Altered Apoptosis in Cancer-Containing Breast

Original article.

Altered Expression of Anti-Apoptotic Proteins in Non-Involved Tissue from Cancer-Containing Breasts

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Abstract

In a previous investigation reduced apoptosis was identified in normal breast tissue from cancer-containing breasts away from the cancer in comparison to age-matched normal breast from women without cancer. The hypothesis for this study was that defects in expression of apoptotic regulatory and DNA repair proteins would facilitate persistence of genetic alterations and predispose to breast cancer development.

Using immunohistochemistry normal breast from 120 age-matched women (58 with breast cancer, 62 without) was analysed for proliferation, apoptosis, bcl2, BAX, caspase 3, Hsp27, Hsp70, BRCA1, ATM and BARD1. All assessments were performed without knowledge as to whether it was a cancer case or control. A significant difference was found for apoptotic index which was higher in controls ($p<0.02$). There was no change in apoptotic and proliferation index with age for cancer cases unlike controls. Higher expression of bcl2 ($p=0.001$) and Hsp27 ($p=0.001$) in was found in normal breast from cancer-containing breast in comparison to controls. There were no differences in the other proteins.

Apoptosis has been found to be reduced in normal breast in a separate cohort of women with breast cancer, along with increased expression of the anti-apoptotic proteins bcl2 and Hsp27. These alterations in apoptotic regulation would enhance tumour development. Further studies are needed to examine the value of these proteins as risk markers.

Key Words:

Apoptosis; Bcl2; Breast cancer development; Hsp27; Normal breast

Introduction

The development of breast cancer is a complex molecular process with progressive changes from normal to hyperplasia, atypia, in situ carcinoma to invasive carcinoma [1]. That there could be progression from benign proliferative hyperplasia and atypical hyperplasia to carcinoma comes from two lines of evidence: the finding of an increased incidence of hyperplasia and atypical lesions in cancer-containing breasts [2-4]; the increased risk of developing breast cancer if various histological changes are present in benign biopsies, with the risk increasing with atypia [5-11]. Alpers and Welling [4] proposed that the factors promoting the development of carcinomas had a field effect. The concept of field cancerisation, introduced in 1953, proposed that cumulative genetic alterations resulting from long term exposure to carcinogens greatly increases the propensity for tumour development [12] and recent molecular findings support this model [13].

Breast carcinomas arise from terminal ductal lobular units [3]. These normal breast structures will, therefore, be the site of initial genetic alterations. If there are molecular and phenotypic differences in normal breast lobules in breasts in which a cancer has developed then this could indicate that there is an endogenous predisposition resulting in sporadic breast cancer and/or that carcinogens have a low level widespread effect, with greater localised effects resulting in cancer development.

Genetic changes have been found in normal breast adjacent to breast cancers, distant from the cancers and in women without breast cancer [14-20], although the genetic abnormalities found in normal cells show little or no overlap with those of neoplastic lesions [16, 17]. The

changes may indicate defects in the mechanisms for maintaining genetic integrity, leading to increased risk [1, 20].

In previous studies from our group differences have been identified in normal tissue of cancer- containing breasts (NTCCB) in the expression of proteins involved in the regulation of growth and apoptosis [21-23], along with a reduction in apoptosis [24]. This could lead to the preservation of genetically aberrant cells. In the present study a separate series of normal breast tissue from cancer-containing breasts and from breasts without cancer has been examined for the expression of apoptotic regulatory and DNA repair proteins, along with cell proliferation and apoptosis. Our hypothesis is that defects in these response mechanisms would facilitate persistence of genetic alterations, so predisposing to breast cancer development. The presence of any changes could lead to the identification of new markers of susceptibility.

Materials and Methods

Patients

A total of 120 patients were studied, with 107 being analysed for all markers. They comprised 58 women with breast cancer (cases) and 62 with no evidence of cancer (controls) (55 and 52 studied for all markers, respectively). They were stratified into three age groups: ≤ 39 years, 40-49 years, ≥ 50 years, with relatively equal numbers of cases and controls in each group. None of the cancer patients had received chemotherapy, endocrine therapy or radiotherapy prior to surgery. The control group comprised women having reduction mammoplasties or excision of histologically benign lesions and had no history or evidence of

breast cancer. Neither group had a strong family history of breast cancer. Ethical approval was given by Leicestershire, Northamptonshire and Rutland Ethical Committee (reference LREC 6927).

