ORIGINAL ARTICLE

Inverse Correlation between P53 and Bcl-2 Expression in Breast Carcinoma of Malaysian Patients

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ABSTRAK

Gen penindas p53 dan proto-onkogen Bcl-2 mengkodkan fosfoprotin nuklear dan protin membran mitokondria yang terlibat dalam pelbagai fungsi sel. Kedua-dua protin mempunyai hubung kait dengan tapak jalan kematian sel berprogram disamping memberi informasi prognostik karsinoma payudara. Objektif kajian ini adalah untuk menentukan kaitan onko-protin p53 dan Bcl-2 pada karsinoma payudara dan hubungannya dengan umur pesakit, saiz tumor, peringkat kanser dan gred histologi. Lima puluh sembilan kes kanser payudara dari Pusat Perubatan Universiti Kebangsaan Malaysia (PPUKM) telah dikaji menggunakan teknik imunohistokimia. Keputusan menunjukkan 45.8% (27/59) kanser payudara adalah imunopositif untuk p53 dan 40.7% (24/59) adalah imunopositif untuk Bcl-2. Terdapat korelasi signifikan di antara ekspresi Bcl-2 dengan peringkat awal kanser (p=0.01). Variabel lain tidak menunjukkan kaitan signifikan. Kaitan songsang didapati di antara p53 dan Bcl-2 (p=0.001). Hasil keputusan mencadangkan regulasi menurun Bcl-2 oleh p53 dalam karsinogenesis kanser payudara.

Kata kunci: korelasi songsang, p53,Bcl-2, karsinoma payudara, regulasi menurun

ABSTRACT

The tumour suppressor gene p53 and the proto-oncogene Bcl-2 encode respectively, for a nuclear phosphoprotein and for a mitochondrial membrane protein involved in multiple cellular functions. Both proteins are linked to programmed cell death pathways and provide prognostic information on breast carcinoma. Our aim is to study the expression of p53 and Bcl-2 oncoproteins in breast carcinoma and correlate with patients' age, tumour size, disease stage and histological grade. Fifty nine cases of breast carcinomas from Universiti Kebangsaan Malaysia Medical Centre (UKMMC) were studied with the immunohistochemical method. Our results

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showed 45.8% (27 of 59) and 40.7% (24 of 59) of the breast carcinomas were immunopositive for p53 and Bcl-2 respectively. There was significant correlation between Bcl-2 expression with early tumour stage (p=0.01). No significant relationship was seen with other variables. Results also showed an inverse relationship between p53 and Bcl-2 expression (p=0.001). These findings indicate a down regulation of Bcl-2 by p53 in breast carcinogenesis.

Keywords: inverse correlation, p53,Bcl-2, breast carcinoma, downregulation

INTRODUCTION

In Malaysia, breast cancer is the commonest cancer in all ethnic groups, accounting for 30.4% of newly diagnosed cancer cases in Malaysian women (Lim et al. 2003). The incidence of breast carcinoma increases from year to year and the risk rises with increasing age.

Until now, the true aetiology of breast carcinoma is still unknown. However, studies have shown that it is a multifactorial and multigenic disease. Genetic factors are one of the risk factors that have been identified or believed to cause breast carcinoma. Several studies have been done to identify the role of biologic tumour markers in predicting response towards treatment of breast carcinoma. Of all the biologic tumour markers that have been identified, the relationship between p53 and bcl-2 protein expression has become the focus of attention.

Breast carcinogenesis involves at least one initiation stage and promotion or progression stage. It can be associated with epigenetic alterations and genetic response which controls gene regulation. Among these alterations, deactivation of p53 gene is considered the most common genetic change (Schmitt et al. 1995).

