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ARTICLE

# Bax Protein Expression in DCIS of the Breast in Relation to Invasive Ductal Carcinoma and Other Molecular Markers

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**This study describes the incidence of Bax protein expression in a series of 106 cases of breast cancer including 56 cases of ductal carcinoma in situ (DCIS) and 50 cases of invasive ductal carcinoma (IDC). Relationships of Bax expression to the histological grades of DCIS & IDC, and to the expression of Ki67, ER, p53, cerbB2 & Bcl2 are described. The expression of Bax, Ki67, ER, p53, cerbB2 and Bcl2 proteins is determined immunohistochemically. Cases were regarded positive for Bax, Bcl2 and cerbB2 when they showed either moderate or strong staining for these markers. The nuclear stains (Ki67, ER, and p53) were quantified in terms of percentage positive cells and cases for ER and p53 were considered positive when more than 10% cells were labelled. DCIS were graded histologically as well (n=18), intermediately (n=18), and poorly differentiated (n=20) Invasive ductal carcinoma was graded as grade I (well-differentiated) n=7, grade II (intermediate) n=24 and grade III (poorly differentiated) n=19. 65/106 cases (61%) were Bax positive including 37/56 (66%) of DCIS and 28/50 (56%) of IDC. Bax expression did not correlate to increasing histological grades of either DCIS or IDC. It did not correlate to Ki67, ER, p53 or cerbB2 but positive correlation was seen with Bcl2 (p= 0.003). Bcl2 immunostaining displayed a negative correlation with increasing histological grades both of DCIS and IDC (p=0.026), (p=0.041) respectively. There was a trend of negative correlation of Bcl2 with Ki67 (p=0.062). It correlated positively with Bax (p=0.003) and ER (p<0.0001). Results suggest that the regulation of apoptosis is important in ductal carcinoma in situ of the breast as well as invasive ductal carcinomas. Bcl2 is associated with good prognostic markers in both DCIS and IDC, whereas the regulation of Bax is complex and does not necessarily correlate with mutant p53.** (Pathology Oncology Research Vol 6, No 4, 256–263, 2000)

*Keywords:* breast cancer, ductal carcinoma in situ, Bax, Ki67, oestrogen receptor, p53, cErbB2, Bcl2

## *Introduction*

Bax is a 21 kD protein with extensive amino acid homology with Bcl2.[1,2] The protein is encoded by six exons and has been shown to undergo alternative splicing leading to at least two cytoplasmic forms.[1,3] Bax has been shown to form heterodimers with Bcl2 and the ratio of Bcl2 to Bax determines the survival or death of cells following an apoptotic stimulus such as removal of growth factor.[1,4]Stimulation of Bax synthesis also appears to be a result of wild type but not mutant p53 activity.[5] More recently, it has been suggested that dysregulation of apoptosis due to imbalances in Bax/Bcl2 levels may contribute to the pathogenesis of breast cancer.[3] Bax has been suggested as a good prognostic marker in node negative breast cancer.[6] There is little information available on the significance of Bax expression in DCIS and its various histological grades in comparison to IDC and its histological grades.

The Bcl2 gene, located on chromosome 18 (18q21), encodes a 26kD protein, which appears to play a key role in cell regulation by inhibiting apoptosis. Abnormalities of the Bcl2 gene were first discovered in human follicular lymphoma in which the gene is translocated to the immunoglobulin heavy chain locus of chromosome 14.[7] Expression of the Bcl2 protein product has been documented in a variety of normal human tissues including breast epithelium.[8,9] Bcl-2 protein is also expressed in invasive

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breast carcinoma and is associated with well-differentiated tumors and positive oestrogen receptor (ER) status.[10-13]

Abnormalities of the p53 tumor suppressor gene, which also plays a role in cell regulation[14] are common in all forms of cancer.[15,16] Recent studies have demonstrated an inverse relationship between p53 and Bcl2 protein expression in breast cancer and other solid tumors.[17-19] The altered expression of p53 in breast carcinomas is associated with high grade, ER negative tumors and been reported to be prognostically significant in breast carcinomas.[20]

The oncogene cerbB2 (HER2/neu) is located on chromosome 17q21 and encodes for a 185 kD membrane protein with tyrosine kinase activity,[22] which has a certain homology to epidermal growth factor receptor (EGF-R).[23] Amplification and over expression is found in 20–30% of breast carcinoma cases and is associated with worse prognosis,[24,25] low ER content, high histopathological grade[26,27] and shortened survival.[28]

Ki67 is a cell cycle associated antigen, expressed in all phases of the cell cycle– except $G_0$. The monoclonal antibody Ki67 was first described in 1983 by Johannes Gerdes and colleagues, who suggested that it might be used as a marker for proliferating cells.[29] It is useful in the identification of hormone insensitivity in breast cancer and in the prediction of tumour growth rates and patient survival,[30,31] therefore it is useful prognostically.[32] It has been shown that elevated levels of this antigen are associated with earlier breast cancer recurrence,[31] shorter survival time and disease free interval[33] as well as a poorer response to therapy.

