasmacytic hyperplasia as described by Nalesnik *et al.* in 1988 (Nalesnik *et al.*, 1988), in which there is no oncogene or tumour suppressor gene alteration but shows multiple EBV infection events. Second, polymorphic B-cell hyperplasia or polyclonal B-cell lymphoma, with a single EBV infection event, but also lacking in oncogene and tumour suppressor gene alterations. Finally the third group of monoclonal disease with single EBV infection event, and frequent alteration in the N-ras or p53 genes. Cytogenetic analysis showed trisomy 9, 11 and BCL-6 alterations in large cell immunoblastic lymphomas, and MYC translocation in small cell, non-cleaved Burkitt-like tumours. In PTLD associated with EBV (>90%), the latent gene proteins, EBNA-2 and LMP1 were expressed in all cases. The patterns of viral antigen expression do not vary with the histology, clonality and cytogenetic group of the tumour. Hence, the suggested model for the pathogenesis of PTLD is that EBV drives the early proliferation and cytogenetic alterations evolve later. But unlike endemic Burkitt's lymphoma, the viral proteins are probably required for maintaining the transformed state, and are therefore expressed.

(iv) *Immunodeficiency (AIDS)-related lymphoma*

Similar to PTLD, the pathology ranges from polyclonal lymphoproliferation to monoclonal lymphomas. However, AIDS-lymphomas are not uniformly EBV-associated (Lyons and Liebowitz, 1998). The average rate of virus association is 40%, with the exception of primary CNS lymphoma of immunoblastic type, which are about 100% associated. The data so far are most consistent with the hypothesis that multiple inciting events can lead to AIDS-related lymphoma, EBV infection being one.

(v) *Hodgkin's lymphoma*

An association of EBV and Hodgkin's lymphoma was proposed from early epidemiological and serological studies. Evidence point towards increased risk of developing Hodgkin's lymphoma from patients who had EBV-associated infectious mononucleosis, and presence of abnormally high titres of antibodies against EBV antigens prior to after the diagnosis of Hodgkin's lymphoma (Mueller, 1987; Mueller *et al*, 1989; Johansson *et al*, 1970; Evans and Gutensohn, 1984; Gutensohn and Cole, 1981; Henle and Henle, 1979; Kvale *et al*, 1979). In 1985, Poppema *et al*. demonstrated the presence of EBNA-1 in the nuclei of the RS cells, in a case of mixed cellularity subtype of HD. However, it was the advancement in DNA molecular techniques on tissue biopsies in the late 1980s that enabled confirmation of the presence of the virus in the tumour. In 1987, Weiss *et al*. reported the presence of EBV in neoplastic tissue of Hodgkin's lymphoma, and subsequently demonstrated to be localised in the neoplastic Reed-Sternberg cells by in situ hybridisation method (Weiss *et al*, 1987; Weiss *et al*, 1989; Coates *et al*, 1991). Interesting patterns of EBV association in Hodgkin's lymphoma had since been reported. A higher association rate is seen in the mixed cellularity subtype of Hodgkin's lymphoma than in nodular sclerosis, in children and old patients rather than in young adults, and also in certain geographical locations (Jarret *et al*, 1991; Amstrong *et al*, 1993; Chang *et al*, 1993; Weinreb *et al*, 1996). Within similar geographical locations, they are often related to poorer socioeconomic status (Flavell *et al*, 1999). The presence of monoclonal EBV viral genomes had been demonstrated in Hodgkin's lymphoma by Anagnostopoulos *et al* (1989) and Gulley *et al* (1994). All the involved sites in EBV-positive cases show presence of the virus in the tumour cells, and it remains EBV associated during the course of the disease (Vasef *et al*, 1995; Brousset *et al*, 1994). These

data strongly suggest an aetiological role. Based on the available data, the favoured hypothesis is immune escape from the host CTL response, conferred by EBV, probably via the expression of cytokines. Interleukin (IL)-6 and IL-10 are significantly more frequently expressed in EBV-positive Reed-Sternberg cells than in EBV-negative cells (Herbst *et al*, 1996; Herbst *et al*, 1997). It is postulated that up-regulation of the ILs expression is mediated by LMP1 (Niedobitek, 1996). IL-10 is a pleiotropic cytokine with inhibitory effects on cell-mediated immunity. It inhibits the synthesis of interferon- γ and IL-2, which leads to inhibition of T-cell growth. It also mediates the immune response away from the Th1-type, which renders host T-cell protection ineffective. IL-10 is also capable of down-regulating expression of cytokines and MHC class II in macrophages, which further inhibits antigen-specific T-cell responses (Niedobitek, 1996). It is interesting to note that the presence of EBV in the tumour cells are associated with better prognosis. Glavnia-Durdov *et al* (2001) reported 26% EBV LMP-1 positive expression in their 100 classical Hodgkin's lymphoma cases, and the presence of EBV was associated with longer disease free survival in their young patients of <30 years old and <34 years old, $p=0.02$ and $P=0.05$ respectively. Similar observations were reported by Naresh *et al* (2000) with higher proportions of 10 years relapse free and 10 years overall survival in EBV positive cases. Engel *et al* (2000) made a study on childhood cases, and noted EBV positive patients presented with less aggressive symptoms at diagnosis and had a significant longer median survival. The proposed explanations include possibly a better host CTL response towards LMP-1 and LMP-2 expressed Hodgkin's Reed-Sternberg cells, and LMP-1 in turn have been shown to up-regulate LFA-3, ICAM-1 and MHC-1, hence resulting in increased immune surveillance.

b. *Human Herpes virus-8 (HHV-8)*

In 1994, Chang *et al.* isolated a unique DNA sequences from Karposi's sarcoma (KS) tissue of AIDS patients (Chang *et al.*, 1994). This DNA sequence being characteristic of the herpes virus family was named HHV-8. This study showed that the virus sequence was present in 90% of the KS tissue and 15% of non-KS tissue in corresponding AIDS patients, and not found in DNA of non-AIDS patients. This virus was found in almost all epidemic (AIDS-related) as well as the classic and endemic KS in subsequent studies. Presence of virus sequence in the peripheral mononuclear cells and seroconversion in HIV-positive patients is predictive of subsequent appearance of KS lesions. In addition to KS, this virus is also found to be closely associated with the rare "primary effusion lymphoma", PEL (Caesarman *et al.*, 1995; Carbonne and Gaidano, 1997), usually in HIV-positive patients, and multiple myeloma. Although the rare variety of body cavity lymphoma exhibits "null"-cell phenotype, it has been shown to have Ig gene rearrangement and to express CD45. The virus genome is known to encode homologues of bcl-2, chemokine receptor (the G-protein-coupled receptor, GPCR), interleukin-6 and cyclin D. However, the exact mechanism(s) of oncogenesis is yet to be elucidated. Cheng *et al.* in 1997 found that the KS bcl-2 blocks programmed cell-death (Cheng *et al.*, 1997), which may be responsible for a step towards malignant transformation due to inappropriate prolongation of cell survival. On the other hand, Arvanitakis *et al.* (Arvanitakis *et al.*, 1997) showed that HHV-8 GPCR is functional, and the signal cascade set in motion results in cellular proliferation. These investigators proposed that HHV-8 GPCR may therefore play a direct role in oncogenesis, by causing abnormal growth and/or transformation. On the other hand, the virus encoded cyclin is also functional,

which can contribute to cellular growth and transformation through activation of the cell cycle (Lyons and Liebowitz, 1998). In a recent study on the role of HHV-8 in PEL, it was suggested that virus infection alone is insufficient for tumour development, and that lesions in the cellular genes may be required for tumorigenesis (Gaidano *et al*, 2000).

1.4 OBJECTIVES OF THESIS

Previous studies of Malaysian lymphomas show a high frequency of EBV association in childhood Hodgkin's lymphoma and NK/T-cell lymphomas in the adult (Peh *et al*, 1997; Peh 2000), and only subtype A virus is present in a small number of T-cell lymphoma (Peh *et al* 1995). Hence, this thesis embarks on studies with the following objectives:

1. To elucidate the pattern of EBV association in non-Hodgkin lymphoma in West Malaysian children, for comparison with the adult pattern in the same medical centre studied earlier.
2. To determine the EBV association pattern of Malaysian Burkitt's lymphoma and whether this disease is more akin to endemic or sporadic type, since Malaysia is situated in the Tropical belt with high rainfall and malaria is endemic in some parts of the country.
3. To determine if the patterns of EBV association and subtypes of lymphoma in the state of Sabah have changed from an earlier study on cases seen 15 years earlier.
4. To determine the characteristic EBV subtype pattern in a wider spectrum and larger numbers of EBV associated diseases presented at different anatomical sites.

5. To determine the virus status in sequential biopsies of cases with known EBV association.

Chapter 2

Pattern of Epstein-Barr virus Association in Childhood Non-Hodgkin's

Lymphoma: Experience of University of Malaya Medical Centre

2.1 INTRODUCTION

Published data consistently show different patterns of malignant lymphomas observed in Asian and Western populations (Kadin *et al*, 1983; Harrington *et al*, 1987; Ng and Chan, 1988; Shih and Liang, 1991; Ho *et al*, 1984; Ji and Li, 1992). Substantial difference in the frequencies of subtypes of non-Hodgkin's lymphoma is also evident across geographical regions (Anderson *et al*, 1998). T-cell non-Hodgkin's lymphoma is more common in the Far East (Shih and Liang, 1991; Ji and Li, 1992; Anderson *et al*, 1998), and Epstein-Barr virus (EBV) associated NK/T-cell lymphomas of the nasal and nasal-type are more common in the East Asians (Anderson *et al*, 1998; Jaffe *et al*, 1996; Aozasa *et al*, 1989; Ho *et al*, 1990; Chan *et al*, 1987; Harabuchi *et al*, 1990; Chiang *et al*, 1996) and Peru (Arber *et al*, 1993) than the United States and Europe (Jaffe *et al*, 1996; Weiss *et al*, 1992b). The reason for these differences may be related to the environmental or host genetic and cultural factors. A recent study on the Asian migrants and their descendants to the United States shows that only some of the differences in disease pattern change (Herrinton *et al*, 1996). Hence, it appears that the incidence of some varieties of malignant lymphomas can be influenced by exposure to social-environmental factors, while others are strongly linked with the host characteristics. In a separate study on adult non-Hodgkin's lymphoma in Malaysians, it was clearly shown that host ethnicity influences the subtypes of lymphomas (Peh, 2001). In addition, the study on EBV associated nasal NK/T-

cell lymphoma in these patients indicate predilection of the disease in ethnic Chinese (Peh *et al*, 1995).

Malignant lymphoma is a relatively common group of cancers seen in children. It accounts for approximately 10% of all childhood cancers in most reported series (Ho *et al*, 1984, Agnarsson and Kadin, 1995), and often ranks third in relative frequency, after acute leukemias and brain tumours (Ho *et al*, 1984; Arber *et al*, 1993). However, childhood non-Hodgkin's lymphomas often constitute only small proportion of all patients with the disease in any one-centre studies (Gall and Mallory, 1942). Some series record a relatively higher incidence of Hodgkin lymphomas than non-Hodgkin's lymphomas in their population (Jaffe *et al*, 1998). The pattern of childhood non-Hodgkin's lymphomas usually differs from the adults, in that, the common subtypes are usually aggressive, "high-grade" varieties, such as precursor (lymphoblastic) and Burkitt's lymphomas with a higher propensity to be leukemic or widely disseminated at the time of diagnosis (Cossman *et al*. 1980; Wilson *et al*. 1984). The large cell lymphomas, particularly anaplastic large-cell is common in children whereas follicular lymphomas or other indolent subtypes are rare (Ho *et al*, 1990; Nair and Krishnaswami, 1990; Walter *et al*, 1991; Atra *et al*, 1998).

More recent published reports indicate a higher risk of developing lymphomas in children of certain ethnic origins, such as, Asian origin living in the United Kingdom (Varghese *et al*, 1996), Hispanic in Florida, United States (Wilkinson *et al*, 2001), and children of subcontinental origins (Revesz *et al*, 1995). These reports proposed that the difference is probably related to the underlying aetiological factors of these diseases. The temporal trends in the incidence of childhood leukemias and lymphomas suggest a role of infection for

the increase in acute lymphoblastic leukemias and Hodgkins' lymphomas, and environmental factors other than infections for NHL (McNally *et al*, 2001). Flavell *et al* (2001) reported a strong association of EBV and Hodgkin's lymphoma in South Asian children from the United Kingdom, and also demonstrated that socioeconomic deprivation increases the risk of EBV-associated diseases which is independent of ethnicity. The observations made on EBV association and Hodgkin's lymphomas in Malaysian patients of various ethnic origins and age groups support this notion (Peh *et al*, 1997). Thus far, HHV-6 is not implicated in Hodgkin's lymphoma in children (Shiramizu *et al*, 2001).

The pattern of non-Hodgkin's lymphomas in Malaysian adults had been studied previously (Peh, 2001), but not that of the childhood group. Since malaria is endemic in Malaysia in view of the tropical climate and high level of rainfall, similar to many tropical African countries, the subtype pattern and the role of EBV in childhood lymphoma is therefore also of special interest. This study aims to determine the disease pattern of children from patient population in a single medical centre in West Malaysia, the University Hospital Kuala Lumpur, for direct comparison to the data of the adults from the same medical centre (Peh, 2001).

2.2 MATERIAL AND METHODS

The total number of confirmed childhood (aged <15 years) malignant lymphomas confirmed retrievable from the archives in the Department of Pathology, University of Malaya, between the period of 1988 to 1992 was 35 NHL and 9 HL (Table 1). The ratio of NHL:HL was 4:1. In order to achieve a

better reflection on the pattern of this group of diseases in children, all cases of lymphomas from this age group were retrieved for review and analysis from the files, dating back from 1971. The method of study was similar to that carried out for the adults (age 15 years and above) for ease of direct comparison (Peh, 2001). In brief, the H&E stained slides were first examined and the lesions initially categorised broadly into Hodgkin and non-Hodgkin's, or non-lymphoma groups. They were subsequently reconfirmed or excluded from the group with the aid of immuno-phenotype profile. A total of 69 patients were re-confirmed as non-Hodgkin's lymphomas and 34 as Hodgkin's disease. The 34 cases of HL were studied separately and published (Peh *et al*, 1997).

The confirmed cases of NHL were sub-classified according to the WHO proposed list on neoplastic diseases of the haematopoietic lymphoid tissue (Jaffe *et al*, 1998). Of the cases of NHL, 60 had sufficient material for further immuno-phenotyping into T-, B- and null-cell categories. T-phenotype was determined by tumour cell expression of CD3 or CD45RO (UCHL1) and CD43 (MT1) with absence of B-cell antigen, and the expression of any or in combination of the following: CD20 (L26), CD79 α , monotypic κ or λ , for B-cell lymphomas. When the tumour cells did not express any of the above antigens, the lesion was classified as null-cell phenotype. All cases with lymphoblastic lymphomas morphology were reconfirmed as precursor cell type by positive phenotype expression of TdT. Cases with poor histomorphology due to bad processing techniques or crush artifacts were categorized as unclassified in the respective T- and B-cell groups. The recruited cases with adequate material were also probed for the presence of EBV genome by in situ hybridization technique for the presence of EBER (EBV-encoded early nuclear RNAs).

The demographic data of these patients were extracted from the patient information provided on the request forms accompanying the biopsy specimen.

Immunohistochemistry

A standard three stage immunoperoxidase were performed using LSAB®+ kit (Dako, Denmark) and a panel of lymphoid antibodies mostly from Dako (Denmark) unless otherwise specified: CD3, CD10, CD15 (Leu-M1, Becton-Dickinson, USA), CD20 (L26), CD21, CD30 (BerH2), CD43 (MT1, compliment from Professor S Poppema), CD45RO (UCHL1), CD56 (1B6, Novocastra, UK), CD57 (Leu-7, Becton-Dickinson, USA), CD68 (PGM1), TdT, α -kappa, α -lamda, CD79 α (a gift from Professor D. Mason), CD5 and CD23 (a gift from Dr. Imam). Antigen retrieval with microwave treatment was used in most instances, and occasionally pressure cooker heat treatment or pre-digestion in trypsin were employed (Appendix).

In-situ hybridization

The materials were tested for the presence of EBV by using EBV encoded RNAs (EBER) *in-situ* hybridization with the use of fluorescein isothiocyanate (FITC)-labeled oligonucleotide (NCL-EBV, Novocastra, United Kingdom) according to the protocol of the manufacturer. Visualisation was achieved with incubation of antibody to FITC tagged with an enzyme (alkaline phosphatase), followed by application of substrates for the color reaction to take place. The substrate of choice was NBT/BCIP (4-nitroblue-tetrazolium chloride/5-Bromo-4 chloro-3-indolyl-phosphate). Malaysian EBV-positive naso-pharyngeal carcinoma cases were used as external positive control for the staining technique. A lymphoma is regarded as EBV associated if majority of the tumour cells strongly expressing EBER.

2.3 RESULTS

Demographic data

There were 50 male and 19 female patients in this series. The male-female ratio is 2.6:1. The ethnic distribution is as follows: Malays 14, Chinese 46, Indian 8 and none-of-the above 1. The M:C:I ratio is 1:3.3:0.6. The age of presentation of these cases is as follows:

<i>Age</i>	<i>No</i>	<i>%</i>
<5 years	18	26
5 to <10 years	29	42
10 to <15 years	22	32
Total	69	100

There is no observed progressive increase in the incidence of non-Hodgkin's lymphomas with age.

Lymphoma subtypes

The ratio of HL to NHL in children was 1:2 (34:69). Of the 60 cases of NHL phenotyped, there were a total of 22 (22/60, 37%) cases of T-cell lymphomas and 35 (35/60, 58%) cases of B-cell lymphomas and 3 (3/60, 5%) cases of null-cell lymphomas (Table 2). The T-cell lymphomas were composed of 14 (56%) precursor type (lymphoblastic lymphoma/leukemia) and 11 (44%) mature types. The latter consisting of 5 anaplastic large cell lymphoma (ALCL), 2 peripheral T-cell lymphoma (PTCL)-unspecified and 1 natural killer (NK)/T-cell lymphoma. This latter case had disease involving both the nose and the skin. The 3 cases of null-cell lymphomas had anaplastic large cell morphology and is grouped under T-cell lymphoma as proposed by WHO classification. All these 8 cases of T/null-cell ALCL express ALK protein (Fig.1).

The 35 cases of B-cell lymphomas were composed of 7 (20%) precursor type (lymphoblastic lymphoma/leukemia) and 28 (80%) of mature types. The latter group consisted of 22 cases of Burkitt's lymphomas, 5 large cell type of which one case expressed CD30 in majority of the tumour cells, and one case in which it was not possible to be categorized due to poor morphological detail. The CD30-positive tumour did not express ALK protein.

The 3 most common morphological subtypes of lymphoma occurred were lymphoblastic 23 (14 T, 7 B and 2 non phenotyped), Burkitt's lymphoma 25 (22 B and 3 non phenotyped) and anaplastic large cell lymphoma 9 (5T, 3 null, 1 non phenotyped). Indolent types of lymphomas appear not to occur in children in this series.

35 cases (51%) presented the disease primarily in the lymph nodes, with or without extra-nodal involvement. The remainder cases had diagnostic tissue excised from the extra-nodal site. The common extra-nodal site was the intestinal tract for B-cell (Burkitt's) lymphoma and mediastinum for T-cell (lymphoblastic) lymphoma.

Ethnic group distribution and EBV association

In this series of childhood non-Hodgkin's lymphomas, the ethnic distribution of T-cell types for Malay:Chinese:Indian was 5:17:3 (1:3.4:0.8) and B-cell types was 7:23:4 (1:3.3:0.6). The 3 cases of null-ALCL were 2 Chinese and 1 Malay. EBV associated lymphomas are more prevalent in B-cell lymphomas (24%, 8/34) than T-cell lymphomas (14%, 3/22). The commonest EBV associated lesion was Burkitt's lymphoma (29%, 6/21). The ethnic distribution of M:C:I in this subtype of lymphoma was 1:2.8:0.6 (5:14:3). However, there was increased risk of EBV association for BL in the Chinese (5/13, 38%) when compared to

non-Chinese (1/8, 12.5%) patients. The increased risk of EBV association in the Chinese was also present in T-cell lymphomas (3/15, 20%), when compared to non-Chinese (0/7, 0%), i.e. all cases of T-cell lymphomas associated with EBV infection is present in the Chinese. The one and only case of precursor T-cell lymphoma associated with EBV infection was presenting in the inguinal lymph node of a 4 year-old female Chinese patient.

2.4 DISCUSSION

The subtype frequencies of this series of Malaysian childhood lymphomas distinctly differ from that of the adult pattern from the same medical centre (Table 3), in that, lymphoblastic, Burkitt's and ALCL accounted for only approximately 6%, 5% and 1% respectively of the total NHL cases in the adult. However, the pattern seen in Malaysian children is rather similar to childhood lymphomas reported elsewhere in the world outside tropical Africa, with 3 dominant groups comprising of lymphoblastic type, Burkitt's lymphoma and anaplastic large cell lymphoma (Cossman *et al.* 1980; Wilson *et al.* 1984). Burkitt's lymphoma is common in children even in non-endemic regions in the world (Philip *et al.*, 1982; Wright *et al.*, 1997), but this subtype is exceedingly higher in the tropical Africa. Burkitt's lymphoma was reported to account for 47% and 68% of all childhood cancers during the period between 1960s to 1980s in countries like Nigeria and Uganda (Stiller and Parkin, 1996). It appears that endemic malaria in this country did not increase the incidence of Burkitt's lymphoma. This is not unexpected since the University Hospital Kuala Lumpur is situated in the Federal Territory, providing health care facilities mainly to urban populations. Anopheles mosquito, the vector for malaria parasites does not flourish in this urban environment, and

hence malaria does not pose a major health problem to town and city dwellers. It is also reflected by the relatively lower EBV association rate in this category of lymphoma (6/21; 28.5%), which differs from the high EBV association rate of endemic BL in tropical Africa (Magrath 1990; Tosato *et al*, 1995).