The p53 gene is a tumour suppressor gene which is located on the short arm of chromosome 17 at 17p13.1. It encodes the 53kDa nuclear phosphoprotein expressed by normal cells and contains many important biological functions. This includes growth and cellular transformation, cell cycle and genomic stability. It also regulates apoptosis which is important after DNA damage. Abnormalities identified in the p53 tumour suppressor gene are some of the molecular incidences seen in human and animal neoplasms (Hainaut et al. 1998). P53 is involved in regulation of cell proliferation, directing apoptosis and promoting chromosomal stability. Any disturbances in these functions play an important role in carcinogenesis (Levine 1997). Changes in the p53 gene cause it to lose its functions as a cell growth regulator.

Somatic mutation of p53 is one of the molecular abnormalities which is frequently seen in carcinoma (Coles et al. 1992; Osborne et al. 1991). Previous studies have shown that somatic gene mutation of p53 was present in 30% to 50% of breast carcinoma. It has been proven that protein overexpression of mutant p53 gene is associated with a poor response towards chemotherapy and poor prognosis in patients with

breast carcinoma (Davidoff et al. 1991; Kovach et al. 1996; Isola et al. 1992; Silvestrini et al. 1993; Thor et al. 1992). Bcl-2 is a human proto-oncogene and is located on chromosome 18 at 18q21. Bcl-2 produces the membrane integral protein which is located on the endoplasmic reticulum membrane, nuclear envelope and outer mitochondrial membrane. The location of Bcl-2 suggests that it may be involved in some of the pathways of metabolic function of mitochondrial membrane (Hockenbery et al. 1990).

Bcl-2 was first discovered in follicular and diffuse lymphomas possessing t(14;18) chromosomal translocation and has been established as a key regulator of apoptosis (Kroemer 1997). A decreased expression of Bcl-2 protein was shown to be associated with a poor clinical outcome in breast cancer (Silvestrini et al. 1994; Joensuu et al. 1994; Gasparini et al. 1995).

An inverse relationship between p53 and Bcl-2 has been shown in primary breast carcinomas, and the two oncoproteins may interact in the regulation of apoptosis (Silvestrini et al. 1994; Gasparini et al. 1995; Barbareschi et al. 1996). Recently, p53 has been shown to be inactivated by alterations other than mutation of TP53 itself (Abdel-Fatah et al. 2010). More importantly, completely inactive p53 pathway exhibited unfavorable clinicopathologic features, bad prognosis and poor outcome despite receiving systemic adjuvant therapy (Abdel-Fatah et al. 2010). However, studies investigating the relationship between p53 and Bcl-2 have mainly been in breast cancers involving the western population. To date, there have been no studies performed on the significance of p53 and Bcl-2 expression in breast carcinomas in Malaysian women.

Based on these observations, we aim to study the expression of p53 and one of its downstream genes, Bcl-2 and its role in tumorigenesis of breast carcinomas in Malaysian patients. We also aim to correlate the findings with other prognostic variables and clinicopathological data.

MATERIALS AND METHODS

Breast Cancer Specimens

This is a retrospective study utilising paraffin embedded tissue (PET) blocks from 59 cases of breast carcinomas (53 infiltrating ductal carcinoma, 2 ductal carcinoma in situ, 2 mucinous carcinoma, 1 squamous cell carcinoma, 1 papillary carcinoma) at Universiti Kebangsaan Malaysia Medical Centre (UKMMC). This study was approved by the Ethics Committee of UKMMC. Clinical data of the patients were obtained from the Medical Records Unit, UKMMC. Only cases with complete medical data were selected. Haematoxylin and eosin stained slides from the tumour of each of the cases were reviewed. Sequential sections for further immunohistochemical studies were determined after selection of the appropriate tumour section.

Immunohistochemical Study

Three μm thick sequential sections from PET blocks were cut and placed

on poly-L lysine coated slides. The slides were then placed in an oven for 30 minutes at 55°C. The tissue sections were then dewaxed in xylene and rinsed in graded alcohol. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Subsequently the slides were rinsed in running water before being subjected to Target Retrieval Solution for 20 minutes at 97°C. For p53 staining, a Target Retrieval Solution of normal pH was used while a high pH was used for Bcl-2 staining. The slides were then allowed to cool for 20 minutes and washed in TBS. The primary antibody is then dropped onto the tissue sections before being incubated for 30 to 60 minutes, followed by a rinse in TBS. The slides are then incubated for 15 minutes with link antibody. Subsequently streptavidin and peroxidase were applied for 15 minutes. The immunoprecipitate was visualized by treating with Diaminobenzidine (DAB) and counterstaining with haematoxylin.