Determination of ER status is an important parameter in the clinical management of breast cancer.[34-37] Expression of Bcl2 in breast cancer in vivo is strongly correlated to that of ER and both are predictive for response to endocrine therapy.[38] This study investigates the incidence of Bax expression in DCIS and IDC relative to their histological grades and also the relationship between Bax protein and the expression of other biological markers including Ki67, ER, p53, cerbB2 and Bcl2.

## *Materials and Methods*

### *Case Selection*

The breast cancer specimens were retrieved from the Histopathology Department Archives between 1985 and 1995. The 106 cases comprised 56 DCIS and 50 IDC. Of DCIS cases, 30 were pure DCIS as there was absence of any associated invasive component and no past history of breast cancer in either ipsilateral or contralateral breast, whereas 26 had associated invasive carcinoma. Within the invasive ductal carcinoma group, 37 cases were histologically proven lymph node negative and 13 were lymph node positive. All patients were diagnosed and treated at the Royal Free Hospital, London and were identified initially using the SNOMED Diagnostic Retrieval System. Age at presentation ranged from 40 to 70 years (median 56 years). All patients were treated by mastectomy or local excision with or without radiotherapy.

### *Histological Grading of DCIS and IDC*

DCIS was classified as well differentiated (n=18), intermediately differentiated (n=18) and poorly differentiated (n=20) according to the published criteria of Holland et al.[39] In cases in which more than one histological grade was identified, DCIS was graded according to the highest nuclear grade.

Invasive ductal carcinomas were classified according to the Elston and Ellis[40] grading system, as grade I (n-7), grade II (n=24) and grade III (n=19).

### *Immunohistochemistry*

Three µ thick, formalin-fixed, paraffin wax embedded sections were immunostained using primary antibodies to Bax, Ki67, ER, p53, cerbB2 and Bcl2 as described in *Table 1.*

Sections were dewaxed in xylene and rinsed in graded alcohols. Endogenous peroxidase was blocked by incubation in (1%) hydrogen peroxide for 15 minutes followed by rinsing in distilled water. Subsequently sections were subjected to antigen retrieval by heating in a microwave oven in (10mmol/litre) citrate buffer (pH6) for all antibodies except Bax which was pressure cooked, then washed in tris buffered saline (TBS). Non specific staining was blocked by treating with (10%) normal goat serum for 15 minutes. Sections were incubated with primary antibodies for 60 minutes each followed by washing with TBS and application of secondary biotinylated antimouse/rabbit antibody (DAKO) as appropriate at a dilution of 1:100 for 30 minutes. Once again, sections were washed in TBS and finally incubated with Streptavidin-biotin complex reagent (Strept ABC Complex/HRP Duet, DAKO) for 30 minutes. The immunoprecipitate was visualised by treating with diaminobenzidine tetrahydrochloride (Sigma Chemicals Co.) and counterstaining with haematoxylin. Negative controls were run with each batch by replacing the primary antibody with TBS. Positive controls for each antibody (see *Table* 1) were included on each occasion that staining was performed.

Tonsil was used as a positive control for Ki67 and Bcl2, colon for Bax and known cerbB2, ER and p53 positive cases of breast and prostate cancer were used as positive controls for cerbB2, ER and p53 respectively.

### *Staining Characteristics and Assessment of Staining*

Ki67, ER and p53 staining was nuclear and the percentage of positive tumor cells with these markers were determined by counting 1000 cells per case. Cases for

*Table 1.* **Primary antibodies used in the study**

| *Anitgen* | *Source* | *Name* | *Type* | *Pretreatment* | *Dilution* | *Positive Control* |
|---|---|---|---|---|---|---|
| Bax | Novabiochem | Ab-1 #PC66 | Rabbit polyclonal | Pressure cooking for 90 seconds | 1/20 | Colon |
| ki67 | Immunotech, Marseilles, France | MIBI | Mouse monoclonal | Microwaving for 10 minutes | 1/50 | Tonsil |
| ER | DAKO | ID5 | Mouse monoclonal | Microwaving for 20 minutes | 1/100 | Known positive breast cancer |
| p53 | DAKO | DO-7 | Mouse monoclonal | Microwaving for 10 minutes | 1/50 | Known positive prostate cancer |
| cerbB2 | DAKO | – | Rabbit polyclonal | Microwaving for 20 minutes | 1/400 | Known positive breast cancer |
| Bcl2 | DAKO | 124 | Mouse monoclonal | Microwaving for 10 minutes | 1/50 | Tonsil |