T-cell lymphomas are commonly seen in the ethnic Chinese (17/25; 68%), a pattern similar to that observed in the adults (Table 3). The predilection for the Chinese is seen more distinctively in the mature subtypes (9/11; 81.8%). The subtype composition of T-NHL again differs in these 2 age groups, in that, the precursor type is more common in children but NK/T-cell lymphomas are not (Table 2 and 3). Similar to other reports, the commonest type in the mature T-cell category in this series is ALCL, known to be closely associated with expression of ALK, and had been reported to carry good prognosis in children (Shiota *et al*, 1995; Aoun *et al*, 1998; Gascoyne *et al* 1999). However, mature (peripheral) T-NHL accounted for 44% of all T-NHL in this series, which is similar to that reported elsewhere (Agnarsson and Kadin 1995). The higher prevalence of T-NHL in Asians is also reflected in childhood group. There is no immediate apparent explanation for this phenomenon.

As a result of the differences in subtype composition of T- and B-cell lymphomas in children and the adults, the overall EBV-association rate also differs. In children, the EBV association rate in T-NHL is 13% whereas it is 36% in the adults, i.e. almost 3X higher in the older age group (Table 3). This is because the commonly EBV associated NK/T-cell lymphomas are uncommon in children, whereas ALCL and lymphoblastic lymphomas, which are not commonly associated with EBV constitute the major subtypes in the T-NHL category (D'Amore *et al*, 1996). A reverse pattern is true for B-cell lymphoma. Due to the

relative common occurrence of Burkitt's lymphoma in children but not in the adults, the EBV association rate of B-NHL as a group in children is 3X higher than in the adults (24% and 7% respectively). However, the EBV association rate of Burkitt's lymphoma in children and adults do not differ significantly (27% and 38% respectively).

In conclusion, the pattern of childhood lymphomas is relatively similar to children elsewhere in the world outside regions with endemic Burkitt's lymphoma. The EBV association rate of T- and B-NHL differ between children and adults from the same medical centre, and this difference is the result of dissimilar subtype composition of NHL in these 2 age groups. The relatively low EBV association rate in childhood NHL differ from the experience of classical Hodgkin's lymphoma from the same medical centre (Peh et. al, 1997), in that, EBV is present in almost all cases of Hodgkin's lymphoma. Hence, EBV possibly plays a vital role in the development of classical Hodgkin's lymphoma in children in the developing country, but its importance in the pathogenesis of non-Hodgkin's lymphoma e.g. Burkitt's lymphoma needs further evaluation. In Hodgkin's lymphoma, the pathogenetic hypothesis is supported by more recent report on the expression of RCAS (Receptor-binding Cancer Antigen SiSo cell)-1 in the Reed-Sternberg cells, believed to be the pathway for evasion of host immune system by the EBV infected tumour cells (Oshima *et al*, 2001).

In conclusion, the subtype patterns of childhood lymphoma in Malaysian children differ from the adult counterpart, resulted in different EBV association rates in the T- and B-cell lymphoma categories. However, the subtype pattern of childhood lymphomas in this series follow closely the pattern reported elsewhere

in the world, except for higher frequency of T-NHL, which appears to be rather characteristic of Asian lymphomas.

Table 1: Age group distribution of malignant lymphomas**Years 1988 – 1992**

Category	Children	Adult	Total
	<15 years	≥ 15 years	
HL	9	15	24
NHL	35	173	208
Total	44	188	232

Table 2: Sex and ethnic distribution of non-Hodgkin's lymphoma in Malaysian childhood patients

Lymphoma Subtypes	No.	Sex		Race				Site of presentation
		m	f	M	C	I	O	
Precursor T=	14	10	4	3	8	3	0	9LN, 5 mediastinum
EBER +ve	1			0	1	0		
Test no	14			3	8	3		
Mature T=	11	7	4	2	9	0	0	6LN, 2 s/c tissue
ALCL(T/null#)	8	5	3	1	7	0	0	
EBER +ve	0			0	0			
PTCL- unspecified	2	2	0	1	1	0	0	2LN
EBER +ve	1			0	1			Nose and skin
Test no.	2			1	1			
NK/T	1	0	1	0	1	0	0	
EBER +ve	1				1			
Test no.	1				1			
Total	25	17	8	5	17	3		
EBER +ve	3			0	3	0		
Test no	23			5	17	3		
Precursor B=	7	5	2	1	6	0	0	4LN, 3testes
EBER +ve	0			0	0			6LN, 8gut, 4others, 4upper aerodigestive
Test no.	7			1	6			
Mature B=	28	21	7	5	14	3	0	
BL	22	17	5	5	14	3	0	4LN, 1PNS
EBER +ve	6			1	5	0		
Test no.	21			5	13	3		
Large cell	5	3	2	1	2	1	1	Pleural tissue
EBER +ve	2			1	1	0	0	
Test no.	5			1	2	1	1	
NOS	1	1	0	0	1	0	0	
EBER +ve	0				0			
Test no.	1				1			
Total	35	26	9	7	23	4	1	
EBER +ve	8			2	6	0	0	
Test no.	34			7	22	4	1	
Non phenotyped=	9	7	2	2	6	1	0	2LN
Lymphoblastic	2			0	1	1	0	1LN, 1ovary, 1liver
BL	3			1	2	0	0	1LN, 1skin
Large cell	2			1	1	0	0	2liver
NOS	2			0	2	0	0	

3 cases of null-cell ALCL

C, Chinese; I, Indian, M, Malay, O, other ethnic groups; f, female; m, male; LN, lymph node; PNS, postnasal space; s/c, subcutaneous.

Table 3: T- and B-cell lymphomas in adult: morphological types, sex and ethnic distribution and pattern of EBV association (adopted from Peh 2001).

Lymphoma subtypes	No.	Sex		Race				EBV			Sites
		m	f	M	C	I	O	+ve	Test	%	
Precursor T=	9	9	0	2	6	1	0	0	7	0	5 LN, 3 med mass, 1 skin+pleura
(25%)											
Peripheral T=	27										
(75%)											
Unspecified	10	8	2	2	6	2	0	3	9	33	6 LN, 1 spleen, 1 skin, 1 liver, 1 intestine
AILD	5	3	2	0	3	1	1	3	5	60	4 LN, 1 skin
ALCL	2	2	0	1	1	0	0	0	2	0	1 LN, 1 s/c tissue
NK/T	7	6	1	2	5	0	0	6	7	86	3 nose, 3 nasopharynx, 1 epiglottis
MF	2	2	0	1	1	0	0	0	2	0	2 skin
Unclassified	1	0	1	0	1	0	0	0	1	0	1 liver
Total	36	30	6	8	23	4	1	12	33		
Precursor B=	1	1	0	0	1	0	0	0	1	0	1 med mass
Peripheral B=	120										
Lymphocytic	3	2	1	1	2	0	0	0	2	0	3 LN
Lympho-plasmacytic	3	3	0	0	3	0	0	0	3	0	3 LN
Plasmacytoma	2	2	0	1	0	0	1	0	2	0	1 spine, 1 chest wall
Follicular	15	10	5	5	4	5	1	0	14	0	14 LN, 1 retroperitoneal mass
Mantle cell	3	2	1	1	2	0	0	0	3	0	2 LN, 1 rectum
Burkitt's	8	8	0	0	8	0	0	3	8	38	2 LN, 2 testes, 1 brain 1 maxilla, 1 thigh, 1 med. Mass
Diffuse, large-B	70	43	27	23	38	8	1	4	66	6	31 non LN*, 36 LN, 3 LN + extranodal**
Primary Gastric	10	7	3	0	8	2	0	0	10	0	10 Stomach
MALT: low gr.	1										
high gr.	2										
Large B-cell	7										
Unclassified	6	3	3	1	4	1	0	1	6	20	3 LN, 2 pancreas, 1 brain
Total	121	81	40	32	70	16	3	8	115	7	

med., mediastinal; *: tonsils 5, nose/nasopharynx/nasal sinus 5, tonsil + gut 1, gut 2, brain 3, mediastinum/thymus 2, spleen 2, bone/spine 2, thyroid 1, parotid gland 1, breast 1, bladder 1, soft tissue (thigh 2, neck 1, chest-wall 1, retroperitoneum 1); **: nodal disease spread to intestine 2, nodal disease spread to skin 1.

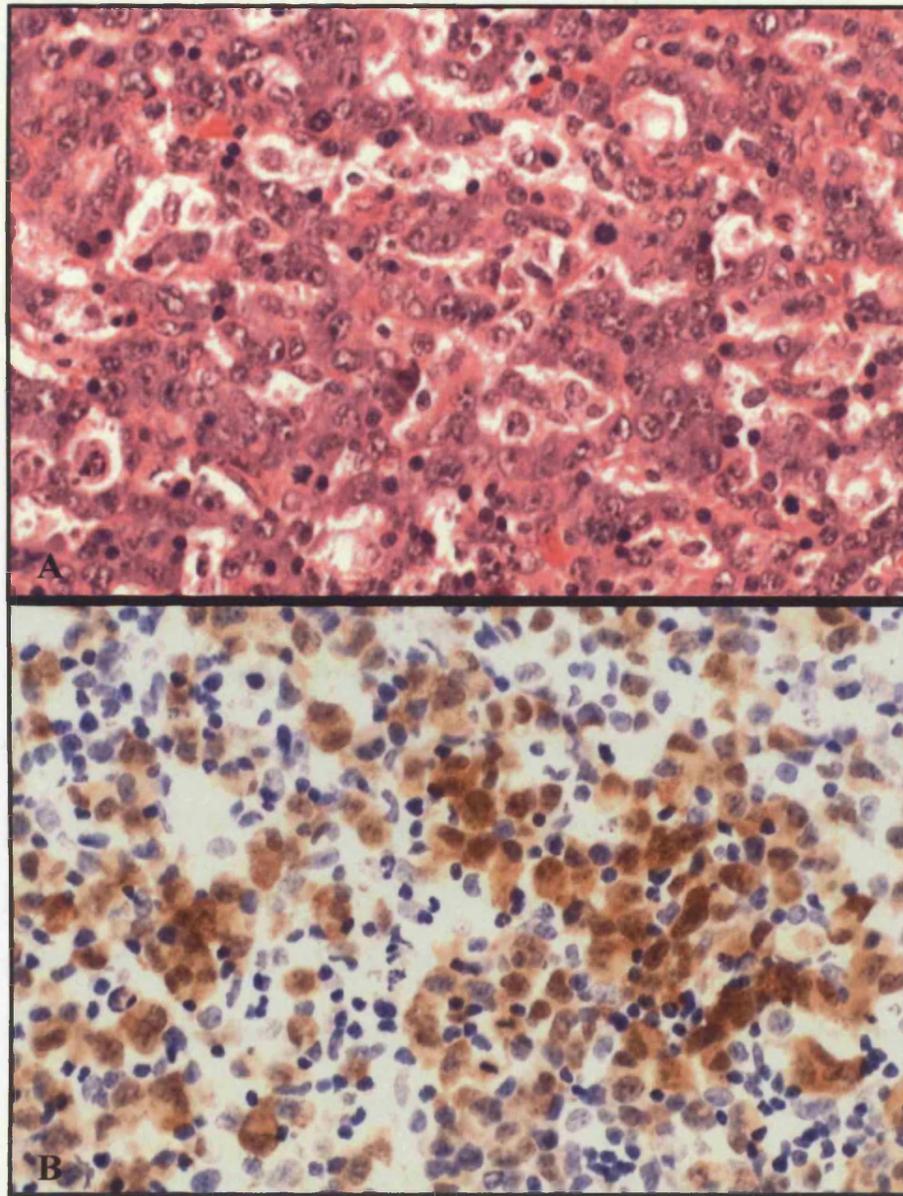


Figure 1: A) Anaplastic large cell lymphoma with cohesive cords of tumour cells with admixed histiocytes and lymphocytes. H&E X 450.

B) Positive nuclear and cytoplasmic expression of ALK protein.

ALK1 immunohistochemical stain X 450

Chapter 3

Epstein-Barr Virus Association Pattern in Burkitt's and Burkitt's-like Lymphomas: Experience of University of Malaya Medical Centre

3.1 INTRODUCTION

Burkitt's lymphoma, a highly aggressive B-cell malignancy is a well-defined entity described by strict morphologic criteria of the proposed World Health Organisation (WHO) classification scheme (Jaffe *et al.*, 1998). This lymphoma group was first described as a rare jaw sarcoma in African children in 1958 by a British surgeon, Dennis Burkitt (Burkitt, 1958). It aroused much interest because jaw tumours of any other histological types were rare in African children. A nations-wide survey in Africa countries revealed that the endemicity coincided with the distribution of rainfall, altitude, and malaria holoendemic or hyperendemic region (Ziegler 1981; Burkitt, 1983). After the description of this African lymphoma by Burkitt, a tumour with similar histology was recognised in other parts of the world (Levine *et al.*, 1975). However, these sporadic cases occur at lower frequency compared to the endemic cases in Africa and Papua New Guinea (Stiller and Parkin, 1996).

Both endemic and sporadic Burkitt's lymphoma carry translocation involving the c-myc gene on chromosome 8 with one of the heavy chain or light chain genes of the immunoglobulin gene. However, they display large variation at the breakpoint on chromosome 8 (Magrath, 1990; Gutierrez *et al.*, 1992; Tosato *et al.*, 1995). Endemic Burkitt's lymphoma almost always breaks at the far 5'-end

upstream of the c-myc gene, while sporadic tumours contain breakpoints within or close to the first exon of the c-myc gene (Gutierrez *et al.*, 1992). In addition, differences with regards to age and site of presentation, and rate of association with Epstein-Barr virus (EBV) are also observed in the endemic and sporadic Burkitt's lymphoma (Magrath, 1990).

The exact role of EBV in the pathogenesis of Burkitt's lymphoma remain uncertain due to the disparity in EBV association in endemic and sporadic types. Studies on Burkitt's lymphoma showed that EBV is detected in almost 100% of the patients in endemic areas, but only approximately 15% to 30% of the patients in sporadic areas (Magrath, 1990; Tosato *et al.*, 1995). It has been postulated that EBV is involved in the first step of pathogenesis (Klein, 1978-79; Goldstein and Bernstein, 1990; Toren *et al.*, 1996), where it immortalises a group of B-lymphocytes. When these EBV-positive lymphocytes are stimulated by environmental agents, such as chronic holoendemic malaria, they then proliferate as a group of polyclonal pre-neoplastic cells. Continuous antigenic stimulation then increases the probability of recombinase error, which ultimately results in chromosomal translocation involving c-myc gene, thence leading to malignancy. Subsequent aberrant events involving other oncogenes or tumour suppressor genes may occur, driving progression to a high grade tumour. Studies showed that a tumour suppressor gene, p53 is frequently mutated in Burkitt's lymphoma (Gaidano *et al.*, 1991; Bhatia *et al.*, 1992).

This current study describes Burkitt's lymphoma from patients in Malaysia with regard to clinical presentation, EBV association, EBV subtype and expression of p53 protein. In addition, the expression of Bcl-2 and retinoblastoma (pRb), and Ki67.

3.2 MATERIALS AND METHODS

Patients' material

Record of cases previously diagnosed as Burkitt's lymphoma were retrieved from the archives. Haematoxylin and eosin (H&E) stained slides of 67 biopsies from 64 patients were reviewed for confirmation and classification according to the criteria from the WHO proposed classification of lymphoid neoplasm. Of these 67 biopsies, 5 (from 5 patients) were reclassified as diffuse large B cell lymphoma and hence were excluded from further analysis. Seven cases (from 7 patients) from the remaining 59 patients were without tissue blocks for immunohistochemical stain. The demographic data of these patients were obtained from the information sheet volunteered by the clinicians. Serial 3 μ m sections of the remaining 55 biopsies from 52 patients with available tissue blocks were used for further analysis by immunohistochemical staining and *in situ* hybridisation.

Immunohistochemical staining

Immunohistochemical stain was performed with a panel of antibodies, mostly from Dako (Denmark) unless otherwise specified, which include CD3, CD20 (L26), bcl-2 (clone 124), p53 (clone DO-7), pRb (clone G3-245, PharMingen) and Ki67. Antigen epitopes were retrieved for all of the antibodies by the microwave heat-inducing method. A three-step immunoenzymatic staining method was used to localise the antigens for CD3, CD20, and pRb. Peroxidase-labelled Avidin-Biotin Complex (ABC) system (Dako, Denmark) was used for CD3, and CD20, and peroxidase-labelled Streptavidin-Biotin Complex (StrepABC) was used for pRb. Bcl-2 and p53 were localised using a two-step staining procedure by peroxidase-labelled polymer in DAKO EnVisionTM+

System (Dako, Denmark). The colour was developed using DAKO liquid DAB⁺ substrate-chromogen system. The tissues were then lightly counterstained with haematoxylin.

Staining value for other antibodies, CD20, CD3, and Bcl-2 was recorded as either positive or negative. For the evaluation of p53, and pRb staining, the average nuclear staining intensity was scored as “+” (weak), “2+” (moderate), and “3+” (strong). Eye-ball estimation of the percentage of positive tumour cells for these stains was performed. Cases with less than 10% of the tumour cells expressing these proteins were considered as negative expression.

In situ hybridisation

The presence of EBV was detected by *in situ* hybridisation (ISH) technique, with fluorescein-conjugated EBV oligonucleotide probes (NCL-EBV, Novocastra, United Kingdom) for EBV early RNAs (EBER). Alkaline phosphatase-conjugated rabbit anti-FITC was then added, followed by introduction of substrate, 4-nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate (NBT/BCIP). The tissue sections were counterstained with Meyer haematoxylin. A known EBV-positive nasopharyngeal carcinoma was used as an external positive control for assessment of adequacy of each batch of staining procedure.

DNA Extraction

DNA was extracted from formalin-fixed, paraffin-embedded tissues. Section of 5- μ m thick was deparaffinised by adding xylene, followed by two washing with absolute alcohol and air-dried. Tissue was digested by adding 200 μ l of digestion buffer containing 200 μ g/ml Proteinase K (Roche Molecular Biochemicals, Germany) and 1X PCR buffer (GIBCO BRL, USA). The mixture

was incubated at 55°C overnight. Proteinase K was inactivated by heating at 95°C for 10 minutes, and the cell debris was pelleted by centrifugation at 10,000rpm for 10 minutes. The supernatant containing DNA was used directly for PCR amplification.

EBV Sub-typing

EBV sub-typing was carried out for all the cases that the virus was detected by *in situ* hybridisation. Amplification of the EBNA-2 gene was performed according to a nested PCR procedure, by using the two primer pairs: a) the outer primer pair (Kunimoto *et al.*, 1992) (sense: 5' TTT CAC CAA TAC ATG AAC C 3'; anti-sense: 5' TGG CAA AGT GCT GAG AGC AA 3'); and b) the inner primer (sense: 5' CAA TAC ATG AAC CRG AGT CC 3'; anti-sense: 5' AAG TGC TGA GAG CAA CCG MC 3'). These primer pairs generate products of 368-bp for subtype A, and 473-bp for subtype B virus. Amplification was performed with 1µl of DNA sample in a 50-µl reaction mixture containing 2.5U of *Taq* DNA polymerase (Gibco BRL, USA), 1.5mM MgCl₂, 0.2mM dNTP mix (Biotools, Spain), and 0.5 µM of each primer in 1X PCR buffer (Gibco BRL, USA). *Taq* DNA polymerase was added after a hot-start step, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes, with the final extension at 72°C for 5 minutes. Nested-PCR using the inner primer pair was carried out with 1 µl of the PCR product under the same conditions and annealing temperature at 60°C for 30 cycles.

Detection of t(14;18) by PCR

Detection of t(14;18) by PCR was performed on cases that showed Bcl-2 protein expression using immunohistochemistry. A nested-PCR described by

Gribben *et al.* (1991) were employed to amplify the major breakpoint region (mbr) of *bcl-2* gene. The 50- μ l reaction mixture containing 2U *HotStarTaq* DNA polymerase (QIAGEN, Germany), 3.0mM MgCl₂, 0.4mM dNTP mix (Biotools, Spain), and 0.5 μ M of each primer was subjected to the following conditions: 15 minutes at 95°C to activate the *HotStarTaq* DNA polymerase, followed by 30 cycles of 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C. The size of the PCR products ranges from 100bp to 300bp. A cell line with known mbr breakpoint, RL-7 (kindly provided by Dr John C Chan of University of Nebraska Medical Center, USA) was used as a positive control. PCR products were electrophoresised on 2% agarose gel (GIBCO BRL, USA) containing 5 μ g/mL ethidium bromide (GIBCO BRL, USA), and was visualised on a UV illuminator.