Controls

Known p53 and Bcl-2 positive breast carcinomas were used as positive controls for p53 and Bcl-2 respectively. Negative controls were run with each batch by replacing the primary antibody with normal saline.

Interpretation of Results and Staining Characteristics

Each case was assessed by two observers without the knowledge of patient's clinical data. The tumour cells showed positive nuclear staining with p53. Cases were considered positive for each marker when at least 5% of the cells showed positive staining with the relevant antibody (Poller et al. 1993).

Positive Bcl-2 staining was cytoplasmic and tissue sections showing less than 10% of the cells with positive staining were considered negative (Simon & Altman 1994).

Statistical Analysis

The relationship between Bcl-2 and p53 immunostaining, and with other clinicopathological variables were evaluated using the X^2-test. Statistical procedures were performed using the SPSS software version 10.0.1. The statistical significance was defined as $p<0.01$.

RESULTS

Twenty seven of 59 (45.7%) of the cases showed p53 immunopositivity (Table 1, Figure 1). Heterogenous and homogenous distribution pattern were seen with p53 nuclear staining. Eleven out of 27 cases showed heterogenous distribution of p53 immunostaining while 16 cases showed homogenous distribution pattern. Both nuclear and cytoplasmic staining were seen in four cases.

Twenty four of 59 (40.7%) of the cases showed Bcl-2 immunopositivity (Table 2, Figure 2). The distribution pattern for Bcl-2 immunostaining was mostly homogenous. However, five out of 24 cases showed heterogeneous distribution pattern of Bcl-2 immunostaining. Bcl-2 immunostaining was also present in the normal breast epithelial cells adjacent to the tumour cells in five cases.

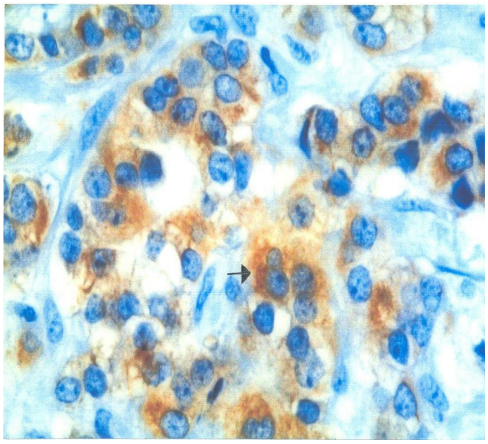

Figure 1. Histological section of IDC showed immunopositivity for Bcl-2 in the cytoplasm (magnification x400)

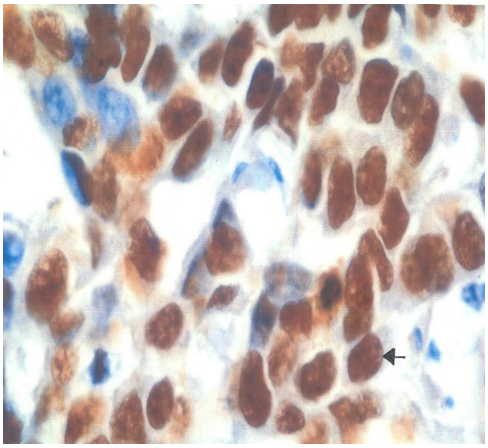

Figure 2. Histological section of IDC showed strong immunopositivity for p53 in the nucleus (magnification x400)

Table 1: Frequency of p53 expression in breast carcinoma (n=59)

P53 expression	Frequency (%)
Positive	27 (45.8)

Table 2: Frequency of Bcl-2 expression in breast carcinoma (n=59)

Bcl-2 expression	Frequency (%)
Positive	24 (40.7)
Negative	35 (59.3)