ER and p53 were considered positive when more than (10%) of tumour cells were labelled.[12,41] For Ki67, range of percent labelled cells was determined for each group and medians were calculated. However, for purpose of statistical analysis the absolute values of these variables were used.

cErbB2 staining was membranous while Bax and Bcl2 staining was cytoplasmic.[42] For Bax and Bcl2 staining, lymphocytes served as internal positive controls. Labelling with these markers was assessed semi-quantitatively as: negative, weak, moderate and strong. Cases were considered positive when they were either moderately or strongly positive. For purpose of statistical analysis scoring was performed as: negative = 0, weak = 1, moderate = 2 and strong = 3. In most of the cases, the staining was homogeneous, but in some tumours a more heterogeneous pattern was observed.

### *Data Analysis*

The relationships between Bax protein expression and the other variables studied were evaluated using the Spearman's rank correlation employing StatView™ statistical software package. Kruskal-Wallis test was used to compare the distributions of Ki67, ER, p53, cerbB2, Bcl2 and Bax among the different histological grades. DCIS and IDC cases were analysed collectively and separately as well. A p value of less than 0.05 was considered significant.

## ***Results***

The tumors were classified according to the World Health Organisation (WHO) histological classification of breast tumours.[43] Of total 106 cases analysed, there were 56 (53%) DCIS and 50 (47%) IDC cases. Out of DCIS, 18 (32%) were grade I well differentiated, 18 (32%) were grade II intermediately differentiated and 20(36%) were grade III poorly differentiated. Out of IDC, 7 (14%) were grade I well differentiated, 24 (48%) were grade II intermediately differentiated and 19 (38%) were grade III poorly differentiated.

30 (54%) of DCIS were pure that is without any associated IDC, whereas 26 (46%) were associated with IDC. 37 (74%) of IDC were histologically proven lymph node negative and 13 (26%) were lymph node positive.

65 of the 106 cases studied (61%) were Bax positive and 41 (39%) were Bax negative.

### *Relationship of Bax Expression to Histological Grades of Breast Carcinoma*

37/56 (66%) of DCIS and 28/50 (56%) of IDC were Bax positive, see *Figures 1* and *2a*. Within different subgroups of the above mentioned two groups, Bax positivity was as followed:

| | | | | | |
|---|---|---|---|---|---|
| DCIS | Grade I | DCIS | 13/18 | = 72% | DCIS = 37/56 = 66% |
| | Grade II | DCIS | 9/18 | = 50% | |
| | Grade III | DCIS | 15/20 | = 75% | |
| IDC | Grade I | IDC | 4/7 | = 57% | IDC = 28/50 = 56% |
| | Grade II | IDC | 15/24 | = 63% | |
| | Grade III | IDC | 9/19 | = 47% | |

Bax expression was not related to the histopathological grades of either DCIS or IDC. Furthermore, there was no significant difference with regards to Bax expression between DCIS and IDC as whole groups.

Bax did not correlate to Ki67, ER, p53, or cerbB2 and showed positive correlation with Bcl2 (p=0.003). Bax expression and positive ER status showed a trend towards positive correlation, although it did not reach statistical significance (p=0.064).