3.3 RESULTS

There are 47 male and 12 female from a total of 59 patients, with a male to female ratio of 3.9:1. Ethnic Chinese is the largest group constituting 52.9% (31/59) of the patients. This is followed by Malay (16/59, 27.1%), Indian (3/59, 5.1%), and others, which include the indigenous groups from Sabah and Sarawak (9/59, 11.9%). The majority are children less than 15 y old (43/59, 72.9%), with a mean age of 15.8 y (Figure 1). The main sites of presentation were the lymph nodes (24/62, 38.7%) and abdominal viscera (19/62, 30.2%). Other common sites included testes and ovary (4, 6.5%), gum and maxilla (4, 6.5%), tonsil (2, 3.2%), nose (2, 3.2%), and brain (2, 3.2%). The remaining 5 (8.1%) biopsies were taken one each from other sites (orbit, bone marrow, soft tissue of neck, upper thigh, infra-spinal region).

Seventeen (17/51, 33.3%) of the patients were infected with EBV (Table 1), and one case was not assessable by ISH for detection of EBER. Among the EBV-positive cases, 11 of 17 were children younger than 15 y, 5 of 17 were adults and one patient lacked information on age. There were 23 childhood and 11 adult tumors not infected by EBV. The ratio of children to adults in EBV-positive and EBV-negative patients was 2.2:1 and 2.1:1, respectively, with no correlation between the age and EBV-association in the tumors.

The association rate with EBV in patients' material was found to be higher in Malay (50%) than in Chinese (25%) compared to zero among Indians. Burkitt's lymphoma from East Malaysia (Sabah and Sarawak) showed a higher association with EBV (4/8, 50%), than those from West Malaysia (13/43, 30%). Subtyping of EBV by PCR showed that all of the EBV-associated tumors were infected with type-A virus, irrespective of the ethnic origin of patients (Figure 2).

Six of the EBV-positive biopsies were taken from abdominal viscera, 4 from lymph nodes, 3 from the gum and maxilla, and one each from the tonsil, brain, upper thigh, and infra-spinal region. The association rate of EBV and site of biopsy was 31.6% (6/19), 16.6% (4/24), and 75.0% (3/4) for abdominal viscera, lymph node, and gum and maxilla, respectively. The EBV-positive rate was significantly higher in biopsies from the gum and maxilla when compared to those from the abdominal viscera and lymph node (Figure 3).

Immunohistochemical staining was performed on 55 biopsies (from 52 patients) with available tissue blocks. Eye-ball estimation of the percentage of positive tumor cells for p53, pRb and Ki67 staining was performed. Staining for Bcl-2 was recorded as either positive or negative. Positive expression was scored when more than 10% of the tumor cells showed positive staining. Of the 55

biopsies analyzed, 7 cases showed negative p53 expression, and 6 cases were unreactive for p53 staining. The remaining 42/49 (85.7%) cases showed a high proportion of the tumor expressing p53 protein at various intensities (Table 2). In 24 (57.1%) of these 42 cases, more than 75% of the tumor cells expressed the p53 protein (Figure 4).

As for pRb expression, 4 cases were considered negative since less than 10% of the tumor cells expressed pRb, another 4 were did not react to pRb stain. Expression of pRb was detected in all of the remaining cases (47/51, 92.2%).

Ki67 expression was assessable in 39 (70.9%) of the cases as 16 (29.1%) were not immuno-reactive. Among the 39 cases, 29 (74.4%) expressed Ki67 in more than 50% of the tumor cells. Ki67 was detected in more than 50% of the tumor cells in all cases expressing Bcl-2. The expression of Ki67 also showed a positive correlation with the expression of p53.

Bcl-2 protein was detected in 9/54 cases (16.7%). The mean age of this group of patients was 19.7 years. The sites of presentation were 3 from the lymph nodes, one each from the tonsil, ovary, intestine, orbit, nose and soft-tissue of the thigh. PCR analysis did not detect the presence of translocation t(14;18) involving mbr in any of these 9 cases, 3 (33.3%) were associated with EBV (Figure 5).

3.4 DISCUSSION

Endemic and sporadic Burkitt's lymphoma share a similar histological appearance, but differ in clinical presentations (Burkitt, 1983; Ziegler, 1981; Magrath, 1990). Both African and sporadic Burkitt's lymphoma frequently involve the abdominal viscera (50 to 60%), however, the jaw is rarely involved in

sporadic Burkitt's lymphoma. African Burkitt's lymphoma peaks between the ages of 5 and 10 y, with a mean age of 7; while the sporadic type peaks at 17 y.

This series revealed only 4 cases (6.5%) with jaw and facial bone presentation. This rate agrees with experiences from the USA (Ziegler, 1981), Europe (Philip *et al*, 1982), Taiwan (Chao *et al*, 1997), and Hong Kong (Chan *et al*, 1995a). The common lymph node presentation (24/62, 38.7%) contrasts sharply with the rate in endemic areas, where fewer than 1% of cases present in the lymph nodes (Tosato *et al*, 1995).

Children younger than 15 y old formed the largest group among our patients. This was similar to reports that Burkitt's lymphoma is one of the most common childhood lymphomas (Philip *et al*, 1982; Wright *et al*, 1997). In Malaysia, Burkitt's lymphoma constitutes approximately 37.0% of childhood NHL (data in preparation for publication), and only about 5.0% of adult NHL (Peh, 2001). The mean age at presentation (15.8 y) in Malaysian Burkitt's lymphoma is intermediate between the pattern for cases from Africa (7 y) and USA (11 y) (4), and in other countries in Asia, such as Taiwan (33 y) (18), Hong Kong (35.5 y) (19), and Japan (28 y) (Miyoshi, 1985).

Almost all endemic Burkitt's lymphomas are associated with EBV. Although more than 75% of patients with sporadic Burkitt's lymphoma are EBV seropositive, the association with EBV in the tumors is low, ranging from 15% to 30% (Magrath, 1990). Moreover, the association of EBV with sporadic Burkitt's lymphoma shows geographical variations. It has been reported to be as low as 5.0% in Europe and the USA (Magrath, 1990; Philip *et al*, 1982), and as high as 73% in Egypt (Anwar *et al*, 1995). Asian countries, such as Taiwan (Chao *et al*, 1997), Hong Kong (Chan *et al*, 1995a), and Japan (Miyoshi, 1985), have reported

association rates of 42.3%, 28.0%, and 13.0%, respectively. This study of Malaysians showed an association of 33.3% that fell within the Asian range.

EBV can be classified into two subtypes based on the polymorphisms in their nuclear antigen (EBNA)-2 genes. It has been reported that type A predominates in Europe, US, and Asia, while type B is restricted to Central Africa, La Réunion, and Papua New Guinea (Young *et al*, 1987; Zimber *et al*, 1986) and in immunocompromised patients (Buisson *et al*, 1994; Borisch *et al*, 1992). EBV subtyping in the current series showed that all of the tumors were infected with type-A virus. It is unknown why Burkitt's lymphoma in other parts of the world is rarely associated with type B virus, although studies have indicated that type B virus is also found in healthy populations, with almost equal frequency in the USA (Sixbey *et al*, 1989), and less commonly in Japan (Sidagis *et al*, 1997). It was suggested that prevalence of type A and type B virus may simply reflect the relative frequency of these viruses in the population (Sixbey *et al*, 1989).

Hence, taking into account all the clinical presentations: (a) low frequency of jaw tumors, but high frequency of lymph node and abdominal involvement; (b) mean age of presentation around 16 y old; and (c) intermediate EBV association rate (33.3%), we concluded that Malaysian Burkitt's lymphoma differs from African Burkitt's lymphoma, and conforms more closely to sporadic Burkitt's lymphoma, despite early EBV infection in the population (Yadav *et al* 1987; Tan and Henle, 1972) and the endemicity of malaria (Rahman *et al*, 1997, Mak *et al*, 1992).

Wild-type p53 protein is present at low levels in cells and has a short half-life. However, mutation of the p53 gene results in conformational changes of the

protein that stabilize the protein and lead to a longer half-life. These mutant p53 proteins therefore will accumulate in the cells, making it possible for them to be detected by immunohistochemical staining (Martinez-Delgado *et al*, 1997; Villuendas *et al*, 1992). Mutations in the p53 gene occur frequently in Burkitt's lymphoma, i.e. 63% of Burkitt's lymphoma cell lines, and 33% of primary tumors (Bhatia *et al*, 1992). Studies have shown that mutation of the p53 gene are more commonly found in high-grade NHL, leading to the hypothesis that p53 is involved in the progression, rather than the initiation, of tumors (Gaidano *et al*, 1991; Villuendas *et al*, 1992). This is in accord with the proposed form of pathogenesis, where the tumor suppressor gene is involved in the final step of the pathogenesis (Klein, 1978-79; Toren *et al*, 1996; Goldstein and Bernstein, 1990). The p53 protein was detected frequently in this series (42/49, 76%). The explanation for the high rate of expression in Malaysian Burkitt's lymphoma awaits mutational analysis of p53 gene.

Functional loss of the translated protein from the retinoblastoma gene, another tumor suppressor gene first described for its involvement in the development of retinoblastoma, is related to a variety of other solid tumors (Benedict *et al*, 1990). Loss of pRb has been reported as one of the most frequently observed abnormalities in lymphoid malignancy (Ginsberg *et al*, 1991; Hangaishi *et al*, 1996). Weide, *et al*. (1994) reported loss of pRb expression in five out of eight (63%) cases of Burkitt's lymphoma. However, loss of pRb was found in only 4 cases (8%) in the current study. This finding is similar to the report by Martinez, *et al*. (1993), where pRb expression was detected in 9/9 cases of Burkitt's lymphoma. Studies of other lymphomas, such as follicular lymphoma (Nguyen *et al*, 1996) and a cohort of 103 low grade and high grade lymphomas

(Geradts *et al*, 1998), also gave similar results. Therefore, we concluded that loss of pRb is an uncommon phenomenon in lymphomas, and it probably does not play a significant role in the pathogenesis of Burkitt's lymphoma.

The *bcl-2* gene on chromosome 18 is involved in t(14;18) translocation, which is present in a large majority of follicular lymphomas (85% to 90%) and 15% to 40% of diffuse large B-cell lymphomas (Knutsen, 1997; Horsman *et al*, 1995). Yano, *et al*. (1992) and Karsan, *et al*. (1993) reported a subgroup of B-cell malignancy that carries both Burkitt-type translocations involving the *c-myc* gene with one of the immunoglobulin heavy or light chain genes, and translocations involving the *bcl-2* gene, t(14;18). In the current series, 9 of the 54 assessable cases (17%) expressed the Bcl-2 protein. However, PCR analysis showed that none of these cases carried the t(14;18) translocation. Although translocation can occur in the minor cluster region (mcr), it is unlikely to influence the results of this series because the involvement of mcr is far less common (Horsman *et al*, 1995). For the purpose of this study, the *bcl-2* expressing tumours are categorized as "Burkitt's-like", since they do not fit the WHO criteria for Burkitt's lymphoma. Their true natures await further investigations. They may be "diffuse large B-cell lymphoma" with unusual morphology, composed of cells with relatively small, regular shaped nuclei that lack cytoplasm, and presence of "starry-sky".

In conclusion, Burkitt's and Burkitt's-like lymphoma in Malaysian patients expresses the same features as the sporadic type, *i.e.* a low rate of jaw involvement, mean age of 16 y old, and intermediate EBV association. Immunohistochemical staining of p53, Bcl-2 and pRb protein expressions showed no disparity from other reports, p53 and pRb proteins are expressed in a large majority of the cases.

Table 1: Association of EBV according to ethnic groups

<i>ETHNIC GROUP</i>	EBV ASSOCIATION			
	Total No. of Patients	Positive (%)	Negative (%)	Association Rate
Chinese	28	7	21	25%
Malay[#]	12	6	6	50%
Indian	3	0	3	0%
Others[*]	8	4	4	50%
Total	51	17 (33%)	34 (66%)	

[#] Does not include one non-reactive case

^{*} Includes the indigenous groups in Sabah and Sarawak

Table 2: Staining pattern for p53 and pRb in Burkitt's and Burkitt's-like lymphoma from Malaysian patients

Staining Value	p53 Expression		pRb Expression	
	No.	%	No.	%
<10%	7	13	4	7
10% $\leq \chi < 25\%$	3	6	1	2
25% $\leq \chi < 50\%$	8	15	6	11
50% $\leq \chi < 75\%$	7	13	9	16
75% $\leq \chi < 90\%$	19	35	19	35
$\geq 90\%$	5	9	12	22
Non-reactive	6	11	4	7
TOTAL	55	100	55	100

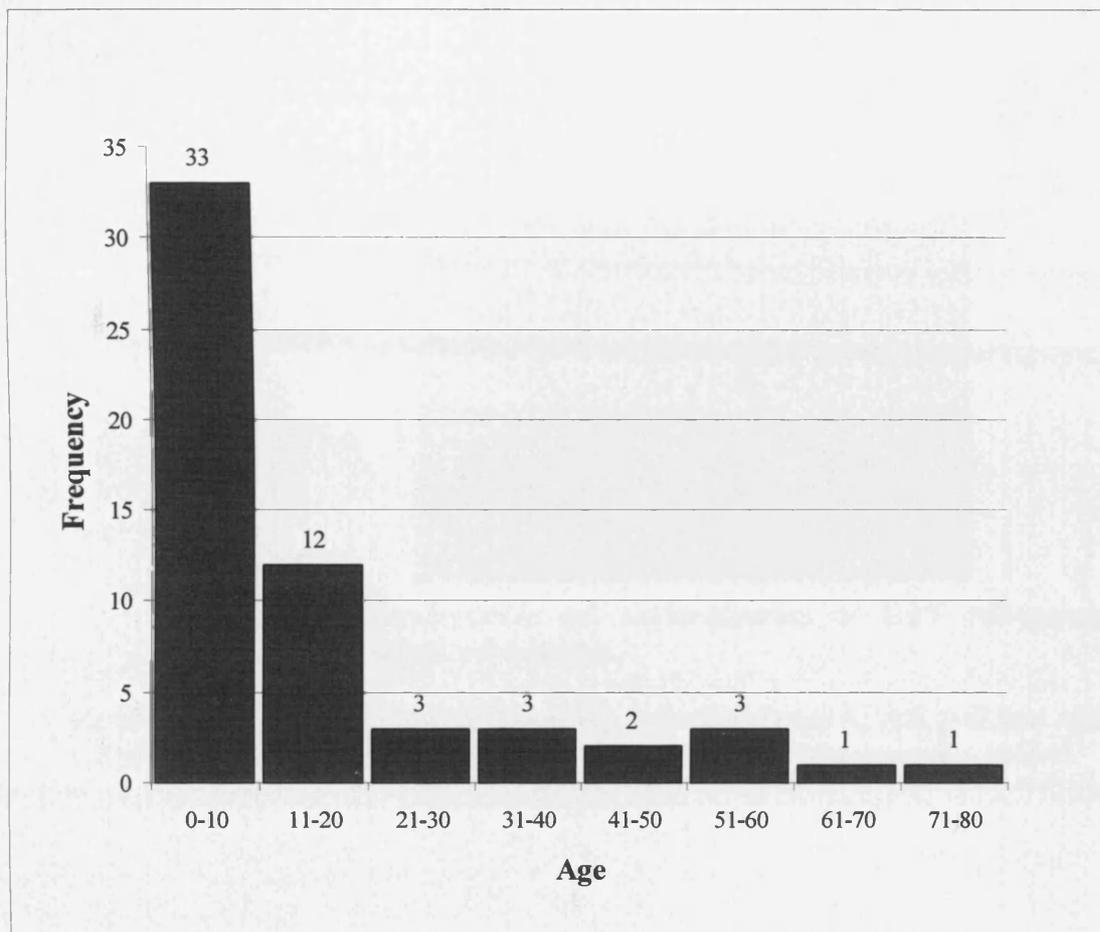


Figure 1: Age distribution of Malaysian Burkitt's and Burkitt's-like lymphoma patients

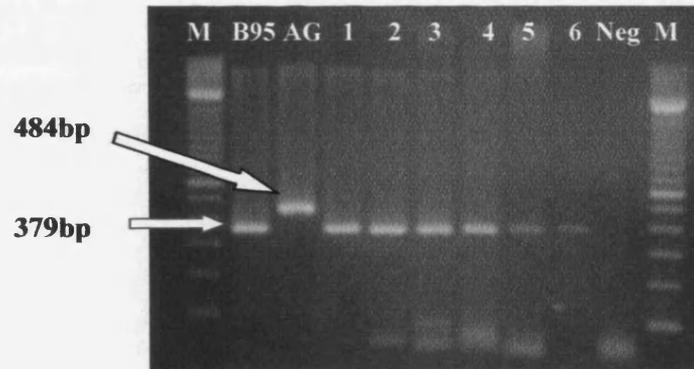


Figure 2: Representative gel electrophoresis of EBV sub-typing in Burkitt's lymphoma.

M, 100-bp DNA ladder; B95, cell line B95.8 for EBV type A; AG, cell line AG876 for type B; 1-6, Burkitt's lymphoma cases; Neg, non-template negative control.

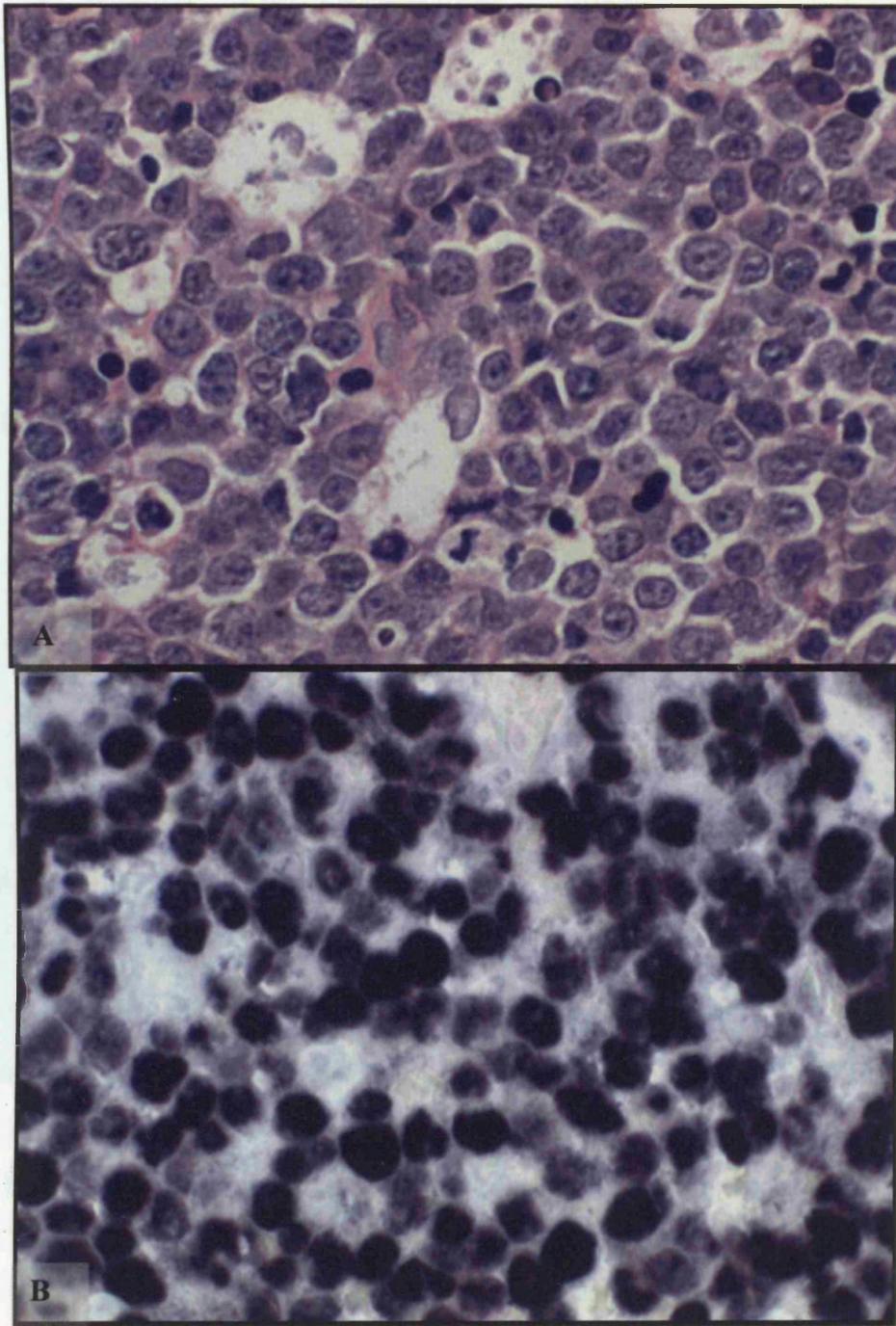


Figure 3: A) Burkitt's lymphoma. H&E X 950. B) Almost all tumour cells are infected with EBV. EBV in situ hybridization X 950.