Tissues

Specimens were received fresh within 30 minutes of excision, sliced and fixed in 4% formaldehyde. Samples from the cancer-containing breasts were taken 4cm or more away from the cancer. Tissues were fixed for 18-30 hours, and processed to paraffin wax using standard protocols. Each sample for analysis was selected on the basis of an adequate number of normal lobules with no evidence of hyperplasia or atypia assessed using haematoxylin and eosin stained sections.

Antibodies

All primary antibodies with their supplier and dilutions used in immunohistochemistry are shown in Table 1. Secondary antibodies and tertiary reagents were from Dako Ltd, UK. Chemicals were from Sigma-Aldrich, UK.

Table 1.

Immunohistochemistry

MIB1 antibody against the Ki-67 antigen was used to assess proliferation and M30 antibody against modified cytokeratin 18 was employed to identify apoptotic cells, both by standard

immunohistochemistry. ATM, BARD1, BRCA1, Hsp27, Hsp70, Bcl2, BAX and Caspase 3 proteins were detected using a standard immunohistochemical technique. Antigen retrieval was by pressure-cooking in 10mM citrate buffer, pH 6.0. Sections were incubated with the relevant primary antibody (Table 1), followed by either biotinylated anti rabbit or mouse immunoglobulin antibody, then StreptABC/HRP complex. Visualisation was by 3, 3'-diaminobenzidine-hydrogen peroxide solution and sections were counterstained using Mayer's haematoxylin.

Controls

The same sample of normal breast tissue from a reduction mammoplasty was included for each antibody for all immunohistochemical staining as a positive control. Negative controls were the substitution of the primary antibody by non-immune serum.

Assessment

All tissues were assessed (AJB, AN G-W) without knowledge as to whether it was from a cancer case or a control, and of the age of the patient.

For proliferation 1000 nuclei of luminal epithelium were counted from at least 6 different normal lobules; Ki-67 positivity was defined as moderate or strong staining. The proliferative index (PI) was the percentage of positive cells. Apoptotic index (AI) was the percentage of cells with strong cytoplasmic reactivity with the M30 antibody, and was counted the same as PI.

For assessment of all other proteins ten normal lobules were identified for each sample and graded according to the extent and intensity of staining. The positive control for each batch of staining was used as the reference point for assessing intensity. Intensity was scored 0 (no staining), 1 (weak), 2 (moderate), 3 (strong). Extent of staining was categorised by proportion (0-25% 0.25; 26-50% 0.50; 51-75% 0.75; 76-100% 1.00). An index for each lobule, between 0 and 3, was generated by multiplying these two scores, and the mean of the 10 lobules gave the index for that tissue.

When all tissues had been analysed the data were linked to the clinical information for statistical analysis.

Statistical analysis

Comparison of cases verses controls was achieved using the non-parametric Mann-Whitney U test. Data relating to the three different age groups was analysed using the Kruskal-Wallis test. Statistical analysis was performed using SPSS 12.01 for Windows.

Results

The correlation coefficient for intra-observer variability for assessment of all markers was between 0.95 and 0.99.

Proliferation

There was a large variance in PI between all samples, with both cases and controls having no detectable proliferation. The range for the control group was greater (0-46%) than the cases

(0-23%) but the median PI was higher for cases than controls (2.2% and 1.55% respectively) (Table 2) although this was not significant ($p=0.79$). The PI of controls showed a significant decline with age (4.5%, 2.9% and 0.8% for the ≤ 39 years, 40-49 years and ≥ 50 years age groups respectively, $p=0.02$). PI for the cases showed no such trend (Table 2).

Apoptosis

The range of AI was similar for both cases and controls but there was a significant difference in the median AI of cancer containing breasts when compared with the controls, the latter having a higher AI ($p=0.043$) (Table 2). Analysis of individual age groups showed an increase in AI with age in controls, although not significant, but no increase with age for the cases (Table 2).

Table 2

Apoptotic Proteins

Cytoplasmic staining was seen in the luminal epithelium for Bcl2, BAX and caspase 3. All tissues showed variations in intensity of staining (Table 3). For all ages combined, the median score for staining for Bcl2 was higher in the case group compared to the control group (2.13 and 1.53 respectively) (Figure 1). This difference was highly statistically significant with a p value of 0.001. When different age groups were considered there was a significant difference between cases and controls for the 40-49 years ($p=0.01$) and ≥ 50 years ($p=0.04$).

Fig1

Expression of BAX was higher in the case group compared to controls, both overall and for the different age groups, although this difference was not statistically significant. There were no differences in the median index for caspase 3 for cases (1.83) and controls (1.84) and no differences in relation to age (Table 3).