Table 3: p53 and Bcl-2 expressions in Breast Carcinoma (n=59)

Bcl-2 expression	P53 expression		P
	Negative	Positive	
Negative	13 (22%)	22 (37.3%)	0.001
Positive	19 (32.2%)	5 (8.5%)	0.001

Results also showed a significant inverse relationship between p53 and Bcl-2 protein expressions in the tumours studied (X^2=10.130[b], df=2, p=0.001). Thirteen of 59 (22%) cases are immunonegative for both p53 and Bcl-2. Only five cases were immunopositive for both p53 and Bcl-2 proteins. Twenty two cases (37.3%) were immunopositive for p53 and immunonegative for Bcl-2. Nineteen cases (32.2%) were immunopositive for Bcl-2 and immunonegative for p53 (Table 3). Neither p53 nor Bcl-2 expression showed any significant correlation with tumour size, age and histologic grade (Table 4).

There was no association seen between p53 expression and individual disease stage (Table 5). On the contrary, a significant association was found between Bcl-2 expression and early stage of disease (Stage 0, I, II). Six of seven (85.7%) patients with early stage disease showed Bcl-2 immunopositivity (p=0.01) while only 34.6% of patients with late stage disease (stage III, IV) showed Bcl-2 immunopositivity (Table 6).

DISCUSSION

p53 and Bcl-2 are known to be involved in the regulation of apoptosis and have

Table 4: Correlation between p53 and Bcl-2 expressions and clinicopathologic characteristics of breast carcinoma

Clinicopathologic characteristics	No of Bcl-2 positive cases (%)	P	No of p53- positive cases (%)	P
Age (yr)				
<50	12 (36.3)	0.447	16 (48.4)	0.636
>50	12 (46.1)		11 (42.3)	
Tumour size (cm)				
< 2	3 (50)	0.717	5 (83.3)	0.06
>2	16 (53.3)		16 (42.1)	
Histologic Grade				
Grade 1	8 (57.1)	0.286	5 (35.7)	0.531
Grade 2	5 (33.3)		7 (46.7)	
Grade 3	5 (31.3)		9 (56.3)	

Table 5: P53 expression with Stage of Disease (n=59)

Stage of Disease	P53 expression	
	Negative	Positive
Early (0, I, II)	5 (71.4%)	2 (28.6%)
Late (III, IV)	27 (51.9%)	25 (48.1%)

p=0.331

Table 6: Bcl-2 expression with Stage of Disease (n=59)

Stage of Disease	Bcl-2 expression	
	Negative	Positive
Early (0, I, II)	1 (14.3%)	6 (85.7%)
Late (III, IV)	34 (65.4%)	18 (34.6%)

p=0.01

been investigated widely in neoplasms (Silvestrini et al. 1994; Watson et al. 1996). The loss of p53 function represents the most common genetic change in human neoplasms. Experimental studies have demonstrated Bcl-2 to be implicated in regulating the cell cycle and proliferation (Watson et al. 1996; Bonnefoy-Berard et al. 2004). Both p53 and Bcl-2 are expressed in breast cancer and have been shown to associate with a series of clinicopathological parameters (Watson et al. 1996). However, the relationship between p53 and Bcl-2 expression and their role in breast tumorigenesis in Malaysian patients has not been reported.

In this present study, there is an overexpression of p53 in 45.8% of the cases. This is consistent with the findings of other previous studies for which the range was between 16% to 58% (Davidoff et al. 1991; Isola et al. 1992; Thor et al. 1992; Bartek et al. 1990; Molina et al. 1998; Allred et al. 1993). The variation of p53 expression in the different studies is probably attributed by the number of samples studied, preferred methodology and the types of antibody that was used.

Studies of p53 expression on ductal carcinoma in situ (DCIS) are limited (Davidoff et al. 1991; Thor et al. 1992; Poller et al. 1993; Walker et al. 1991). In our study, one of the two DCIS cases showed positive expression of p53. This finding is in keeping with previous studies and supports the idea

that abnormal p53 protein is implicated in the early development of breast carcinomas (Walker et al. 1991).