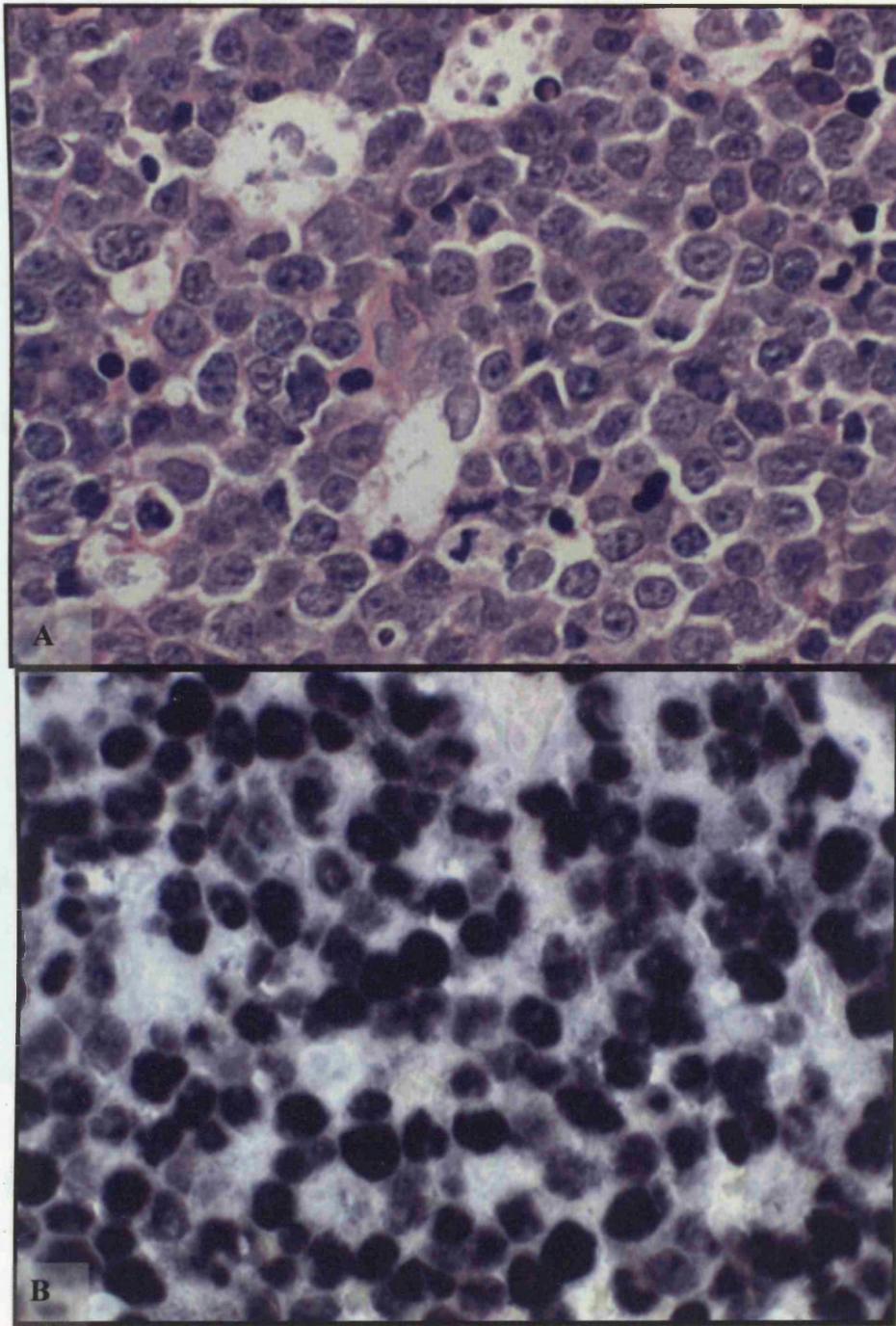


Figure 3: A) Burkitt's lymphoma. H&E X 950. B) Almost all tumour cells are infected with EBV. EBER in situ hybridization X 950.

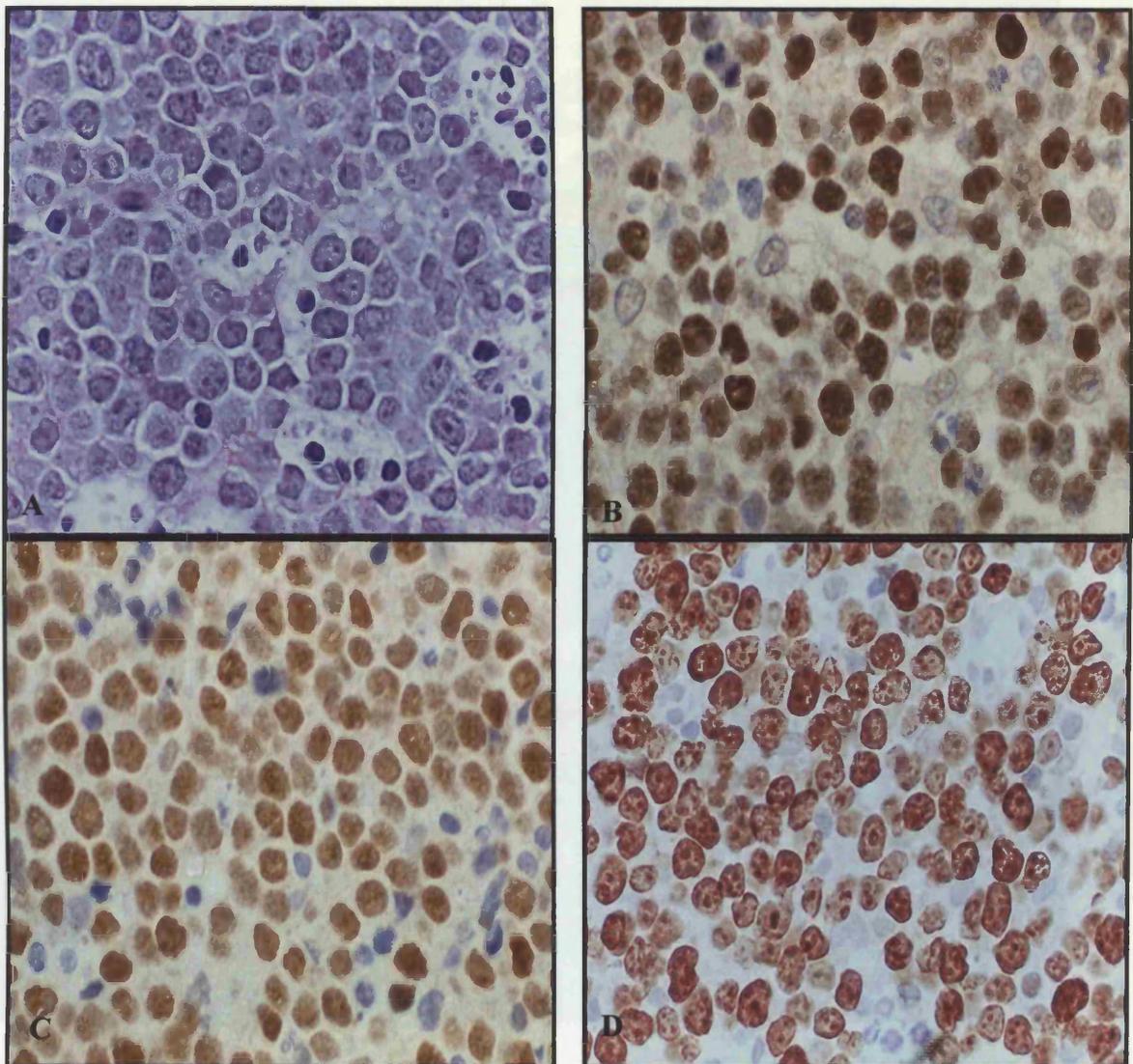


Figure 4: A) Burkitt's lymphoma. H&E X550. B) High percentage of cells show strong nuclear expression of p53 protein. Immunohistochemical stain, X 550. C) Tumour cells express pRb. Immunohistochemical stain, X 550. D) Tumour cells show high proliferation rate. Ki-67 immunohistochemical stain, X 550.

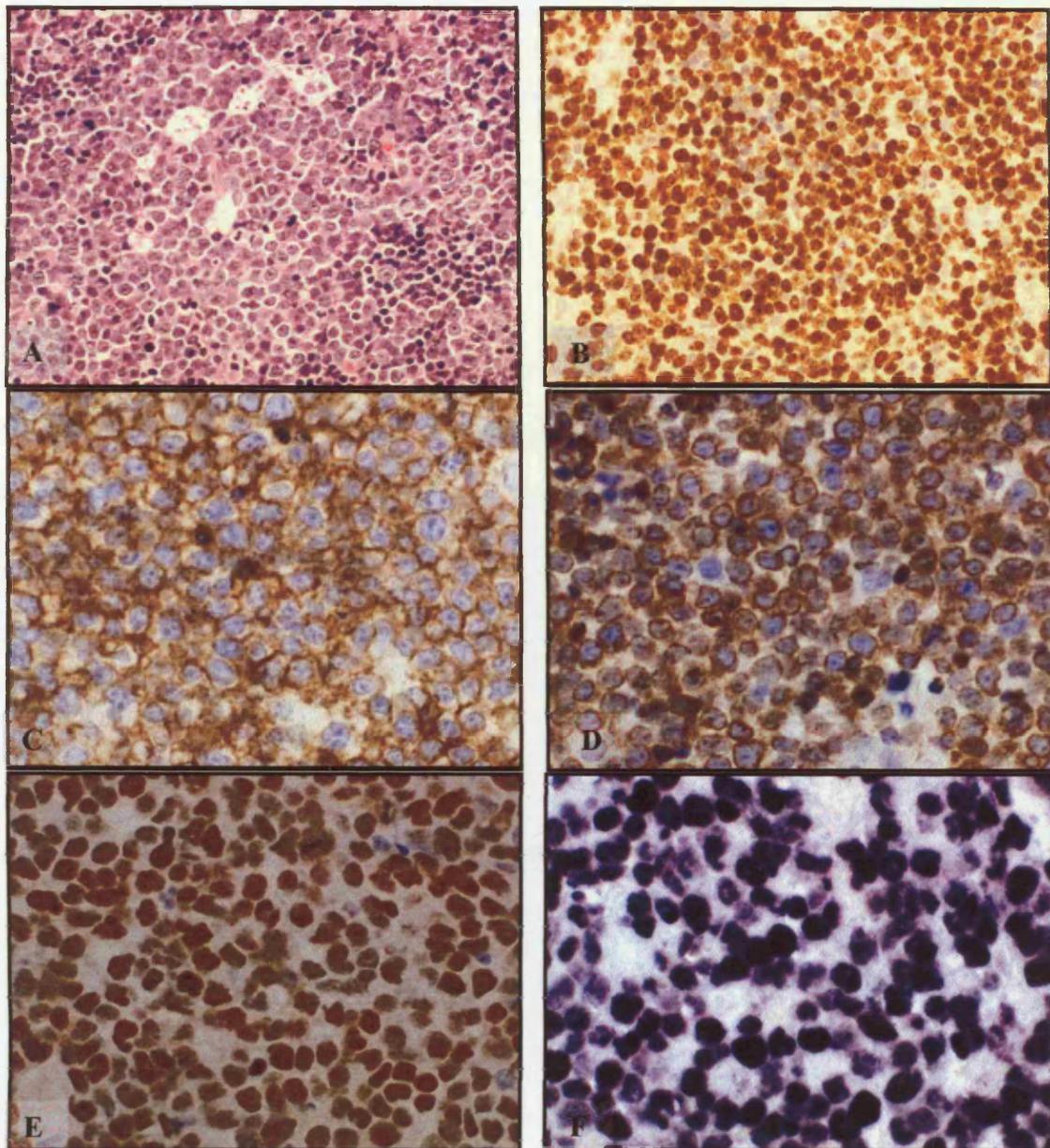


Figure 5: A) Burkitt's-like lymphoma from a 3 year-old, with presentation of disease in the oral nasal region. H&E, X275. B) Tumour with high proliferation rate. Ki67 immunohistochemical stain, X275. C) Tumour cells express CD10. Immunohistochemical stain, X500. D) Tumour cells express bcl-2 protein. Immunohistochemical stain, X500. E) Nuclear positivity for pRb in large majority of cells. Immunohistochemical stain, X500. F) Almost all tumour cells are infected with EBV. EBER in situ hybridization, X500.

Chapter 4

Epstein-Barr Virus and Lymphoma Pattern in East Malaysian Patients

4.1 INTRODUCTION

Malignant lymphoma involves neoplastic transformations of cells that reside predominantly within lymphoid tissues. Both Hodgkin's lymphoma (HL) and non-Hodgkin's lymphomas (NHL) differ in clinical behaviour and response to treatment. NHLs are derived from subpopulations of B and T cells (Hartge *et al.*, 1994), and recent reports also indicate lymphoid derivation of HL (Chan, 2001; Stein and Hummel, 1999). Cell-surface molecules involved in cellular growth and microenvironmental localization provides the basis needed to immunophenotype malignant lymphomas. Advances in immunophenotyping and improvement in the classification of hematological malignancies rendered better epidemiological comparison in the incidence and pattern of lymphoma (Jaffe *et al.*, 1998).

Lymphomas, ranked twelve among all cancers world-wide in 1990s, in which it is more prevalent in males when compared to females (Pisani *et al.*, 2002). Hence, this disease brings about profound personal and economic impact (Pisani *et al.*, 2002). Mortality and incidence rates from NHLs have also been reported to increase in both sexes in most countries over recent decades (Hartge *et al.*, 1994; Devesa and Fears, 1992). These increases have been more significant for extranodal disease, particularly those arising in the brain and in high grade tumors (Hao *et al.*, 1999; Devesa and Fears, 1992). The emergence of acquired immunodeficiency syndrome-related non-Hodgkin's lymphomas has been implicated to cause a rise in NHL in recent years (Hartge *et al.*, 1994). In contrast,

there has been significant decline in HL mortality rates in both sexes since the early 1970s (Hartge *et al.*, 1994).

Viruses, immunosuppression, and certain occupations have been identified as risk factors for the development of NHL (Hartge *et al.*, 1994; Toren *et al.*, 1996). The human T lymphotropic virus type 1 (HTLV-1), induces a specific form of lymphoma called the adult T cell leukaemia/lymphoma (ATL) (Sng *et al.*, 1992; Su *et al.*, 1985; Ho *et al.*, 1986; Blatter *et al.*, 1982), whereas Epstein-Barr virus is implicated in the etiology of Burkitt's lymphoma, HL, some varieties of T-cell lymphoma, HIV-associated and post-transplant lymphoproliferative lesions (Tosato *et al.*, 1995; Baumforth *et al.*, 1999; Anagnostopoulos *et al.*, 1996; Ambinder, 2001; Kawa, 2000).

Hospital-based studies can yield useful information on the epidemiological pattern of lymphomas. Previous study on lymphomas in East Malaysia for a period of 3 years from 1981-1983 revealed that the pattern of lymphomas in East Malaysia concurred with the general pattern in Asia (Chai *et al.*, 1999). This current study on the surgical specimens retrieved from the Department of Pathology, Queen Elizabeth Hospital, Sabah between the year 1997 and 1999 aims to elucidate if the spectrum and pattern of lymphoma in Sabah has since changed 15 years later in the 1990s.

4.2 MATERIALS AND METHODS

Patients' Material

Over a period of three years from January 1997 to December 1999, 125 surgical biopsies were diagnosed as malignant lymphomas in the Department of Pathology, Queen Elizabeth Hospital, Sabah where a total of 25,871 surgical

biopsies were received during this period. The haematoxylin and eosin (H&E) stained slides of these cases were reviewed and lymphoma cases were classified according to the WHO proposed list of lymphoid neoplasms (Peh *et al.*, 1999; Jaffe *et al.*, 1998) with the aid of immunohistochemical stain performed on serial sections of the archival tissue using a panel of antibodies. Confirmed lymphoma cases were also probed for the presence of the Epstein-Barr virus by in situ hybridization detection technique. Patients' demographic data were extracted from the Queen Elizabeth Hospital clinical records. Queen Elizabeth Hospital, Sabah is the major government hospital in Sabah serving a population of 1,863,659 (State Population Report, 1995). It is the only hospital with histopathological laboratory service in the state. Pathological biopsies from other hospitals in Sabah are sent to this hospital for histopathological diagnosis. In the early 1980s pathological biopsies from East Malaysia (Sabah and Sarawak) were sent to the Department of Pathology, University Hospital for histological diagnosis, as there were no pathologists in service in these 2 states. The archival collection constituted the material for the earlier study by Chai *et al.*, 1999. Lymphoma cases from the state of Sabah in the series reported by Chai *et al.*, 1999 were retrieved and reclassified according to the new WHO proposed list of lymphoid neoplasia for the purpose of comparison

Immunohistochemical staining

Immunohistochemical staining of 4 μ m tissue section was performed with a panel of antibodies mostly from Dako (Denmark) unless otherwise specified. The panel of lymphoid antibodies included: CD3, CD5 (Novocastra, United Kingdom), CD10 (Novocastra, United Kingdom), CD15 (Leu-M1, Becton-Dickson, USA), CD20 (L26), CD21, CD23 (Novocastra, United Kingdom), CD30

(BerH2), CD43 (MT1, a gift from S.Poppema), CD45 (LCA), CD56 (Novocastra, United Kingdom), CD57 (Leu-7, Becton-Dickson, USA), CD79, Ki-67, Bcl-2 and Anaplastic lymphoma kinase (ALK). Cases diagnosed as non-lymphoma after careful study with additional antibodies such as epithelial membrane antigen (EMA, Dako, Denmark), S-100 (Dako, Denmark), cytokeratin (MNF116, Dako, Denmark), Mic 2 (Dako, Denmark), melanoma antigen (HMD45, Dako, Denmark) and vimentin (Dako, Denmark), pending the need of individual cases were subsequently excluded from analysis.

In situ hybridization

A non-isotopic in-situ hybridization technique was employed to detect the presence of EBV. The tissue sections were hybridized with fluorescein-conjugated oligonucleotide probe for EBV early RNAs, EBER (NCL-EBV, Novocastra, United Kingdom). Hybridization products were visualized by alkaline phosphatase-conjugated rabbit anti-FITC developed in the substrate, 4-nitro-blue-tetrazolium chloride/ 5-bromo-4-chloro-3-indolyl- phosphate (NBT/BCIP). These tissue sections were then lightly counterstained with Mayer's haematoxylin. A known EBV- positive nasopharyngeal carcinoma case was used as an external control for the staining procedure.

4.3 RESULTS

Of the 125 cases retrieved for this study, 34 were excluded for further analysis due to either absence of tumour in the remaining archival tissue, insufficient tissue for further investigation, or the cases were not reconfirmed as lymphoma. The remaining 91 cases, representing 0.35% of total biopsies of the hospital are composed of 83 (91.2%) NHL and 8 (8.8%) of HL. The ratio for NHL

to HL is 9:1. There are 56 male and 32 female patients, male: female ratio is 2:1. The gender information of three patients are not available in the clinical record. The ages of these patients range from 7 to 84 years, with a mean age of 42.1 years. The age distribution pattern is presented in Figure 1. The highest preponderance is in the age group of 51 to 60 years old, (20 cases, 22.0%). Distribution in the major ethnic groups showed 27 Kadazan, 7 Malay, 8 Chinese, 8 Bajau and 6 Dusun. The remaining 30 are from other minor indigenous groups (Indian, Murut, Indonesian, Pribumi, Bugis, Brunei, Jawa, Filipino, Timor, Sungai, Rungus, Nocano). The ethnic identification is missing in 5 cases. The distribution of different lymphoma subtypes according to the ethnic group is listed in Table 1. Diffuse large B-cell lymphoma (DLBCL) is uniformly the commonest subtype, constituting approximately 50% of lymphoma in all these ethnic groups.

Table 2 summarizes the distribution of NHL and HL subtypes according to age group and EBV association. Of the 83 cases of NHL, 66 (79.5%) are confirmed B-cell type, 13 (15.7%) T-phenotype, 1 (1.2%) null cell type and one case is unclassified. DLBCL is the most prevalent group among the B-NHL, a total of 43 cases (65.1%), followed by Burkitt's (BL) and follicular lymphomas (FL) of 7 cases (10.6%) each. There are 6 cases (9.1%) of mucosa-associated lymphoid tissue (MALT) type, in which 5 (7.6%) originate in the stomach. All the FL cases show CD10 expression in the neoplastic follicles where as none of the MALT cases expressed CD10. Common B-NHL in children (< 15 years) are BL (3 cases) and lymphoblastic lymphoma (1 case). None of the peripheral T-cell lymphoma (PTCL) unspecified and natural killer (NK)/Tcell type are diagnosed in children. There are 4 cases of nodular sclerosis HL (NSHL) , 2 cases of mixed cellularity (MCHL) and 1 case each of lymphocyte rich (LRHL) and lymphocyte

depleted HL (LDHL). All HL, except one case of MCHL are from patients in older age group (≥ 15 years).

The main sites of disease presentation are in the lymph nodes in 43 cases (47.2%) and extranodal sites in 42 (46.1%). The sites of presentation for six cases cannot be determined. All the HL cases presented in lymph node. The majority of lymph node biopsies were from the head and neck region (20, 22.0%), followed by superficial nodes of the axillary and inguinal regions (11, 12.1%), and mesentery (2, 2.3%). The sites of the remaining 10 (11.0%) cases are not indicated. The common extranodal sites were gastrointestinal tract (14), tonsil (4), oral cavity (4), testis (2), spine (2), nose (2), and others (14). All the 14 cases of lymphoma from the gastrointestinal tract are B-cell NHL: DLBCL (4), gastric MALT-type (5), FL (4), and B-NHL NOS (1).

EBV are detected in 6/8 (72.5%) of HL and 8/83 (9.6%) of NHL cases (Table II). Three of 43 (7.0%) DLBCL, 2 of 7 BL (28.6%) are EBV associated. Three of 13 T-cell NHL are EBV associated (23.1%), in which 2 are NK/T cell type and 1 PTCL unspecified subtype. Ethnic Kadazan (6/14) has the highest rate of EBV association in their lymphomas (DLBCL 2; NSHL 1; MCHL 1; BL 1; NK/Tcell 1), followed by Malay (PTCL unspecified 1; LRHL 1), one case each from the ethnic Indian (NSHL), Murut (DLBCL), Bajau (BL), Jawa (NK/Tcell), Chinese (NSHL) and 1 case of MCHL without information of ethnicity.