DNA Repair and Heat Shock Proteins

Nuclear staining was seen for BRCA1, BARD1 and ATM. There was partly cytoplasmic and partly nuclear staining for Hsp 70, and cytoplasmic reactivity for Hsp 27.

The median index for BRCA1 was slightly higher in normal tissue from cancer-containing breast, with the difference greater in the young age group but this was not significant. Similarly ATM had a slightly higher staining index in cases, more so in the < 50 year olds but this was not significant. Staining was less for BARD1 in the case group, but was not significant.

Differences were found for Hsp 27 with greater staining in normal tissue from cancer-containing breasts (median 1.37) compared to normal tissue from women without cancer (median 0.80), which was highly significant ($p=0.001$) (Figure 2). The difference was most marked in women in the youngest age group. Hsp 70 had a slightly lower staining index in the cases but this was not significant.

Table 3

Figure 2

Discussion

This study has confirmed our previous findings [24] that there is a significantly lower level of apoptosis in the non-involved normal tissue of cancer-containing breasts in comparison to age-matched breast tissue from women without cancer. The present study was of a different cohort and used a different method to identify apoptosis (antibody against cleaved cytokeratin 18 as compared to *in-situ* end labelling). The differences in apoptotic index were present for all age groups but were greater in the ≥ 50 year old group. Allan *et al* [25] also found a reduced apoptotic index in breasts from women with fibrocystic change and carcinomas, but for the premenopausal age range.

In contrast there was no significant difference in the proliferative indices for the two groups, although interestingly there was no decline in proliferation with age in the cancer-containing breasts, whilst there was a significant decline in the control group. Information about menstrual cycle and the use of oral contraceptives/hormone replacement therapy was not available but this applies to all tissues studied. The continued proliferation with age in the cancer-containing breasts suggests that there is altered sensitivity to, or increased synthesis of growth promoting factors in those breasts. In normal breast Ki-67 positive cells and estrogen receptor (ER) positive cells are mutually exclusive [26]. The percentage of Ki-67 positive/ER α positive cells is significantly increased in proliferative lesions and correlates with the level of risk [27] in normal breast from cancerous and non-cancerous breasts. In a previous study [21] we found no differences in the expression of ER α between cancer-containing and non-cancerous breasts. Therefore, the reduced apoptosis and continued proliferation with age may be independent of expression of ER.

In order to evaluate the reduced apoptosis in cancer-containing breasts the expression of apoptotic regulatory proteins was examined. A significant difference was found in expression of the anti-apoptotic protein bcl-2, with higher levels in the cancer-containing breast, but no differences in the pro-apoptotic protein bax and in the effector caspase 3. Bcl-2 acts by promoting cell survival; the balance between pro-survival proteins and their BH3 ligands regulates tissue homeostasis [28]. Over-expression of a pro-survival protein such as bcl-2 can impair apoptosis and hence be critical in tumour development [28]. Gain of bcl-2 has been shown to be more potent than loss of bax in regulating mouse mammary epithelial cell survival [29]. There is cyclical variation in bcl-2 expression in normal breast, with maximal expression at the end of the follicular phase [30]. In vitro, oestradiol can decrease apoptosis in human breast epithelial cells [31]. Bcl-2 is induced in ER positive breast cancer cells by oestradiol [31] and in breast carcinomas there is a significant association between expression of bcl-2 and presence of ER [32]. These various lines of evidence point to the importance of hormonal regulation of bcl-2. The mechanisms leading to higher levels of bcl-2 in cancer-containing breasts is unclear but may relate to response of cells to oestradiol. Increased bcl-2 expression would reduce apoptosis, as found, so allowing DNA damage to persist.

Significant differences were also found for the expression of the heat shock protein Hsp 27, with higher levels in cancer-containing breasts. Hsp 27 is constitutively expressed at low levels and is induced following exposure of cells to environmental and physiological stresses [33]. Hsp 27 has also been shown to have an anti-apoptotic role [34]. It can increase cell survival in response to apoptotic stimuli, activate protein kinase B, an apoptotic inhibitor

[34], and inhibit caspase activation [35]. Therefore, increased expression of Hsp 27 confers a survival advantage to cells and may contribute to the reduction in apoptosis identified. O'Neill et al [36] found an increase in expression in Hsp 27 that related to increased risk of malignant progression of breast lesions. Expression was related to ER α expression. They proposed that differential expression of Hsp 27 modulated the phenotype of epithelial cells and may be an important determinant in initiating or promoting a population of human mammary cancers. Our findings support this hypothesis.