We also found that Bcl-2 expression was present in 40.7% of the cases. This finding is in keeping with the findings of other previous studies for which the range of expression varies between 38.7% and 81.2% (Joensuu et al. 1994; Charpin et al. 1998; Berardo et al. 1998; Coppola et al. 1999). The variation of Bcl-2 expression in the different studies is probably attributed by tissue sampling (paraffin or frozen section), method of analysis, and the positive cut- off point. In this study, Bcl-2 expression was identified in almost all of the normal breast epithelia adjacent to the tumours. This suggests that Bcl-2 expression is required to protect normal breast epithelial cells from apoptosis and ensure the survival of the breast epithelium (Zhang et al. 1997).

Both of the DCIS cases in our study had shown Bcl-2 expression whereas only 35.8% of the invasive breast carcinomas had shown Bcl-2 expression. A significant decrease in Bcl-2 expression was observed during the evolution from normal breast epithelial cells to ductal carcinoma in situ (DCIS) and from DCIS to invasive breast carcinomas. This present result indicates that the reduction in Bcl-2 expression is associated with carcinogenesis and progression of breast carcinoma. This phenomenon was also reported in other studies (Zhang et al. 1997; Leek et al. 1994). The down regulation of Bcl-2 is necessary to induce apoptosis of cancer cells for the maintenance of increased cell turnover and the balance between tumour growth and apoptosis. Although we could not find any significant correlation between Bcl-2 expression and DCIS due to the limited number of DCIS cases studied, our findings suggest that DCIS may share smilar molecular pathways to that of an invasive carcinoma. Of note, the lack of Bcl-2 expression was seen more in recurrent DCIS (Provenzona et al. 2003), suggesting the role of Bcl-2 in the progression and malignant transformation of DCIS.

The present study had demonstrated a significant inverse correlation between p53 and Bcl-2 expressions in breast carcinoma. A significant inverse correlation was also demonstrated in other studies (Kroemer 1997; Silvestrini et al. 1994; Joensuu et al. 1994; Gasparini et al. 1995; Barbareschi et al. 1996) while no correlation was found in others (Bonnefoy-Berard et al. 2004; Nohara et al. 2001; Lau et al. 2001; Sgambato et al. 2000). Haldar and colleagues had shown that the overexpression of mutant p53 in breast cancer (MCF-7) cell line induced the down regulation of Bcl-2 at both the protein and mRNA levels (Haldar et al. 1994).

We have also demonstrated that 37% of p53 immunopositive breast cancer were Bcl-2 immunonegative. In addition, we have also found that lack of Bcl-2 expression was also more associated with tumours of late stage. A combination of p53 overexpression and lack of Bcl-2 expression was more likely to be seen in aggressive tumours with reduced five year survival (Rolland et al. 2007).

In this study, there is a significant correlation between Bcl-2 expression and early tumour stage of the disease

in which the tumours tend to be better differentiated. A relative reduction of Bcl-2 expression in the late stages of breast carcinoma suggests a downregulation of Bcl-2 with tumour development. This finding indicates that Bcl-2 expression in early breast carcinoma is associated with a good prognosis. Our results are in accordance with a study done by Leek et al. (1994) who had reported that loss of Bcl-2 expression was associated with poor outcome and that its expression was associated with improved survival (Berardo et al. 1998, Callagy et al. 2006). Bcl-2 has also been shown to be a powerful independent prognostic marker for patients with early breast cancer independent of adjuvant therapy (Dawson et al. 2010).

CONCLUSION

The present study demonstrated an inverse relationship between p53 and Bcl-2 protein expressions indicating that overexpression of mutant p53 induces downregulation of Bcl-2 in breast carcinogenesis. Our results also demonstrated that Bcl-2 is expressed in the early stages of breast cancer. The combined Bcl-2 overexpression and loss of p53 expression are useful markers in predicting good prognostic outcome in patients with breast cancer.

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