4.4 DISCUSSION

The HL: NHL ratio in this series concurs with earlier study of 1:9 by Chai *et al* (1999) indicating there is no significant change in the pattern of lymphoma in Sabah (Table 3). In both these series from Sabah, the frequency of HL is low,

similar to other reports from Asia that HL is uncommon (Su *et al.*, 1985; Seow *et al.*, 1996; Mancer, 1990; Ho *et al.*, 1984). There is a two fold increase in NHL cases in Sabah compared to the previous series and this appears to echo reports from Western countries of a rise in NHL (Hartge *et al.*, 1994; Devesa and Fears, 1992) in which the incidence rates rose by about 50% or more in less than two decades (Hartge *et al.*, 1994; Devesa and Fears, 1992). The incidence of NHL in Asia was also reported to increase but in a more modest rate (Hartge *et al.*, 1994; Su *et al.*, 1985). This increase in the incidence of NHL was believed to be partly due to AIDS (Hartge *et al.*, 1994; Su *et al.*, 1985; Peh *et al.*, 2001). Other factors implicated include occupational exposures to chemical agents and viruses (Hartge *et al.*, 1994; Pearce and Bethwaite, 1992; Zahm *et al.*, 1992; Scherr *et al.*, 1992; Blair *et al.*, 1992). Occupational statistics of Sabah inhabitants (1991) showed a high proportion of them being involved in agricultural and animal husbandry, which accounted for 41.8% of the employed population, and in the remaining employed population, 23.5% is working as industrial labourers who are also exposed to chemical agents (Sabah State Population Report, 1995). However, a larger cohort and a longer period of study are required to confirm if the two-fold increase is significant.

Asia reported high frequency of high-grade DLBCL and low frequency of FL when compared to the West (Hartge *et al.*, 1994; Seow *et al.*, 1996; Anderson *et al.*, 1998). The ratio of high grade DLBCL to low grade FL in Sabah is 3.2:1 in the 1980's and 6.1:1 in the 1990's. The low incidence of FL were also reported in other Asian countries (Table 4). Harrington *et al* (1987) proposed that the prevalence of high grade diffuse lymphomas are related to environmental factors associated with agricultural activity. Others suggested that delay in patients

seeking medical care may explain the high ratio of DLBCL to FL (Harrington *et al.*, 1987), since FL can progress into a more aggressive DLBCL by acquiring secondary genetic alterations (Elenitobo-Johnson *et al.*, 1998; Dalla-Favera *et al.*, 1994; Zelenetz *et al.*, 1991). BL does not constitute a big proportion of NHL in both the earlier and present Sabah series. The association of EBV with this disease is less than 30%. Although the rarity of occurrence of BL had been reported in Hong Kong (Ho *et al.*, 1984), it is unexpected since malaria is endemic in Sabah (Hii *et al.*, 1985; Lim, 1992).

T-cell lymphoma constituted only 17.1% of the total NHL cases in this series, which is comparable with reports from Klang (Peh *et al.*, 2000), Singapore (Eletinobo-Johnson *et al.*, 1998), East Malaysia (Chai *et al.*, 1999), University Kebangsaan Malaysia (Mancer, 1990) and Thailand (Intragumtornchi *et al.*, 1996), but differ from reports of high incidence of T-NHL in Taiwan (Su *et al.*, 1985), Hong Kong (Ho *et al.*, 1986) and Japan (Hinuma *et al.*, 1982). It appears that there is a decline in the occurrence of T-NHL when compared to the earlier series of 27.5%. Similar observation was also noted in another study on East Malaysian cases from the state of Sarawak (Peh *et al.*, 2001). The reason for this phenomenon is not immediately apparent, and it also contradict reports that T-NHL is on a rise (Ho *et al.*, 1986; Lee *et al.*, 1999).

Ethnic Kadazan constitute highest numbers of lymphoma cases (30%) when compared to other ethnic groups. However, Queen Elizabeth Hospital patient admission data for the year 1997, 1998 and 1999 (Patient's Admission and Census Data, 1997, 1998 and 1999) also reveal higher admission of ethnic Kadazan patients when compared to others (Table 5), eventhough the Sabah State Population Census Report showed that ethnic Kadazan constitutes only 6.1% of

the people in the state (Sabah State Population Report., 1995). It appears that there is higher utilization of the hospital service by Kadazans, and therefore the ethnic distribution of lymphomas may actually reflect patients composition in the hospital rather than disease predilection (Patient's Admission and Census Data, 1997, 1998 and 1999).

In conclusion, lymphoma pattern in Sabah in the 1990s appears to concur with the earlier pattern in 1980s, higher prevalence of DLBCL, and lower incidence of FL and HL, showing a pattern similar to elsewhere in Asia. The current series shows an overall increase in the numbers of NHL in the 1990s but a drop in the proportion of T-NHL, and a low EBV association rate in BL. The reasons for the 2 latter observations are not immediately apparent. A study involving a larger series would be necessary to establish the findings from this study as statistically significant and reliable.

Table 1: Ethnic distribution of lymphoma subtype in Queen Elizabeth Hospital, Sabah (1997-99).

Diagnosis	Ethnic Group							Total
	Malay	Kadazan	Chinese	Bajau	Dusun	Others	NA	
Hodgkin's Lymphoma	1	2	1	-	-	3	1	8
Lymphocyte rich	1		-	-	-	-	-	1
Nodular scleroris	-	1	1	-	-	2	-	4
Mixed cellularity	-	1	-	-	-	-	1	2
Lymphocyte depleted	-	-	-	-	-	1	-	1
Non-Hodgkin's Lymphoma	6	25	7	7	7	27	4	83
Diffuse large B-cell	3	13	5	4	2	15	1	43
Burkitt's	-	2	1	1	1	2	-	7
Follicular	-	1	1	-	1	1	3	7
Gastric MALT type	-	1	-	1	1	2	-	5
MALT type	-	1	-	-	-	-	-	1
B-lymphoblastic	-	1	-	-	-	-	-	1
Plasmacytoma	-	-	-	-	1	-	-	1
B-NHL NOS	-	2	-	-	-	1	-	3
PTCL unspecified	1	-	-	1	1	2	-	5
NK/T cell	-	2	-	-	-	1	-	3
ALCL T cell	1	-	-	-	-	-	-	1
 null-cell	-	-	-	-	-	1	-	1
T-immunoblastic	-	1	-	-	-	1	-	2
T-lymphoblastic	1	1	-	-	-	-	-	2
NHL NOS	-	-	-	-	-	1	-	1
TOTAL	7	27	8	7	7	30	5	91

NA : data not available ; NHL : Non-Hodgkin's lymphoma ; MALT: mucosa-associated lymphoid tissue; PTCL: peripheral T cell lymphoma; NK: natural killer; ALCL: anaplastic large cell lymphoma; ML: malignant lymphoma; NOS: not otherwise specified

Table 2: Distribution of Queen Elizabeth lymphoma cases by age and subtypes (1997-99)

Diagnosis	Subtype	Age			EBV Association *	
		<15 yrs No. (%)	> 15 yrs No. (%)	NA No. (%)		
Hodgkin's Lymphoma (N=8,8.8%)	Lymphocyte rich		1(1.3)		1/1(100.0)	
	<i>Nodular sclerosis</i>		3(3.8)	1(33.3)	3/4(75.0)	
	<i>Mixed cellularity</i>	1(11.1)	1(1.3)		2/2(100.0)	
	Lymphocyte depleted		1(1.3)		0/1(0.0)	
Non-Hodgkin's Lymphoma (N=83, 91.2%)	(a) B cell					
	<i>Diffuse large B-cell</i>		41(52.0)	2(66.7)	3/43(7.0)	
	Burkitt's	3(33.3)	4(5.1)		2/7(28.6)	
	Follicular		7(8.9)		0/7(0.0)	
	<i>Gastric MALT type</i>		5(6.3)		0/5(0.0)	
	<i>MALT type</i>		1(1.3)		0/1(0.0)	
	<i>B-lymphoblastic</i>	1(11.1)			0/1(0.0)	
	<i>Plasmacytoma</i>		1(1.3)		0/1(0.0)	
	<i>B-NHL NOS</i>		3(3.8)		0/4(0.0)	
	(b) T cell					
	<i>PTCL unspecified</i>		5(6.3)		1/5(20.0)	
	<i>NK/T cell</i>		3(3.8)		2/3(66.7)	
	<i>ALCL T-cell</i>	1(11.1)			0/1(0.0)	
	<i> null-cell</i>		1(1.3)		0/1(0.0)	
	<i>T-immunoblastic</i>	1(11.1)	1(1.3)		0/2(0.0)	
	<i>T-lymphoblastic</i>	1(11.1)	1(1.3)		0/2(0.0)	
	<i>NHL(NOS)</i>	1(11.1)			0/1(0.0)	
	TOTAL		9(100)	79(100)	3(100)	14/91(15.4)

NA : data not available ; NHL : Non-Hodgkin's lymphoma ; MALT: mucosa-associated lymphoid tissue; PTCL: peripheral T cell lymphoma; NK: natural killer; ALCL: anaplastic large cell lymphoma; ML: malignant lymphoma; NOS: not otherwise specified

*EBV association: EBER positive cases out of the total number of cases from each subtype.

Table 3: Comparison of Sabah series from 1981-83 and 1997-99

Diagnosis	Series from Sabah	
	1981-83* No. (%)	1997-99 No. (%)
Hodgkin's lymphoma	4(9.1)	8(8.8)
Lymphocyte predominant	1(2.3)	-
Lymphocyte rich	-	1(1.1)
Nodular sclerosis	1(2.3)	4(4.4)
Mixed cellularity	2(4.5)	2(2.2)
Lymphocyte depleted	-	1(1.1)
Non-Hodgkin's lymphoma	40(90.9)	83(91.2)
Diffuse large B-cell	19(43.2)	43(47.2)
Burkitt's	6(13.6)	7(7.7)
Follicular	2(4.5)	7(7.7)
Gastric MALT type	1(2.3)	5(5.5)
MALT type	-	1(1.1)
B-lymphoblastic	-	1(1.1)
Plasmacytoma	-	1(1.1)
B-NHL NOS	1(2.3)	3(3.3)
PTCL unspecified	10(22.7)	5(5.5)
NK/T cell	-	3(3.3)
ALCL T-cell	-	1(1.1)
null cell type	-	1(1.1)
T-immunoblastic	1(2.3)	2(2.2)
T-lymphoblastic	-	2(2.2)
NHL NOS	-	1(1.1)
TOTAL	44(100)	91(100)

*Adapted from Chai *et al.* Pattern of malignant lymphoma in East Malaysia patients as experienced in University Hospital, Kuala Lumpur. *Malaysian J Pathol* 1999; 21(1): 45-50

Table 4: Frequency of Diffuse and Follicular NHL from different study groups within Asia.

Research Groups	No. of cases	Percentage of all NHL	
		Diffuse No. (%)	Follicular No. (%)
Taiwan (Su <i>et al.</i> , 1985)	90	31 (34.4%)	11 (12.2%)
Hong Kong (Ho <i>et al.</i> , 1986)	69	60 (87.0%)	9 (13.0%)
Sarawak, Malaysia (Peh <i>et al.</i> , 2001)	58	38 (54.3%)	5 (7.2%)
Guangzhou, China (Harrington <i>et al.</i> , 1987)	192	158 (82.3%)	24 (12.5%)
Klang, Malaysia (Peh <i>et al.</i> , 2000)	66	31 (47.0%)	11 (16.7%)
New Delhi, India (Garg <i>et al.</i> , 1985)	238	206 (87.0%)	21 (9.0%)
Thailand (Intragamtornchai <i>et al.</i> , 1996)	1391	1271 (91.4%)	53 (3.8%)
UH, Kuala Lumpur (Peh., 2001)	121	70 (57.9%)	15 (12.4%)

NHL; Non-Hodgkin's Lymphoma, UH ; University Hospital.

Table 5: Percentage of Patients' Ethnic Distribution Admitted in Queen Elizabeth Hospital, Sabah in the year 1997, 1998 and 1999

Year	Ethnic Group						
	Kadazan	Chinese	Malay	Bajau	Murut	Other Pribumis	Others
1997	33.3%	13.2%	4.3%	12.9%	1.3%	15.7%	19.2%
1998	33.0%	13.6%	4.3%	10.2%	1.5%	14.5%	21.9%
1999	33.2%	13.4%	5.1%	11.3%	1.7%	14.4%	19.6%

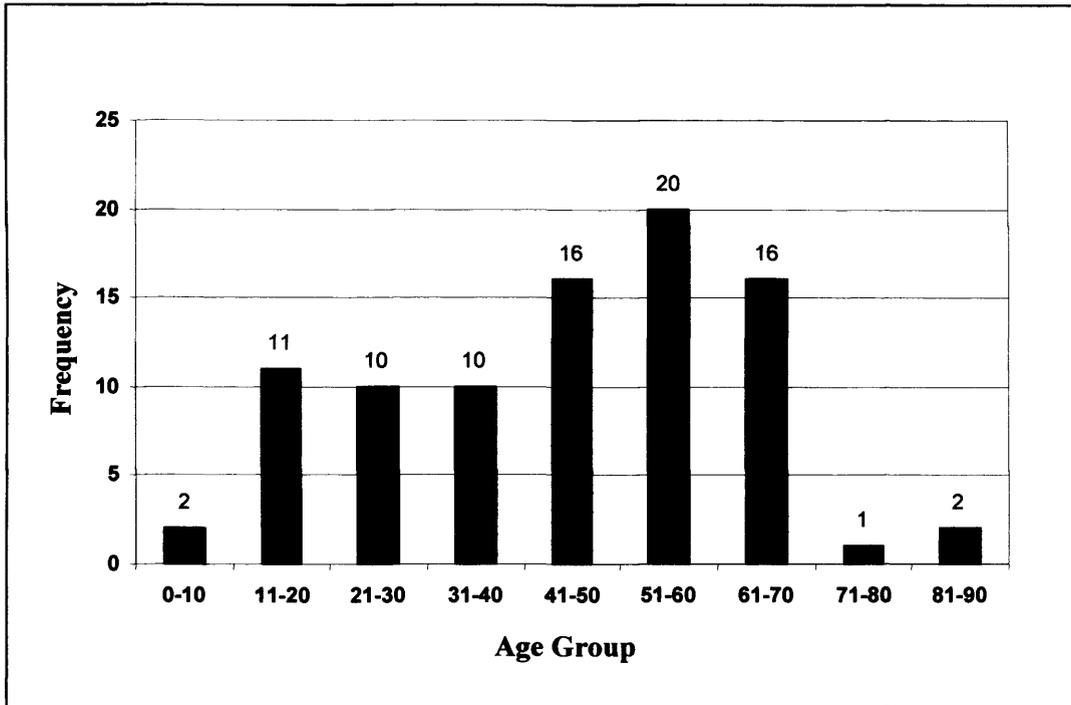


Figure 1: Distribution of Sabah Lymphoma Cases According to Age Groups (1997-1999)

Chapter 5

Epstein-Barr virus subtype pattern in host and diseases

5.1 INTRODUCTION

Epstein-Barr virus (EBV) is a ubiquitous B-cell lymphotropic human gamma-herpes virus, and is carried by a great majority of individuals as a life long asymptomatic infection. It has potent B-cell transforming activity and is strongly implicated in the pathogenesis of several human malignancies, notably Burkitt's lymphoma, nasopharyngeal carcinoma, classical Hodgkin's lymphoma, nasal NK/T-cell lymphoma and lymphomas in immunocompromised patients (Becker, 1980; Lyons and Liebowitz. 1998; Munch, 1998). How the virus contribute to the pathogenesis of such a diverse set of malignancies remains to be determined.

There are two subtypes of EBV, A and B, which are distinguishable based on their genetic polymorphism in the nuclear antigen (EBNA)-2, 3, 4 and 6 genes where the two alleles differ in predicted primary amino acid sequence by 47%, 16%, 20% and 28% respectively (Munch, 1998; Dambaugh *et al*, 1986). Type A virus is ubiquitous, efficiently transforms and immortalises infected B-lymphocytes in vitro, whereas type B virus is reported to be a weaker transformer than type A virus (Rickinson *et al*, 1987; Ring, 1994; Kunimoto *et al*, 1992). EBNA-2 gene is known to function as one of the elements required for the transactivation of the LMP-1 and 2 gene for transforming activities. Studies have shown different frequencies of infections by these two subtypes in different geographical locations, being type A predominance in the European, American and Asian, whilst type B in African countries (Zimber *et al*, 1986; Khanim *et al*,

1996; Young *et al*, 1987) and immuno-compromised patients (Buisson *et al*, 1994; Borisch *et al*, 1992). Recent investigations revealed that type B virus is as prevalent as type A in normal population, not only in the endemic area of Burkitt's lymphoma, but also in the United States of America (Sixbey *et al*, 1989). The possible influence of virus subtypes may have in disease development is not known despite many studies which attempted to correlate EBV strains to various diseases (Khanim *et al*, 1996; Young *et al*, 1987; Buisson *et al*, 1994; Borisch *et al*, 1992; Sixbey *et al*, 1989). Among the techniques that had been employed to study EBV strains included Ebnotyping, a technique applying immunoblotting to study the size polymorphism of the EBNA proteins (Gratama *et al*, 1990), DNA restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) (Kunimoto *et al*, 1992; Falk *et al*, 1995).

PCR is a powerful technique for detection of a specific DNA sequence in a small amount of sample. However, PCR on paraffin embedded tissues may sometimes give ambiguous and inconsistent results due to poorly preserved DNA upon fixation and storage of tissue (Greer *et al*, 1991; Pan *et al*, 1995). Various techniques have been used to enhance the sensitivity and specificity of PCR, for example nested-PCR, hot-start PCR, heat-soaked PCR and touch-down PCR (Pan *et al*, 1995).

Previous study on a small number of Malaysian T-cell lymphoma of the upper aerodigestive tract were all found to be infected by type A virus (Peh *et al*, 1995). In this study, we chose nested-PCR method for determining the EBV subtype pattern in larger numbers and a wider spectrum of EBV associated diseases presented at different anatomical sites.

5.2 MATERIALS AND METHODS

Samples

Formalin-fixed, paraffin embedded archival tissue of EBV infected lymphomas confirmed by *in-situ hybridisation* staining technique, using probe for the EBV early RNAs (EBER, Dako PNA probe, Denmark) were selected for this study. These included a total of 14 Burkitt's lymphomas, 4 T-cell non-Hodgkin's lymphomas (2 peripheral T-cell unspecified, 1 peripheral T-cell angioimmunoblastic type, 1 natural killer (NK)/T-cell type), 3 diffuse large B-cell non-Hodgkin's and 17 classical Hodgkin's lymphomas (12 mixed cellularity, 4 nodular sclerosing, 1 lymphocyte rich). In addition, 14 cases of nasopharyngeal carcinomas, 12 reactive lymph nodes and tonsils with presence of small numbers of EBER-positive mature lymphocytes were also retrieved from the files for comparative study. Cell lines B95.8 (type A positive, EBNA-2A) and AG876 (type B positive, EBNA-2B) were used as positive controls for EBV subtype A and B respectively. Before commencing cutting of tissue sections, the microtome (Leica RM2135, Germany) and working bench in the vicinity was cleaned meticulously with 100% ethanol, followed by 70% ethanol and lastly DNA Away™ (GibcoBRL, USA). This cleaning procedure was strictly adhered to in between cutting of every paraffin block. One 5- μ m thick section was cut from each block with a disposable blade, followed by cleaning procedure, then changing to a new blade and gloves before cutting the next block. One blank block made of paraffin wax only was used in between cutting of every test case, to serve as negative controls and to check for absence of block to block contamination during the cutting procedure.

DNA Extraction

One 5- μ thick tissue section from each case was placed in a sterile 1.5-ml tube, deparaffinised with xylene, re-hydrated in descending concentration of alcohol and then air-dried. The tissue was resuspended and lysed overnight in 200 μ l of digestion buffer containing 1X PCR buffer (GibcoBRL, USA) and 200 μ g/ml Proteinase K (Boehringer Mannheim, Germany) at 55^oC. Digestion was stopped by inactivating Proteinase K at 96 ^oC for 10 minutes. Cell debris was pelleted by centrifuging at 10,000 rpm for 5 minutes. The supernatant was transferred to a new tube and used directly in polymerase-chain-reaction (PCR) procedure. Tubes containing blank block were subjected to similar extraction procedure, along side those containing the tissue sections. DNA extraction from cell lines was performed using Tri-Reagent™ (Molecular Research Center Inc. USA) according to the manufacturer's recommendation.

PCR

Amplification for EBNA-2 gene was performed with some modifications on the one-step PCR as previously described by Kunimoto *et al* (1992). A hot-start nested-PCR was employed to enhance the sensitivity and specificity of detection. Consensus primers were selected from conserved sequences in the U2 region encoding the EBNA-2 gene. A second set of inner oligonucleotide primers was designed for the nested-PCR.