If there is altered expression of DNA repair proteins in breasts in which a cancer develops then this, along with the impaired apoptosis, would contribute towards the development of breast cancers. Decreased protein expression of BRCA1 [37, 38] and ATM [39, 40] has been found in sporadic breast cancers. Reports on expression of these proteins in normal breast are limited. Both BRCA1 and ATM can be identified in the nuclei of normal breast lobules [38, 41]. Conflicting differences have been found in expression between myoepithelial and luminal epithelial cells for ATM in benign lesions [41, 42]. In the present study no significant differences were found for BRCA1, ATM and BARD1 immunoreactivity between normal breast from cancer-containing breasts and non-cancerous breasts. Genomic instability has been reported to be present at a higher frequency in cancer-containing breasts [19, 20] and hypermethylation, which can modify gene expression, of genes involved in growth regulation has been found [18, 43]. A higher frequency of hypermethylation is found in benign lesions [reviewed in 44]. Other approaches may, therefore, be needed to assess whether there are alterations in the DNA repair machinery.

In conclusion, this study has found, in a separate cohort of women, that normal breast from women with breast cancer has reduced apoptosis and that there is significantly higher expression of anti-apoptotic proteins bcl-2 and HSp27. The alterations in apoptotic regulation would enhance tumour development. Further studies are required to examine the value of these proteins as risk markers within larger populations.

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Figures

Figure 1

Box plot showing staining index for bcl 2 for cases and controls in relation to different age groups.

Figure 2

Box plot showing staining index for HSp27 for cases and controls in relation to different age groups.

Table 1**Antibodies used in the study with supplier and dilutions used for immunohistochemistry**

Antibody	Code	Supplier	Dilution
ATM Rabbit polyclonal	OBT 4132	Oxford Biotechnology Limited, UK	1:1000
BARD1 rabbit polyclonal	AHP 874	Serotec Limited, UK	1:200
BRCA1 mouse monoclonal	MS 110	Calbiochem, UK	1:100
HSP27 mouse monoclonal	NCL-HSP 27	Novocastra Limited, UK	1:15
HSP70 mouse monoclonal	NCL-HSP 70	Novocastra Limited, UK	1:30
Bcl-2 mouse monoclonal	NCL-Bcl-2	Novocastra Limited, UK	1:80
BAX rabbit polyclonal	A 3533	Dako Limited, UK	1:30
Caspase 3 mouse monoclonal	CPP-32	Novocastra Limited, UK	1:40
Ki-67 mouse monoclonal	NCL -Ki67 -MM2	Novocastra Limited, UK	1:100
M30 CytoDEATH mouse monoclonal	2 140 349	Roche Molecular Biochemicals Limited	1:100

Table 2**Apoptotic and proliferative indices for case and control tissues**

		Apoptosis		Proliferation	
Age		Range (%)	Median (%)	Range (%)	Median (%)
≤39	Cancer containing breast	0-1	0.23	0-19	2.4
	Control	0-2.5	0.39	0-46	4.5
40-49	Cancer containing breast	0-1.04	0.4	0-23	4.2
	Control	0-1.7	0.48	0-14	2.9
≥50	Cancer containing breast	0-2.8	0.2	0-13	1.1
	Control	0-2.9	0.83	0-22.4	0.8
Overall	Cancer containing breast	0-2.8	0.29	0-23	2.2
	Control	0-2.9	0.47	0-46	1.55

Table 3

Intensity of immunohistochemical staining for case and control groups for all ages

Age Group		ATM	BARD1	BAX	BCL2	BRCA1	HSP27	HSP70	CASPASE 3
≤39 years	Controls	2.67	2.20	1.75	1.48	1.73	0.64	2.21	1.55
	Cases	2.79	2.11	1.83	1.80	1.91	1.46	2.30	1.50
	p value	0.09	0.88	0.65	0.45	0.27	0.01	0.54	0.60
40-49 years	Controls	2.68	2.12	1.72	1.62	1.89	0.92	2.46	1.90
	Cases	2.75	2.27	2.35	2.23	1.88	1.20	2.39	1.50
	p value	0.61	0.48	0.78	0.01	0.56	0.21	0.61	0.45
≥50 years	Controls	2.73	2.18	1.54	1.62	1.84	0.82	2.47	1.90
	Cases	2.65	2.06	1.75	2.30	1.85	1.33	2.31	1.70
	p value	0.36	0.11	0.24	0.04	0.98	0.06	0.49	0.35
All ages	Controls	2.70	2.18	1.70	1.53	1.84	0.80	2.38	1.84
	Cases	2.72	2.11	2.00	2.13	1.88	1.37	2.33	1.83
	p value	0.32	0.58	0.098	0.001	0.37	0.001	0.75	0.49