Amplification of EBNA-2 gene was carried out by using automated PTC-200 Peltier thermal cycler, (DNA Engine, MJ Research, USA). All primers were synthesised by Genosys (Genosys Products, USA). The outer pair of primers (sense: 5' TTT CAC CAA TAC ATG AAC C, at base position 2771-2789; anti-sense: 5' TGG CAA AGT GCT GAG AGC AA, at base position 3149-3130) and

the inner pair of primers (sense: 5' CAA TAC ATG AAC CRG AGT CC, at base position 2777-2796; anti-sense: 5' AAG TGC TGA GAG CAA GGC MC, at base position 3144-3127) flank a 105-bp deletion fragment from the U2 region encoding for EBNA-2A. The amplified product from the EBNA-2A subtype is 368-bp and 473-bp from EBNA-2B.

First amplification was performed with 1 μ l of DNA (supernatant) from tissue samples and blanks, and 0.5 μ l of DNA from cell lines, in a 50 μ l reaction mixture. The reaction mixture contained 1.5mM MgCl₂, 0.2mM dNTPs mix (GibcoBRL, USA), 0.5 μ M of each primer and 2.5U of HotStarTaq® DNA polymerase (Qiagen, Germany) in a standard PCR buffer (Qiagen, Germany). Amplification was performed with first cycle of 95°C for 15 minutes to activate the HotStarTaq® DNA polymerase, as described by the manufacturer, then continued with 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes, with the last extension at 72°C for 5 minutes. The second PCR (nested) was performed with 1 μ l of the product from the first PCR reaction, using the second pair of primers in similar reaction mixture, and amplified for 30 cycles with annealing temperature at 60°C. Non template control, consisting of master mix only, in similar volume of reaction mixture was included in each run of PCR. Cases which were not amplified after nested-PCR were double-checked for DNA integrity by performing a PCR on housekeeping beta-globin (β -globin) gene as described by Greer *et al* (1991).

Agarose gel electrophoresis

PCR products were analysed by gel electrophoresis (E-C Electrophoretic Gel Systems, Florida, USA), using a 2% agarose gel (Gibco BRL, USA) containing 2 μ g/ml ethidium bromide (Gibco BRL, USA) in Tris-borate-EDTA,

pH8.0 (TBE) buffer at 80V. PCR products were visualised under the UV light transilluminator (E-C Apparatus, Florida, USA). Cases exhibiting similar PCR product length when compared to B95.8 were considered infected with EBV type A. In contrast, samples exhibiting a larger PCR product coinciding with fragment size from AG876 were considered to harbour EBV type B. Five random sampling of positively amplified bands were sequenced (by Universiti Putra Malaysia DNA Sequencing Centre, using ABI PRISM 377 dye terminator kit, Perkin- Elmer). All sequenced cases when compared with that obtained with the prototype cell line, B95.8 (Gene Bank 59074) were confirmed specific for the amplified segment.

5.3 RESULTS

When DNA from the test cases and cell lines B95.8 and AG876 were amplified by one-step PCR using the outer set of primers, only the cell lines consistently showed positively amplified bands, observed at 379-bp and 484-bp region from B95.8 and AG876 respectively. Fig. 1A shows PCR products from one- step PCR. Weak bands were seen in the positive controls of AG876 at 484-bp and B95.8 at 379-bp, and weak smears but no specific bands were seen in most of the tested samples. When nested-PCR was performed, positive bands were observed in 100% of tested samples. More specific and higher yield was obtained as observed by the greater intensity of the PCR products and discrete bands in the agarose gel after electrophoresis (Fig. 1B).

All amplifiable cases tested showed infection by EBV type A, irrespective of the disease entities, types of tissue, anatomical sites and age of presentation of the lesions, and the ethnicity of the patients (Table 1). Direct DNA sequencing analysis on selected cases showed PCR fragments similar to the prototype cell

line, B95.8 (data not shown), confirming the specificity of the nested-PCR technique in determining EBV subtype. Three cases of reactive tonsils could not be amplified with the primers for EBNA-2, but were successfully amplified for the housekeeping β -globin gene. In all the PCR reactions, both the one step and nested-PCR yielded specific DNA bands with the positive controls, absence of bands in all non-template controls and negative controls (blank) as shown in Fig. 2.

5.4 DISCUSSION

EBV, a linear double-stranded DNA virus of more than 172 kilobase (kb) pairs in length which encodes more than 85 genes, circularises inside infected cells. In latent infections, six nuclear antigens, (EBNA-1, EBNA-2, EBNA-3 (3a), EBNA-4 (3b), EBNA-6 (3c) and EBNA-LP), three latent membrane proteins (LMP-1, LMP-2a, LMP-2b) and two small nuclear non-coding RNAs (EBER-1 and EBER-2) are expressed depending on the latency type (Lyons and Liebowitz, 1998; Munch, 1998; Dambaugh *et al*, 1986). Regardless of the latency type, type A and B virus, which vary in their frequency of occurrence at different geographical locations had been detected (Kunimoto *et al*, 1992; Zimber *et al*, 1986; Khanim *et al*, 1996; Young *et al*, 1987)

Type A virus was predominant in Turkey (82% of the Hodgkin's lymphomas studied by Durmaz *et al*, 1998) and Egypt (83% of the Burkitt's lymphoma studied by Anwar *et al*, 1995). Martinez *et al* (1998) of Mexico detected type A virus in 87% of their post-transplantation lymphoproliferative disorder, and Borisch *et al* (Borisch *et al* 1992) showed equal proportion of type A and B virus in AIDS related lymphomas. Zimber *et al* (1986) reviewed studies

from various parts of the world and concluded that type A is more prevalent in the United States, Germany, France, North America and Asia, whereas type B virus is commonly encountered in Central Africa, La Reunion and New Guinea. In our study, all amplifiable cases were infected with type A virus. This finding concurs with the reports that type A virus is prevalent in Asia (Zimber *et al* 1986; Tomita *et al*, 1995; Oshima *et al*, 1999; Tomita *et al*, 1996; Sidagis *et al*, 1997; Wu *et al*, 1996), and the finding in Malaysian nasal T-NHL by Peh *et al* (1995). However, in Asian series other than Malaysia, a small number of cases were reported to be infected by type B virus. In Japan, Oshima *et al*, Tomita *et al* (1995, 1996) and Sidagis *et al* (1997) showed 85-97% of their cases, consisting of infectious mononucleosis, Hodgkin's lymphoma, B-NHL, Wiskott-Aldrich syndrome, acquired immune deficiency syndrome (AIDS), nasal B- or T cell lymphomas, gastric carcinomas as well as from the normal population were infected with EBV type A, with 6-13% showing type B virus infection and 3-6% double infections. In Taiwan, Wu *et al* (1996) and Chen *et al* (1992) showed that 95% of their peripheral T-cell lymphomas (PTCL) and 84% of nasopharyngeal carcinomas (NPC) were infected with type A virus, with 5.2% and 16.6% respectively with double infection of type A and B viruses. Type B virus was not detected in our series of EBV associated diseases. This is perhaps due to the absence of cases of AIDS-related lymphomas and post-transplant lymphoproliferative disorders in this cohort of study, or possibly the presence of type B genome is below detectable level in double infection.

Investigations into the polymorphism of other EBV latent genes variants have shown disease-specific association and geographical differences, suggesting different modes of EBV tumourigenesis in different diseases and geographical

areas. For example, Asian NPC cases have higher prevalence of the "f" variant, Xho-1 site mutation and 30-bp deletion in the LMP-1 gene (Khanim *et al*, 1996; Oshima *et al*, 1999; Wu *et al*, 1996; Sandvej *et al*, 1994), whereas Burkitt's lymphoma showed different EBNA-1 variants in different geographic areas (Habeshaw *et al*, 1999). However, EBV subtype has thus far shown no specific association with a specific disease type (Peh *et al*, 1995; Durmaz *et al*, 1998; Anwar *et al*, 1995; Martinez *et al*, 1998; Tomita *et al*, 1995 and 1996; Oshima *et al*, 1999; Sidagis *et al*, 1997; Wu *et al*, 1996). Our results concur with this observation.

Various techniques had been used to subtype the virus, such as Ebnotyping (Gratama *et al*, 1990), RFLP and PCR (Falk *et al*, 1995) for investigation on the aetiology and epidemiology of the virus associated diseases. We demonstrated that one step PCR is a less sensitive method, which could be partly due to poor DNA quality. It is known that fixation and storage can degrade DNA to a variable degree and sizes that cannot be consistently amplified, nor visualised in the agarose gel (Greer *et al*, 1991; Pan *et al*, 1995). Using a nested-PCR procedure, the sensitivity of the PCR technique on paraffin embedded tissue samples has been remarkably increased, even for a small sample size, such as one 5µm thick tissue section. In addition, using consensus primers from the conserved sequences in the U2 region encoding for EBNA-2 in the nested-PCR protocols is less tedious and costly when compared to using general primers from the EBNA-2 region followed by specific primers for the EBV type A and type B in the second PCR, as described by Durmaz *et al* (1998), Martinez *et al* (1998), Tomita *et al* (1995) and Oshima *et al* (1999). Three cases of reactive tonsils were not amplifiable even after nested-PCR for EBNA-2. The failure of amplification in these three cases

could be due to very low amount of EBV genome present in the DNA extracted from the whole tissue section, where only occasional small lymphocytes are positive for EBER.

In conclusion, nested-PCR has increased the sensitivity of EBV subtype detection. Type A virus is the sole pathogenic virus detected in Malaysian cases of EBV-associated diseases.

Table 1: EBV Typing in the Malaysian EBV associated diseases

Disease Type	Number of cases	Ethnic Group				Site of Biopsy (No. of cases)	EBER Positive (No. of Cases)	EBV Typing (No. of Cases)
		M	C	I	O			
Reactive Lymph Nodes / Tonsils	12	7	2	3	-		small lymphocytes (12)	A (9)†
<i>Childhood</i>	5	3	-	2	-	Tonsils (2), LN: inguinal (1), axillary (1), submental(1)	Small lymphocytes (5)	A (3)
<i>Adult</i>	7	4	2	1	-	Tonsils (4), LN: cervical (1), axillary (1), unspecified (1)	Small lymphocytes (7)	A (6)
Nasopharyngeal Carcinoma*	14	3	11	-	-	PNS (7), FOR (6), tonsil (1)	+ (14)	A (14)
Burkitt's Lymphoma	14	3	10	-	1		+ (14)	A (14)
<i>Childhood</i>	8	3	5	-	-	Intestine (3), liver (1), infrascapular mass (1), tonsil (1), LN: cervical (1), submandibular (1)	+ (8)	A (8)
<i>Adult</i>	6	-	5	-	1	maxilla (2), thigh (1), brain (1), peritoneal mass (1) LN: cervical (1),	+ (6)	A (6)
Hodgkin's Lymphomas	17	6	5	6	-		+ (17)	A (17)
<i>Childhood</i>	7	3	2	2	-	LN: cervical (6), para-aortic (1)	+ (7)	A (7)
<i>Adult</i>	10	3	3	4	-	LN: cervical (4), supraclavicular (2), unspecified (1); neck gland (2), mediastinal mass (1)	+ (10)	A (10)
T non-Hodgkin's lymphomas*	4	2	-	1	1	LN: cervical (2), jejunum (1), nasal (1)	+ (4)	A (4)
B non-Hodgkin's lymphomas	3	-	2	1	-		+ (3)	A (3)
<i>Childhood</i>	1	-	-	1	-	LN: inguinal (1)	+ (1)	A (1)
<i>Adult</i>	2	-	2	-	-	LN: cervical (1), axillary (1)	+ (2)	A (2)

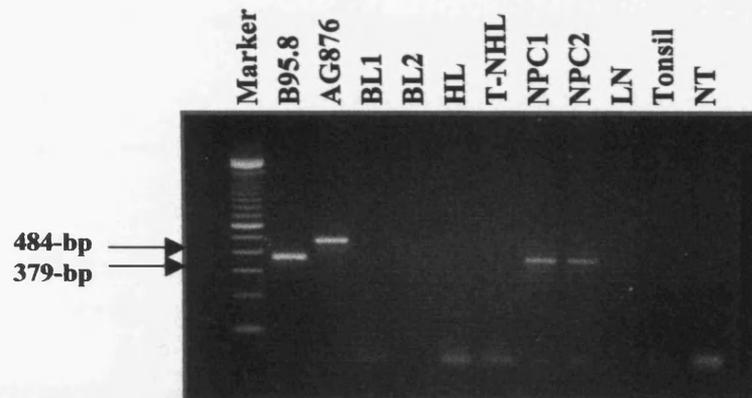
Abbreviation: M, Malay; C, Chinese; I, Indian; O, Others; LN, lymph node; PNS, post nasal space; FOR, fossa of rosacae muller

Childhood: age < 16 years old, adult: age ≥ 16 years old

†: 3 other cases not amplifiable for EBNA-2 gene, but β-globin gene were amplifiable.

*: all cases were from the adults age group

1A.



1B.

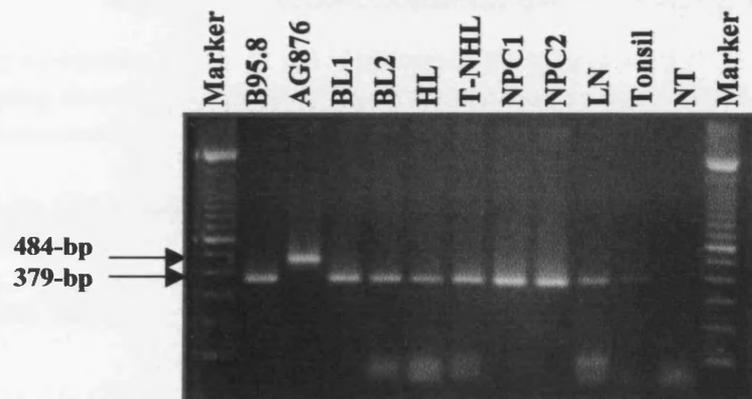


Figure 1: (A) EBV typing by one-step PCR showing no bands from most of the cases. Weak bands were seen in the positive controls at 379-bp for cell line B95.8 (EBV type-A) and 484-bp for cell line AG876 (EBV type-B) and NPC cases. (B) EBV typing by nested-PCR. Specific and intense bands were observed from all cases after nested PCR except for three cases of reactive tonsils which were not able to amplify. All cases tested showed presence of EBV type A coinciding with the position of positive band from B95.8.

BL, Burkitt's lymphoma; HL, Hodgkin's lymphoma; T-NHL, T-non-Hodgkin's lymphoma; NPC, nasopharyngeal carcinoma; LN, reactive lymph node; NT, non-template control

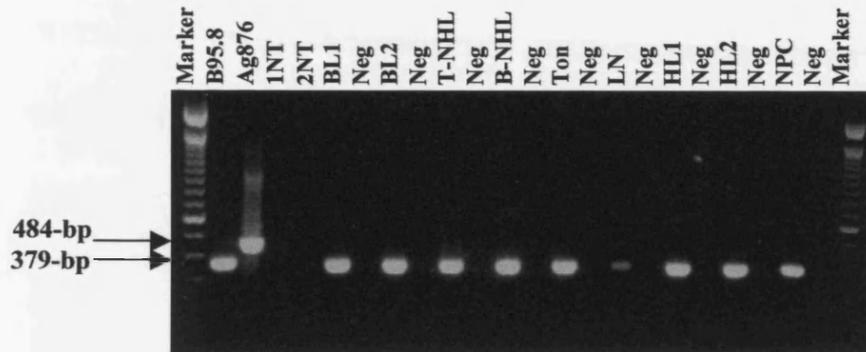


Figure 2: Representative figure of gel electrophoresis after nested-PCR for EBV subtyping showing no block to block contamination and no PCR carry-over contamination.

Marker, 100-bp DNA ladder; BL, Burkitt's lymphoma; HL, Hodgkin's lymphoma; T-NHL, T-non-Hodgkin's lymphoma; NPC, nasopharyngeal carcinoma; LN, reactive lymph node; NT1, non-template control from first PCR; NT2, non-template control carry-over from first PCR; Neg, negative controls (blank)

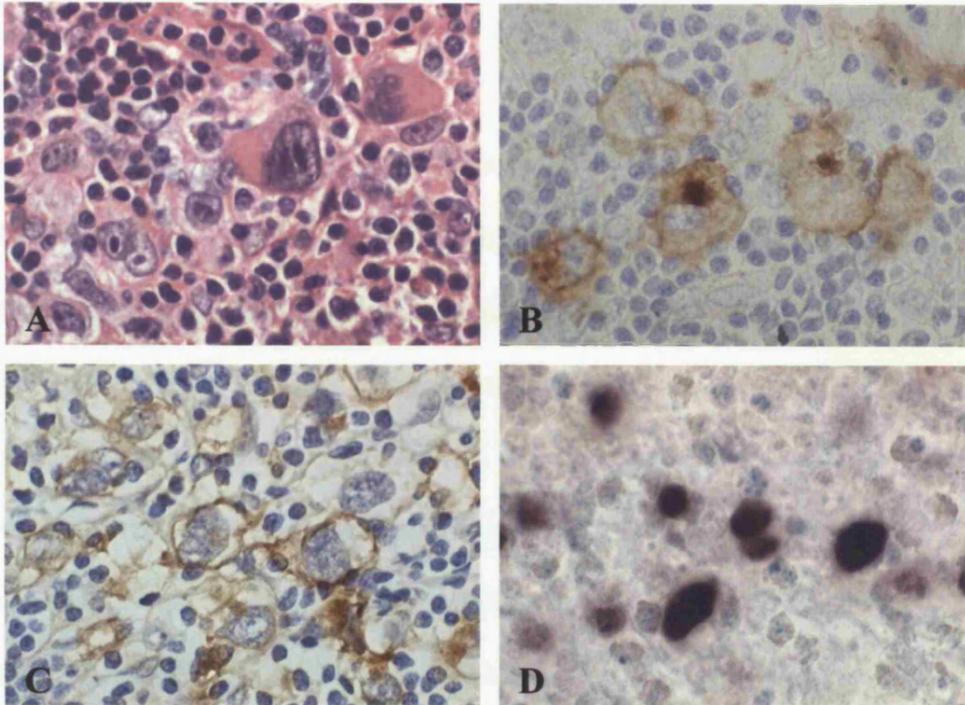


Figure 1: A) Classical Hodgkin's lymphoma. H&E, X 600. B) Reed-Sternberg cells express CD15. Immunohistochemical stain, X 600. C) Reed-Sternberg cells express CD30. Immunohistochemical stain, X 600. D) Nuclear expression of EBER. In situ hybridization, X 600.

Chapter 6

Status of virus in sequential biopsy material of Malaysian Lymphomas: shedding light on the possible role of Epstein-Barr virus in Hodgkin's disease

6.1 INTRODUCTION

Following early seroepidemiological studies that linked the Epstein-Barr virus (EBV) with Hodgkin's lymphoma (HL) by Levine *et al.*, (1971), subsequent studies using various techniques such as southern blot and DNA amplification technology, immunohistochemical staining method and in situ hybridization revealed frequent association of EBV and HL. The frequency of association had been reported to be subtype dependent, being more common in the mixed cellularity subtype than nodular sclerosis (Pallesen *et al.*, 1991; Murray *et al.*, 1992; Delsol *et al.*, 1992, Chan *et al.*, 1995b; Tomita *et al.*, 1996). Within a positive case, vast majority of the Hodgkin, Reed-Sternberg cells express EBERs and LMP-1. This latter latency gene protein had been identified to be essential for immortalization of B-lymphocytes (Kaye *et al.*, 1993) and possess transforming properties, as demonstrated in rodent fibroblast (Wang *et al.*, 1985). In view of the above findings, EBV was suspected to play an aetiological role in the pathogenesis of HL. Further evidence that EBV could be involved in the pathogenesis of HL included the monoclonal origin of EBV infection by analysis of the terminal repeats of the EBV genome (Weiss *et al.*, 1989; Anagnostopoulos *et al.*, 1989; Gledhill *et al.*, 1991). The evidence was strengthened by the observation that in patients with EBV-associated HL, all involved sites were EBV positive and remained positive during the course of the disease (Vasef *et al.*, 1995; Brousset *et al.*, 1994), with the exception of one report of a case of nodular

sclerosing HL with disappearance of EBV in the relapse tumour by Delecluse *et al* (1997). This study aimed to investigate the EBV status of available sequential biopsy material obtained from similar and different anatomic sites in the Malaysian HL patients.

6.2 MATERIAL AND METHODS

Patients' material

Two groups of diseases, HL and NK/T-cell lymphomas with sequential biopsy specimen were retrieved for the study: 10 HL patients with a total of 25 biopsies and 7 NK/T-cell lymphoma patients with 15 biopsies were retrieved from the files. The demographic data and information on the sites and dates of tissue biopsy was retrieved from the information volunteered by the attending doctors, on the original request forms which accompanied the patients' biopsy material.

Immunohistochemical staining

The available stained tissue sections inclusive of all immunohistochemically stained slides from all the biopsy material were reviewed. Further sections were made from the corresponding blocks for more immunohistochemical study with additional antibodies whenever deemed necessary to complement those available, for confirmation of diagnosis and subtyping of lymphomas. The staining method is as described in earlier chapters (see appendix).

EBER in situ hybridization

The detection of Epstein-Barr virus encoded RNAs (EBER) was performed using fluorescein isothiocyanate (FITC)-labeled oligonucleotide (NCL-EBV, Novocastra, United Kingdom) according to the protocol of the manufacturer (see

appendix). Visualisation was achieved with incubation of antibody to FITC tagged with an enzyme (alkaline phosphatase), followed by application of substrates for the color reaction to take place. The substrate of choice was NBT/BCIP (4-nitroblue-tetrazolium chloride/5-Bromo-4 chloro-3-indolyl-phosphate). Malaysian EBV-positive naso-pharyngeal carcinoma cases were used as external positive control for the staining technique. A lymphoma is regarded as EBV associated if majority of the tumour cells strongly express EBER.

6.3 RESULTS

Hodgkin's Lymphoma

The presenting age of these patients ranged from 13 to 49 years. In 3 patients, the 2 surgical biopsies were not obtained more than 2-weeks apart (cases 7, 8 and 9 in table 1). One patient had 2 biopsies performed within 3 months interval (case 10) and the remainder 6 patients (cases 1-6) had subsequent biopsies which were excised minimally 1½ years from the time of the initial diagnostic biopsies (Table 1). Based on the information of the sites and interval of the biopsies, the 2 cervical lymph node specimen from case 9 was considered as repeat biopsy from the same disease presentation. Likewise, biopsy tissue from cases 7, 8 and 10 were regarded as from different sites of presentation of the same disease, whereas the various specimens subsequent to the initial diagnostic biopsies in cases 1 to 6 were regarded as relapse lesions. In 5 cases (case 3, 6, 7, 8 and 10), EBERS were shown to be present in the HRS cells of the initial diagnostic material (Figure 1), and the virus was again found to persist in the subsequent tumour biopsies. It was shown to be for as long as after 9 years in one case (case 3). The cases without the presence of EBER in the HRS cells remained

virus free in the relapsed tumours (4 cases), and for as long as 5 years in case 1. Irrespective of the sites of the biopsy material, the virus status in the HRS cells of the sequential specimen was similar to the original biopsies. Except for minor variations in the morphology of the tumour, the histological subtypes of the lesions in these sequential biopsies also remain unchanged.

NK/T-cell lymphoma

Seven cases of EBV-associated NK/T-cell (6 nasal: case 1 to 6, and 1 nasal-type from the intestine: case 7) were found to have persistence of EBV in subsequent and repeat biopsies (more than 3 months apart) as presented in Table 2. Case 1 showed that 3 years later, the recurrence in a distant site, pleura, also exhibited the expression of the virus genome. These tumours show expression of CD3 and CD56 by the tumour cells, and many of these cells also express EBER in their nuclei. The morphology of the lymphomas can vary from biopsy to biopsy, with some tumours showing extensive necrosis, and some fragments infiltrated with a mixture of acute and chronic inflammatory cells. The tumour cells also vary in appearance from cases to case, some with large round to oval nuclei, others have cigar-shaped, crooked nuclei.

6.4 DISCUSSION

The findings in these 10 cases of Malaysian HL and 7 cases of NK/T-cell lymphomas with sequential biopsies reconfirm the observations of other studies, with the exception of one report (Delecluse *et al.*, 1997). The persistence of the virus at different time intervals, and irrespective of the sites of biopsy material strongly suggests that the virus was present very early in the development of the disease. The persistent presence of this virus is probably necessary for maintaining the growth of these tumour cells. In this study, the non-EBV-

associated HL remained EBV negative in the biopsy specimen from all sites and at various time intervals, which further support the contention that EBV is not likely to be secondarily acquired, either due to susceptibility of the tumour to the virus infection or other unknown reasons. Other researchers who studied the terminal repeat profile of the viral genome found a clonal pattern of episomal EBV in HL (Weiss *et al.*, 1987; Staal *et al.*, 1989; Gledhill *et al.*, 1991; Brousset *et al.*, 1994). Hence, it is in support of the pathogenetic role of the virus, or its conferment of growth advantage for the tumour. Similar findings were also obtained from the investigation on NK/T-cell lymphoma (Ho *et al.*, 1990). In addition, the analysis of LMP-1 gene polymorphism by PCR and DNA sequencing (Brousset *et al.*, 1994; Vasef *et al.*, 1995) had also demonstrated similar clone of the virus in the initial disease and in relapse. Together, these findings are in support of EBV playing an important role in the pathogenesis of EBV-associated HL, and against the notion that EBV is an innocent bystander in the disease. Similar conclusion can be made for NK/T-cell lymphoma.

In the case report by Delecluse *et al.* (1997), a 5 year-old boy who presented with multiple cervical and supraclavicular enlarged lymph nodes and pleural effusion, due to mediastinal extension had remission of the disease following treatment, but relapsed a year later. The initial biopsy showed expression of viral latent protein (LMP)-1, and was absent in the relapse tumour, while the histological subtype remained unchanged. Similarly, other sensitive techniques such as in situ hybridization for EBER and PCR also revealed presence of the virus in the initial lesion but became undetectable in the relapse material. To this, the authors hypothesized that in overtly malignant cells, such as the case discussed, the virus was not required for the persistence of neoplastic growth, and

hence may be lost. The explanation seems plausible since this phenomenon was previously observed in *in vitro* experiment with EBV-positive Burkitt's lymphoma cell line Akata, which resulted in subclones that had lost the viral genome (Shimizu *et al.*, 1994). Hence, the notion of further genetic alterations, e.g. somatic mutations in the tumour may substitute the virus for neoplastic growth. If this hypothesis holds true, it may explain the absence of EBV in some HL cases. However, this hypothesis is not supported though not totally excluded by the observations made on 2 separate groups of diseases, both known to be closely associated with EBV, since the sample size for this study is relatively small.

In conclusion, the persistence of EBV in EBV-associated lymphomas and the absence of EBV in EBV-negative lymphomas from sequential biopsy study is supportive of the notion of early introduction of the viral factor in favour of the growth and or the development of these diseases.

Table 1: Association of Epstein-Barr virus in sequential biopsies of classical Hodgkin Lymphoma (N=10)

Case No.	Age*	Sex	Race	Biopsy year	HL Subtype	Site	EBER
1	40	f	C	1985	NS	Spleen	-
				1989	NS	LN (infrascapular)	-
				1990	NS	LN (axillary)	-
2	25	m	M	1990	NS	LN (cervical)	-
				1992	NS	Mediastinal mass	-
3	13	m	I	1974	NS	LN (cervical)	+
				1979	NS	LN (cervical)	+
				1980	NS	Liver	+
				1980	NS	Retroperitoneal mass	+
				1983	NS	LN (inguinal)	+
4	40	m	M	1980	NS	LN (supraclavicular)	-
				1982	NS	LN (site unspecified)	-
5	40	f	I	1978	MC	LN (cervical)	-
				1982	MC	LN (cervical)	-
6	30	m	M	1989	NS	LN (paraaortic)	+
				1990	NS	LN (cervical)	+
				1993	NS	LN (cervical)	+
7	35	f	C	1978	MC	Liver	+
				1978	MC	LN (cervical)	+
8	20	m	I	1974	NS	LN (supraclavicular)	+
				1974	NS	LN (parasplenic)	+
9	49	m	I	1972	MC	LN (cervical)	-
				1972	MC/NS	LN (cervical)	-
10	23	f	M	1993	NS	LN (cervical)	+
				1994	NS	LN (supraclavicular)	+

f, female; m, male; C, Chinese; I, Indian; M, Malay; MC, mixed cellularity, NS, nodular sclerosis; LN, lymph node

Table 2: Association of Epstein-Barr virus in sequential biopsies of NK/T-cell lymphomas (N=7)

Case No.	Age *	Sex	Race	Biopsy year	Site	EBER
1	38	m	M	1991	Nasal septum	+
				1994	Pleura	+
				1994	Right maxillary sinus	+
2	48	m	C	1993	Inferior turbinate	+
				1994	Nose	+
3	34	m	C	1994	Lateral wall of nose	+
				1994	Nose	+
4	56	f	M	1999	Nose	+
				1999	Nose	+
5	40	f	M	1997	Maxilla	+
				1998	Post nasal space	+
6	58	f	C	1982	Inferior turbinate	+
				1983	Nasopharynx	+
7	48	m	C	1996	Lymph node	+
				1996	Large gut	+

f, female; m, male; C, Chinese; I, Indian; M, Malay

Chapter 7

Summary, conclusions and future perspectives

7.1 SUMMARY

The “evolution” of lymphoma, not of the disease but the origination of nomenclature to the present day concept has taken more than 150 years to crystallize. Classification schemes have traditionally been developed separately for Hodgkin’s disease and lymphomas other than Hodgkin’s disease (non-Hodgkin’s lymphoma). Advancement of knowledge indicated a large majority of Hodgkin’ disease cases possess B cells characteristics with aberrant hypermutation of immunoglobulin gene, suggesting biological properties of follicular-centre cell type. Hence, the consensus is to rename it Hodgkin’s lymphomas. These newer techniques also enable sub-classification of Hodgkin’s and non-Hodgkin’s lymphomas into further subtypes with specific phenotype, genotype and cytogenetic characteristics, and into 2 main streams of T and B-cell lymphomas for non-Hodgkin’s group. The new WHO classification scheme adopts the practical approach of listing the well-defined entities based on current morphological, immunologic and genetic profiles, incorporated newer entities that did not exist in the older classification schemes, and added on relevant clinical information of the these entities. The studies of lymphoma entities in Malaysian patients using this new classification system enables one to determine if the pattern of lymphoma observed is in anyway unique to this multi-ethnic patient populations, and allow meaningful comparison of data with others that adopt comparable system of classifications.

In the pursuit of aetiological agents of malignant lymphomas, 2 families of viruses appear to be important, the retrovirus and the human herpesvirus. Human

T-cell leukaemia virus-1 (HTLV-1), a T-cell (CD4) trophic retrovirus closely related to the adult T-cell lymphoma-leukaemia, first described in Japan (Uchiyama *et al*, 1977), its importance is found to be rather restricted to the South-Western Japan, the Caribbean basin and the sub-Saharan Africa. However, in the past 15 years, there has been massive accumulation of information and knowledge on human herpes virus and their possible pathogenetic roles in malignant lymphomas, the Epstein-Barr virus (EBV) and human herpesvirus-8 (HHV-8). Following its discovery in the tumour cells of Burkitt's lymphoma in the 1964 (Epstein *et al*, 1964), EBV has since been shown to be closely associated with several other cancers such as nasopharyngeal carcinoma, T-cell and NK/T-cell lymphomas, immunodeficiency/AIDS-related lymphomas, post-transplant lymphoproliferative disorders and Hodgkin lymphoma. There is convincing evidence that this virus is not just an innocent bystander, although the exact pathogenetic role(s) of EBV in each lymphoma type has yet to be elucidated. In most instances, the virus is shown to be clonal, based on the study of terminal repeats sequence of the viral episomal form by Southern blot analysis.

Studies on non-Hodgkin's lymphoma in 69 Malaysian children (Chapter 2) showed disease pattern rather similar to reported Western series and elsewhere in other parts of the world. Burkitt's lymphoma, lymphoblastic lymphoma and diffuse large cell lymphoma (large proportion are the CD30-positive anaplastic large cell type) forming the 3 major groups. The childhood pattern therefore differs from the adults from similar multi-ethnic population (Table 3, Chapter 2 adopted from Peh, 2001). The difference in subtype composition results in different overall EBV association rates for T- and B-cell lymphomas, since EBV association is subtype dependent. Hence, EBV association is 3X higher in the B-

cell lymphomas in children, whereas it is the reverse for T-cell lymphomas when compared to the adult data (Table 3, Chapter 2). NK/T-cell lymphoma which contributes to the high percentage of EBV association rate in T-NHL in the adults, is noted not a common occurrence in children. Similar to most other reports, there is also a high frequency of ALK1 expression in anaplastic large cell lymphomas in Malaysian childhood cases (100%), and anaplastic large cell lymphoma constitutes 50% (8/16) of large cell T- and B-cell lymphomas combined (Table 2, Chapter 2). Hence, one may speculate that half the children with diffuse large cell lymphomas will have good clinical outcome.

Despite being in the tropics with high rainfall and endemic malaria infection in the country, there is no observed extreme high frequency of Burkitt's lymphoma nor EBV association rate in this disease in Malaysian children such as that reported in tropical African countries. However, University Hospital Kuala Lumpur (University of Malaya Medical Centre) is located in the urban district where malaria is not endemic. From these preliminary findings, one speculates that the Burkitt's lymphoma seen in Malaysian are probably of the sporadic type. Hence, further studies and investigations into the aspects of EBV association rate and Burkitt's lymphoma encountered in Malaysian patients are presented in chapter 3, and in chapter 4, the frequency of Burkitt's lymphoma in the malaria endemic state of Sabah.

The study on a total of 59 cases of Burkitt's lymphomas collected from a few hospitals scattered in the peninsular Malaysia and East Malaysian states of Sabah and Sarawak suggest occurrence of sporadic type of Burkitt's lymphoma (Chapter 3). There is a low rate of jaw involvement of this disease, a mean age of 16 years at presentation, and the EBV association rate is intermediate (33%). The

viral association is stronger in East Malaysian cases (50%) than West Malaysian (30%). However, the disease presentation in other aspects do not differ between these 2 groups of patients, and from other reports, in that there is a male preponderance (male:female ratio is 3.9:1), and the 2 main sites of presentations are the abdominal viscera and the lymph nodes. There is also strong expression of p53 and pRb proteins in large proportion of cases. Small numbers of cases (9 in the series) can express bcl-2 protein, 3 of them are EBV associated.

Previous studies on Malaysian lymphomas revealed that EBV were very closely associated with several subtypes of lymphomas in Malaysia, namely the nasal NK/T-cell lymphomas, childhood classical Hodgkin's lymphomas and mixed cellularity Hodgkin's lymphoma (Peh *et al*, 1995; Peh *et al*, 1997; Peh, 2001). Although there was some suggestion of ethnic predilection of EBV association in Hodgkin's lymphoma (Peh *et al*, 1997), more frequently in the Indians than non-Indians, this ethnic predilection for EBV in Indian is not further substantiated in other subtypes of lymphomas. In the case of Burkitt's lymphoma, EBV association is more commonly observed in the ethnic Malay and the East Malaysian indigenous populations, and the site of presentation of the tumours, ie gum and maxilla (Chapter 3).

Lymphomas, ranked twelve among all cancers world-wide in 1990s, hence, this disease has profound personal and economic impact (Pisani *et al.*, 2002). Mortality and incidence rates of non-Hodgkin's lymphomas have also been reported to increase in both sexes in most countries over recent decades (Hartge *et al.*, 1994; Devesa and Fears, 1992). In contrast, there has been significant decline in HL mortality rates in both sexes since the early 1970s. Viruses, immunosuppression, and certain occupations have been identified as possible

factors for the changes observed (Hartge *et al.*, 1994; Toren *et al.*, 1996). An earlier study on lymphomas in East Malaysia from years 1981-1983 revealed that the pattern of lymphomas in East Malaysia concurred with the general pattern in Asia (Chai *et al.*, 1999). Another study was deemed timely to elucidate if the spectrum and pattern of lymphoma in Sabah has since changed 15 years later in the 1990s. Queen Elizabeth Hospital, Sabah is the major hospital in Sabah serving a population of 1,863,659, and is the only hospital with histopathological laboratory service in the entire state. Pathological biopsies from other hospitals are sent to this hospital for histopathological diagnosis. The data from Queen Elizabeth Hospital, Sabah would therefore be representative of the disease pattern in the state of Sabah. Based on the study of 91 reconfirmed cases, lymphoma pattern in Sabah in the 1990s appears to concur with the earlier pattern in the 1980s (Table 3, Chapter 4). There is higher prevalence of diffuse large B-cell lymphoma and lower incidence of follicular lymphoma and Hodgkin's lymphoma, again, a pattern similar to elsewhere in Asia. There was an overall increase in the numbers of NHL in the 1990s (to almost twice the numbers) but a drop in the proportion of T-NHL which are commonly EBV associated, from 25% in the 80s to 16.5% in the 90s. There is also a low EBV association rate in Burkitt's lymphoma in the state of Sabah (28.6%). The reasons for the 2 latter observations are not immediately apparent. In particular, the relative rarity of Burkitt's lymphoma is rather unexpected since malaria infection is endemic in of Sabah. However, a study involving a larger series would be necessary to establish if the findings from this study are statistically significant and reliable.

There are two subtypes of EBV, A and B, which are distinguishable based on their genetic polymorphism in the nuclear antigen (EBNA)-2, 3, 4 and 6 genes

(Munch, 1998; Dambaugh *et al*, 1986). Type A virus is ubiquitous, predominates in the European, American and Asian countries, it efficiently transforms and immortalises infected B-lymphocytes in vitro. Whereas, type B virus is a weaker transformer than type A virus (Rickinson *et al*, 1987; Ring, 1994; Kunimoto *et al*, 1992). Type B virus was initially reported to be confined to African countries and immuno-compromised patients previously (Zimber *et al*, 1986; Khanim *et al*, 1996; Young *et al*, 1987; Buisson *et al*, 1994; Borisch *et al*, 1992), but recent investigations revealed that it is as prevalent as type A in normal population (Sixbey *et al*, 1989). An earlier study on a small number of Malaysian T-cell lymphoma of the upper aerodigestive tract were all found to be infected by type A virus (Peh *et al*, 1995). Using a sensitive nested-PCR method, EBV subtype pattern study was performed on a larger number and a wider spectrum of EBV associated diseases presented at different anatomical sites. A total of 38 lymphomas (14 Burkitt's lymphomas, 4 T-cell non-Hodgkin's lymphomas, 3 diffuse large B-cell non-Hodgkin's and 17 classical Hodgkin's lymphomas), 14 cases of nasopharyngeal carcinomas, and 12 reactive lymph nodes and tonsils were studied. Cell lines B95.8 (type A positive, EBNA-2A) and AG876 (type B positive, EBNA-2B) were used as positive controls for EBV subtype A and B respectively. In all these samples, only EBV type A was detected, irrespective of the anatomical sites of the biopsy material, age group, sex and ethnicity of the patients (Table 1, Chapter 5). This finding differs from other Asian series, where type A virus was present in 85-97% of their cases (Oshima *et al*, 1995; Tomita *et al*, 1996; Sidagis *et al*, 1997), with 6-13% showing type B and 3-6% double infections. Similarly in Taiwan, small numbers of double infections were reported

(Wu *et al*, 1996). This is perhaps due to the absence of AIDS-related lymphomas and post-transplant lymphoproliferative disorders in this cohort of study.

In one report by Delecluse *et al* (1997), a case of nodular sclerosing Hodgkin's lymphoma which was EBV associated in the initial presenting biopsy, was later found to be non-virus associated in the relapsed tissue by several detection techniques. However, the relapsed tissue contains tumour of similar morphology as the initial presentation. The authors proposed that when tumour cells are overtly malignant, virus may not be required for the persistence of neoplastic growth, and hence may be lost. If that is the case, it may explain the absence of detectable virus gene in EBV-negative cases. The study on 2 small series of EBV associated lymphomas (10 cases of classical Hodgkin's lymphomas and 7 cases of NK/T-cell lymphomas), indicated persistence of the virus in sequential biopsies, irrespective of the time interval nor the site of presentation of the relapse lesions (Table 1 and 2, Chapter 6). In cases where there was no EBV gene detectable at initial diagnostic material, the relapse material remained virus free (Table 1, Chapter 6).

7.2 CONCLUSIONS AND FUTURE PERSPECTIVES

Variations in lymphoma subtypes exist between geographical regions. Although previous studies reconfirmed East-West differences in non-Hodgkin's lymphoma pattern in the adults of multi-ethnic patient populations from an urban environment in Malaysia, the East-West difference is less distinct in the childhood group from this present study. The reason is not immediately apparent. One may speculate that early onset lymphomas are probably related to the immature host immune status dealing with the effect of widespread risk factors. However, the

subtype frequency which differs significantly in certain diseases between ethnic groups in Malaysia, such as the more common occurrence of classical Hodgkin's lymphoma in the Indian, EBV associated NK/T-cell lymphomas in the Chinese adults, is again reflected in EBV association of T-NHL in Chinese Malaysian children. However, the relatively small number of cases in the series does not provide meaningful statistical analysis. It is probable that the predilection of some EBV associated T-cell lymphomas to the ethnic Chinese in Malaysia is more real than apparent since published literature also indicates that ethnic Chinese is at risk of developing EBV nasopharyngeal carcinoma (Prasad and Rampal, 1992). This may in part explain the higher incidence of T-NHL in this series when compared to Western data. All these findings are in support of the importance of host factors in determining the final outcome of an infection by potentially an oncogenic virus. Data from published literature shows that B-cell lymphomas with a high degree of EBV association (except for endemic Burkitt's lymphoma) are almost exclusively seen in immunodeficient patients, suggesting that host immunity is of importance for the development of EBV-related B-cell lymphomas. A likely explanation for this phenomenon may be that immunogenic epitopes are differently expressed or recognized in various EBV-infected lymphocyte sub-populations, and host immune status determines how these infected cells can be kept under surveillance.

Although in a small number of paediatric Burkitt's lymphoma cases there was a suggestion of predilection of EBV association in the Chinese when compared to non-Chinese, this phenomenon disappeared when the study was expanded to a larger series of the disease. Despite the high rainfall and endemic malaria infection in rural Malaysia, and in the state of Sabah, there is no evidence of an excessive high frequency of Burkitt's lymphoma and EBV association rate

in this disease in Malaysia. The clinical presentations of the disease also support the pattern of sporadic Burkitt's lymphoma. This may be due to probable higher socioeconomic environment in Malaysia when compared to tropical African countries, since EBV association in Burkitt's lymphoma was found related to low socioeconomic status of the patients (Anwar *et al*, 1995). However, further investigation into the socioeconomic status of Malaysian cases need to be carried out for a more definitive conclusion.

Even though the pathogenetic mechanism of EBV is not well understood for individual type of EBV associated lymphoma, there is strong indication that the presence of EBV in these tumours did not occur just by chance. It was demonstrated by the sequential biopsy study that there is persistence of the virus gene in EBV associated cases, and secondarily acquired EBV did not occur in non-EBV associated tumours. Moreover, only one subtype of virus (Type A) is detected in EBV associated tumours and reactive lymphoid tissue. The latter finding is probably indicative of the predominance of this viral subtype in Malaysia.

In an earlier study of patient material from Malaysian peripheral T-cell lymphoma with EBV infection, the results show high frequency of 30-bp deletion at the carboxyl-terminal of the LMP-1 gene. This transforming gene remains a subject of interest, due to the observation that tumors associated with this mutation pattern were more aggressive in behavior in previous report (Kingma *et al*, 1996). It remains to be unraveled, if this behavior is the direct effect of the mutated gene, such as in driving cell towards aggressive proliferation, anti-apoptosis effect, or participate indirectly via immune escape mechanism (Triveli *et al*, 1994). Whether or not the occurrence of LMP-1 gene mutation pattern is

similar in healthy individuals and patients, in both the by-stander infected B-lymphocytes and diseased material from an individual, and also their pattern in the various ethnic groups remain a fascinating subjects for future studies.

Future direction of investigation on the role of EBV may therefore be more beneficially focused on eliciting in situ host and tumor cells interaction pattern, in the presence or absence of virus, and the virus immunogenic status in clinical material from different ethnic groups with similar disease pattern. There are newer technologies available for cell isolation, such as the micromanipulator and lazer-capture microdissectors, which are useful tools to achieve accurate and stringent sample selection. Cloning, tissue culture and transfection techniques can similarly be applied to investigate the effect of various viral genes on cell proliferation and apoptosis pathways in tumour cell lines in vitro. Microchip arrays analysis is a promising and comparatively non-tedious screening method, of obtaining large repertoire of molecular information. However, it is necessary to apply these technologies cautiously and sensibly in order not to cause information overload, and accumulation of massive amount of useless and confusing data.

APPENDIX

Methods

Immunohistochemistry

Serial 4 μ m sections were mounted on silanized (3-aminopropyltriethoxysilane) glass slides and stained with a panel of antibodies (Table 1). Based on the principle of antigen-antibody reaction, a 3-stage immunoperoxidase staining technique was performed, without or with antigen retrieval steps by proteolytic enzyme digestion, microwave or high pressure cooker heat pre-treatment (Perkin and Kjeldberg, 1993). It was thought that during the process of aldehyde fixation, some antigenic epitopes may be masked by cross-linking of protein molecules (Huang *et al.*, 1976). Hence, enzyme digestion (Curran and Gregory, 1977; Curran and Jones, 1978; Mepham *et al.*, 1979) and heat treatment by microwave (Shi *et al.*, 1991) or high-pressure cooking (Norton *et al.*, 1994; Miller *et al.*, 1995) re-expose these antigens (retrieved) for antigen-antibody reaction. Table 1 represents antibodies adopted, the source and concentration used and the method of antigen retrieval employed.

Table 1: The choice of antibodies and methods of antigen retrieval used in the immunohistochemical staining technique

Antibody	Dilution	Antigen retrieval	Buffer	Source
CD3 (polyclonal)	1:50	Microwave	Citrate buffer, pH6.0	Dako
CD4, (1F6)	1:20	Microwave	Dako high pH	Novocastra
CD5 (NDL-CD5)	1:50	Microwave	EDTA, pH8.0	Novocastra
CD8, (C8/144B)	1:50	Microwave	Dako low pH	Dako
CD10 (270)	1:50	Microwave	Dako high pH	Novocastra
CD15 (leuM1)	1:50	Microwave	Citrate buffer, pH 6.0	Becton Dickinson
CD20 (L26)	1:500	microwave	Citrate buffer, pH6.0	Dako
CD21 (IF8)	1:100	0.1% trypsin	0.1% CaCl ₂ , pH7.8	Dako
CD23 (1B12)	1:50	Microwave	Citrate buffer, pH6.0	Novocastra
CD30 (BerH2)	1:50	Microwave	Citrate buffer, pH6.0	Dako
CD43 (MT1)	1:50	Microwave	Citrate buffer, pH6.0	compliment of S. Poppema
CD45RO (UCHL1)	1:500	nil		Dako
CD56 (1B6)	1:50	Microwave	Citrate buffer, pH6.0	Novocastra
CD57 (leu 7)	1:50	Microwave	Citrate buffer, pH6.0	Becton Dickinson
CD68 (PGM1)	1:50	nil		Dako
CD79a (JCB117)	1:20	Microwave	Citrate buffer, pH6.0	compliment of D. Mason
TdT (339)	1:50	Microwave	EDTA, pH8.0	Immunotech
TIA-1 (2G9)	1:500	Microwave	Dako high pH	Dako
ALK (ALK1)	1:25	Microwave	Dako high pH	Dako
EMA (E29)	1:50	0.1% trypsin	0.1% CaCl ₂ , pH 7.8	Dako
Cytokeratin, MNF116	1:500	0.1% trypsin	0.1% CaCl ₂ , pH 7.8	Dako
kappa	1:500	microwave	Citrate buffer, pH6.0	Dako
lambda	1:500	microwave	Citrate buffer, pH6.0	Dako
Bcl-2 (C124)	1:50	microwave	Citrate buffer, pH6	Dako
TARC (polyclonal goat anti human TARC)	1:400	microwave	EDTA, pH8.0	RandD System, USA

In situ hybridization

4µm thick tissue sections were mounted on glass slides precoated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO, USA). The source of probe for the detection of Epstein-Barr virus encoded RNAs (EBERs) is from Novocastra (NCL-EBV, Novocastra, United Kingdom). The staining procedure was according to the protocol of the manufacturer. Malaysian EBV-positive nasopharyngeal carcinomas were used as positive controls for the staining procedure.

In brief, the sections were dewaxed in xylene and cleared in alcohol, followed by enzyme digestion of the tissue sections. The concentrations of proteinase K routinely applied for the digestion process were 5 µg/ml, with increased or diminished strength depending on the adequacy of the digestion state of the tissue on individual case. The probes in hybridization solution was then incubated at 37°C for a minimum of 2 hours, which was followed on by stringent washing steps. The detection steps were similar in principle to routine immunohisto-chemistry. Antibodies to FITC tagged with an enzyme (alkaline phosphatase) were applied, followed by application of substrates for the color reaction to take place. The substrate of choice was NBT/BCIP (4-nitroblue-tetrazolium chloride/5-Bromo-4 chloro-3-indolyl-phosphate). The former gave a purplish-blue and the latter a red color reaction. A light counter-staining with Haematoxylene or nuclear fast red was performed to provide better morphological details of the cells under studied.

Protocol of EBER in situ hybridisation (ISH):

1. Dewax and bring section to water
2. Air dry tissue section
3. Place slides in humidity chamber
4. Add 150µl proteinase K, 5µg/ml at room temperature 30 min
5. Wash slide in Tris buffer 2X 5 min
6. Air dry tissue section
7. Place slides in humidity chamber
8. Add approximately 50µl of EBER probe onto section
and cover with cover slip, incubate at 55°C 1 hr 30 min
9. Immerse slides in preheated stringent wash solution,
at 55°C, with frequent shaking 30 min
10. Wash with Tris buffer 2X5 min
11. Place slides in humidity chamber
12. Add 100-200µl anti-FITC, room temperature 30 min
13. Wash with Tris buffer 2X 5 min
14. Wash with distilled water 5 min
15. Place slides in humidity chamber
16. Add 100-200µl substrate, NBT/BCIP, room temperature 30- 60 min
17. Counter stain section in Mayer's Haematoxylin
18. Mount section in Aqueous mounting medium, Clear-Mount

*Polymerase chain reactions (PCR):**a. Tissue sectioning for DNA extraction*

Paraffin sections were cut with new disposable blades that were changed between samples. Extreme precaution was observed in the cutting of tissue sections for EBV sub-typing to prevent contamination of tissue from case to case. The semi-automated microtome (Reichert-Jung) was cleaned thoroughly with 100% ethanol and followed by 70% ethanol before use. For each case, the required number of section was cut and placed into labeled microcentrifuge tubes with sterile disposable tooth-pick or Pasteur pipette. A new blade and changing of the gloves were strictly observed for the cutting of each block. The cutting area of the microtome was cleaned with 70% ethanol between each specimen, to avoid transferring of tissue from case to case. Between cases, 2 tubes of a blank paraffin block (only paraffin wax without tissue, prepared specially in a clean environment devoid of contamination with any tissue) was cut between each test case to serve as negative case control for the DNA extraction and PCR steps.

b. DNA extraction

Several DNA extraction methods were used for the isolation of DNA from routinely processed paraffin-embedded tissue. They were found to give reproducible results with EBV sub-typing. In addition, two cell-lines, B96.8 (infected with type-A EBV) and AG876 (infected with type-B EBV) were used as reference positive controls for the sub-typing of the virus (compliment of Dr. Sam CK, IPSP, UM)

(i) *Extraction of DNA from paraffin-embedded tissue with incorporated phenol-chloroform step*

In brief, 20µm of tissue section in microcentrifuge tube was treated twice with xylene at 37°C. It was then washed with two changes of absolute alcohol. The tissue was spun down after each step, at 5000rpm for 2 minutes. Xylene and alcohol was discarded with gentle tilting of the tubes to prevent dislodging of the tissue. The de-paraffinized tissue was air-dried. The dried tissue was digested in 0.2ml digestion buffer (20mM Tris-Cl, 1% SDS) containing 2mg/ml proteinase K for 48 hours at 37°C. Following which, phenol-chloroform extraction was carried out. 0.2 ml of phenol-chloroform was added to the sample, the tube was gently inverted several times, and centrifuged at full speed for 5 minutes. The upper aqueous layer was transferred to a new tube without disturbing the interphase. The chloroform-phenol extraction step was repeated on the organic phase. Absolute alcohol was added to the aqueous solution and the suspension was left overnight at 20°C to precipitate the DNA. The DNA was spun down at full speed for 5 minutes. The supernatant was discarded, and the pellet was washed once more with 70% ethanol, then centrifuged as before. The DNA pellet was air-dried, then suspended in TE buffer. It was stored at 20°C before use.

(ii) *Extraction of DNA from paraffin-embedded tissue with modified method by Shibata D (1994)*

5µm of tissue section in microcentrifuge tube was de-paraffinized with 2 changes of xylene at 37°C and cleared in absolute alcohol as described above. 50µl of digestion buffer (100mM Tris-Cl, pH 8, 4mM EDTA pH 8)

with 200µg/ml Proteinase K was added to each tube of air-dried tissue. It was incubated overnight at 37°C. Proteinase K was inactivated by boiling for 10 minutes. The residual tissue was pelleted by centrifugation, and the supernatant was transferred to a new tube. It was stored at -20°C before use. 1µl of the supernatant was used for PCR.

(iii) Extraction of DNA from cell-lines (Rolf et al., 1992)

In brief, cells from culture flasks AG876 and B95.8 were transferred separately to different centrifuge tubes and spun at 5000rpm for 5 minutes. The cell pellets were washed twice with 4ml PBS and spun as above. The pellets were re-suspended in 0.6ml PBS and 0.4ml lysis buffer for incubation at 37°C for 1 hour. At the end of the hour, 15µl of 20mg/ml of proteinase K was added to each tube, incubate at 37°C for another 2.5 hours, until the solution was clear. 0.4ml of phenol-chloroform was added to each tube, inverted several times and centrifuged at 10,000rpm for 5 minutes. 0.3ml of the lower layer of phenol-chloroform was removed and the remainder was spun for another 5 minutes. The upper layer was transferred to a new tube. The DNA was precipitated with 1ml of 100% ethanol overnight at -70°C. The DNA was spun briefly, the supernatant discarded and the pellet re-dissolved in 400 µl sterile distilled water with addition of 40 µl of 2 M NaOAc and 1ml of absolute alcohol to re-precipitate the DNA. The DNA pellet was washed with 80% ethanol. The final DNA pellet was air-dried, dissolved in 20-50 µl of TE buffer overnight at 4°C and stored at -20°C before use.

(iv) *Extraction of DNA from cell-lines using Tri-ReagentTM*

DNA extraction from EBV positive control cell lines (B95.8 and AG876) was performed using Tri-ReagentTM (Molecular research Centre Inc USA). Briefly, 1 ml of Tri-ReagentTM was added to cell pellets to homogenise the cells in a 1.5ml PCR tube. The content was mixed thoroughly by vortexing then incubating at room temperature for 5 minutes. 100 µl of BCP solution which was a phase separation buffer containing phenol was added into the mixture, vortexed to mix and incubated for 15 minutes at room temperature. It was then spun down at 12,000 rpm for 15 minutes at 4° C. Following centrifugation, the mixture separates into 3 layers of lower phenol phase, interphase and an upper aqueous phase. RNA remains in the aqueous phase whereas DNA and proteins are in the interphase and organic phase. For DNA extraction, the interphase and organic phase were carefully pipette out and dispensed into a new 1.5 ml tube. 300 µl of absolute alcohol was added in, mixed thoroughly by inverting the tube several times and incubated at room temperature for 3 minutes. Next, DNA was pelleted by centrifuging at 2000 rpm for 5 minutes at 4° C. Supernatant containing protein was removed while DNA pellet was washed twice in 1 ml of 0.1 M sodium citrate in 10% ethanol. The DNA pellet was then resuspended in 1.5 ml of 75% ethanol for further washing by incubating at room temperature for 20 minutes with periodic mixing and centrifuged at 2000 rpm for 5 minutes. Ethanol was removed and DNA pellet was air-dried at room temperature for 5-10 minutes. The DNA was then dissolved in 600 µl of sterile distilled water. Impurities was spun

down at 12,000 rpm for 10 minutes and the supernatant was transferred to a new tube for use in PCR.

c. *Polymerase chain reaction (PCR)*

(i) *EBV subtyping*

Two steps PCR procedure was performed for EBV nuclear antigen (EBNA)-2 typing of the virus. The PCR was performed in an automated thermal cycler (DNA engine, MJ Research). The primers were synthesized by Genosys Products. The external (consensus) primers flanked the U2 region of the EBNA-2 gene with 105 bp deletion present in Type-1 (Type-A) virus, hence resulted in a 378bp product, while Type-2 (Type-B) virus gave 483 bp product.

The external primers (Kunimoto *et al.*, 1992):

sense: 5' TTT CAC CAA TAC ATG AAC C 3'

at base positions 2771-2789

antisense: 5' TGG CAA AGT GCT GAG AGC AA 3'

at base position 3149-3130

The internal (nested) primers:

sense: 5' CAA TAC ATG AAC C<GA>G AGT CC 3'

at base position 1777-2796

antisense: 5' AAG TGC TGA GAG CAA GGC <TG>G 3'

at base position 3144-3125

< > denotes nucleotide mix

In brief, the outer set of primers underwent 15 min at 95°C, followed on with 30 cycles of 94°C (1 minute) for denaturation, 51°C (1 minute) for annealing and 72°C (2 minutes) for extension, using 2.5U of HotStarTaq

(QIAGEN, Germany). In the design of PCR on every batch of test cases, purified DNA from cell-lines B95.8 and AG876 were included as positive reference controls, the “extract” from blank tissue block and distilled water (in place of DNA template) added to the reaction mix were used as negative controls. The amplification condition of second run PCR using the inner primers was 30 cycles of 94°C (1 minute) for denaturation, 57°C (1 minute for annealing), 72°C (2 minute) for extension, ending with 72°C for 3 minutes on the last cycle and 8°C on hold. The DNA amplification process was carried out in a 50 µl reaction mixture as shown in Appendix, with the conditions of 1.5 mM MgCl₂, 2.5 U of HotStarTaq, 200 µM of each of the dNTP and 50 pmol of every primer in a standard PCR buffer. The nested PCR products were analyzed by gel electrophoresis, using 2% agarose gel in 1X TAE buffer, against standard molecular weight markers for estimation of the molecular size of the amplified products. 10 µl of the reaction product mixed with 3 µl of bromophenol blue was loaded to the wells. The DNA bands were visualized by ethidium-bromide staining.

Protocol of Nested-PCR For EBV Subtyping from Paraffin Embedded Tissue**Preparation of DNA from Tissue section**

1. A known EBV positive case (by ISH) was selected for this purpose.
2. Microtome and the vicinity were cleaned thoroughly using absolute alcohol
3. 5um paraffin sections were cut and placed in the PCR tube.
4. Tissue was then digested with 1X PCR buffer containing 200ug/ml Proteinase K, overnight at 57°C. Prolong digestion if deemed necessary.
5. Proteinase K was inactivated by heating at 96° C for 10 min.
6. Undigested tissue debris was pelleted at 10, 000 rpm for 10 min.
7. Supernatant was transferred to a new PCR tube and used for PCR

Polymerase Chain Reaction**(a) First Amplification**

1. HotStart procedure was employed as below:

PCR reagents	Volume/ 50 ul reaction	Final Concentration
10X PCR buffer with 15mM MgCl ₂	5 ul	1 X (1.5 mM MgCl ₂)
dNTPs Mix	1 ul	200 uM each dNTP
EBV sense primer, 20µM	1ul	0.5 uM
EBV antisense primer, 20µM	1 ul	0.5 uM
Hot Start Tag Polymerase	0.5 ul	2.5U/50 ul
Template DNA	1µl	
sd H ₂ O	40.5µl	

1st amplification cycling parameter

95° C - 15 min
 94° C - 1 min
 51° C - 1 min
 72° C - 2 min
 72° C - 3 min
 8° C - hold

} 35 cycle

(b) Second Amplification

PCR reagents	Volume/ 50 ul reaction	Final Concentration
10X PCR buffer with 15 mM MgCl ₂	5 ul	1 X (1.5 mM MgCl ₂)
dNTPs Mix	1 ul	200 uM each dNTP
Inner Sense primer, A1 20µM	1 ul	-
Inner antisense primer, A2 20µM	1 ul	-
Hot Start Tag Polymerase	0.5 ul	2.5U/50 ul
Template DNA	1 ul from 1 st PCR product	
sd H ₂ O	40.5µl	

2nd amplification cycling parameter

95° C - 15 min	} 30 cycle
94° C - 1 min	
57° C - 1 min	
72° C - 2 min	
72° C - 3 min	
8° C - hold	

Analysis of PCR Product – Gel Electrophoresis

1. PCR products were analyzed by 2% agarose gel electrophoresis, at ~80 V
2. 100 bp DNA ladder and bromophenol blue loading buffer was used as the indicator
3. Each run of gel consisted of DNA ladder, EBV positive control, negative control (omitting DNA template) and blank (extract from “blank” block).

(ii) *Detection of t(14;18) by PCR*

Detection of t(14;18) by PCR was performed using a nested-PCR described by Gribben *et al.* (1991), which was employed to amplify the major breakpoint region (MBR) of *bcl-2* gene. The 50- μ l reaction mixture containing 2U *HotStarTaq* DNA polymerase (QIAGEN, Germany), 3mM MgCl₂, 200 μ mol/L each of dNTPs (Biotools, Spain), and 0.5 μ M of each primer was subjected to the following conditions: 15 minutes at 95°C to activate the *HotStarTaq* DNA polymerase, followed by 30 cycles of 1 minute at 95°C, 1 minute at 55°C and 1 minute at 72°C. The cycling conditions for both external and nested amplifications are similar. The size of the PCR products ranges from 100bp to 300bp. A cell line with known MBR breakpoint, RL-7 (kindly provided by Dr John C Chan of University of Nebraska Medical Center, USA) was used as a positive control. PCR products were electrophoresised on 2% agarose gel (GIBCO BRL, USA) containing 5 μ g/mL ethidium bromide (GIBCO BRL, USA), and was visualised on a UV illuminator.

The external primers:

J_H external: 5' ACC TGA GGA GAC GGT GAC 3'

MBR external: 5' CAG CCT TGA AAC ATT GAT GG 3'

The internal (nested) primers:

J_H nested: 5' ACC AGG GTC CCT TGG CCC CA 3'

MBR nested: 5' TAT GGT GGT TTG ACC TTT AG 3'

PCR reagents	Volume/ 50 ul reaction	Final Concentration
10X PCR buffer (contain 1.5 mM MgCl ₂ ; QIAGEN, Germany)	5 ul	1 X
50mM MgCl ₂	3 ul	1.5 mM
dNTPs Mix (10mM)	1 ul	200 uM each dNTP
MBR primer (external/nested)	1.25 ul	0.5 uM
J _H primer (external/nested)	1.25 ul	0.5 uM
Hot Start Tag Polymerase	0.4 ul	2.0 U
Template DNA	5 ul	
sd H ₂ O	33.1 µl	

Amplification cycling parameter (for both external and nested PCR)

95° C - 15 min
 94° C - 1 min
 55° C - 1 min
 72° C - 2 min
 72° C - 3 min
 8° C - hold

} 30 cycle

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BIBLIOGRAPHY*Data from Chapter 3*

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Data from Chapter 5

- Title:** Frequent Presence of subtype A virus in Epstein-Barr virus-associated malignancies
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- Title:** Spectrum of Malignant Lymphoma in Queen Elizabeth Hospital, Sabah
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- Title:** Pattern of Epstein-Barr virus Association in Childhood Non-Hodgkin's Lymphoma: Experience of University of Malaya Medical Centre
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