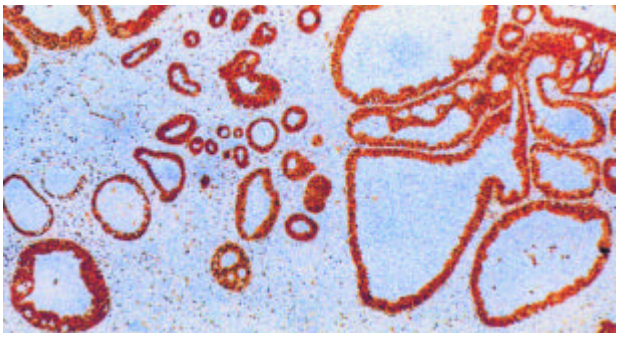

*Figure 1a. Bax Positive Grade I DCIS (x10)*

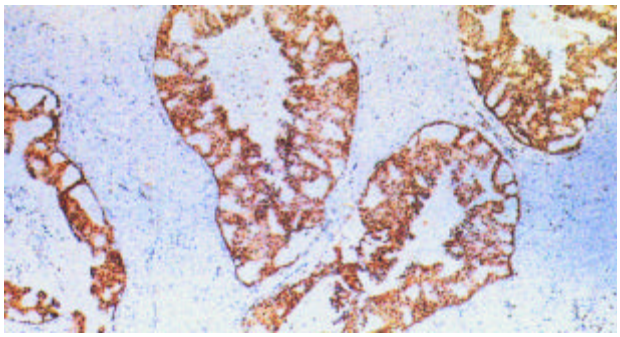

*Figure 1b. Bax Positive Grade II DCIS (x10)*

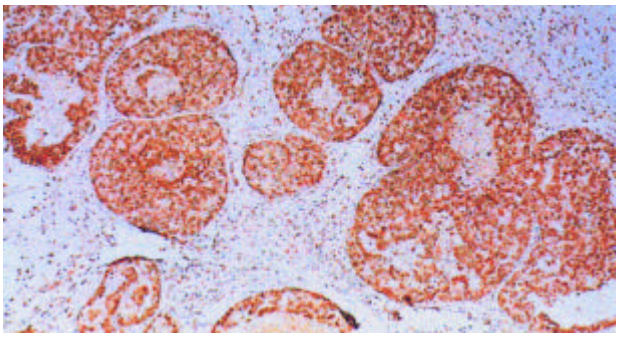

*Figure 1c. Bax Positive Grade III DCIS (x10)*

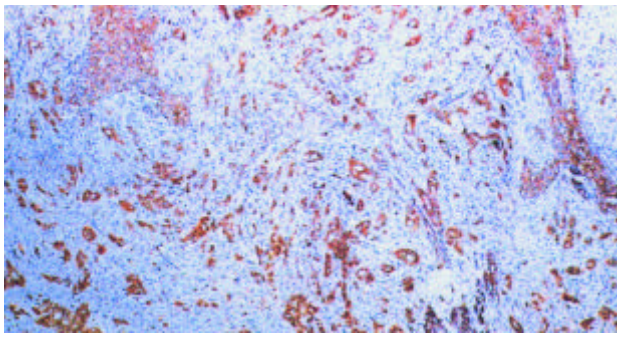

*Figure 1d. Bax Positive Grade I IDC (x10)*

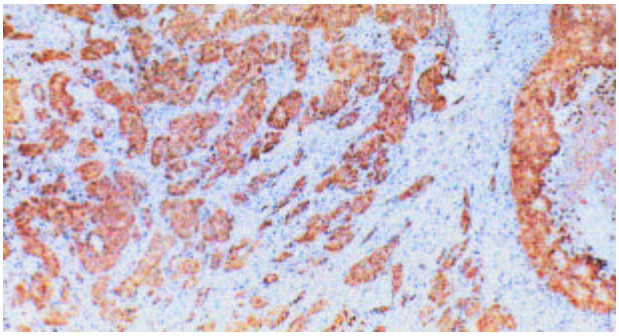

*Figure 1e. Bax Positive Grade II IDC (x10)*

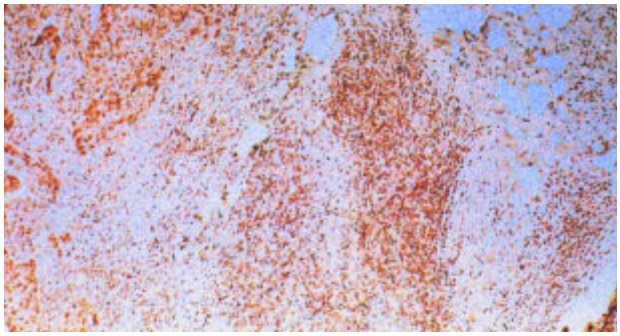

*Figure 1f. Bax Positive Grade III IDC (x10)*

*Relationship of Bax Expression to Ki67*

Ki67 as measured by MIB-1 labelling index showed a significant difference between DCIS and IDC as whole groups (p=0.001) as well as it was significantly correlated to increasing histological grades of both DCIS (p=0.001) and IDC (p<0.0001). The range of proliferating cells was 1 to 49% (median=10) for DCIS and 2 to 80% (median 23) for IDC. The medians for different nuclear grades of DCIS and IDC are illustrated in *Figure 2b* and tabulated in *Table 2*.

Ki67 showed a significant positive correlation with p53 (p=0.001) and cErbB2 (p=0.004) and negative correlation with ER (p<0.0001). There was a trend of negative correlation with Bcl2 (p=0.06). Bax did not correlate to Ki67 (p=0.94). These correlations were similar to above when evaluated within 56 DCIS cases and 50 IDC cases separately (p values not shown).

*Relationship of Bax Expression to ER Status*

ER positivity was seen in 74/106 (70%) of all cases including 41/56 (73%) of DCIS and 33/50 (66%) of IDC, as illustrated in *Figure 2c* and tabulated in *Table 2*.

ER expression was correlated to well differentiated DCIS (p=0.04) and IDC (p=0.02), although there was no significant difference between DCIS and IDC as whole groups.

ER expression correlated negatively with Ki67, p53 and cerbB2 (p<0.0001, p=0.018, p=0.02 respectively) and positively with Bcl2 (p<0.0001).

*Relationship of Bax Expression to p53*

Overall positivity was 28/106 (26%) for p53 with 14/56 (25%) of DCIS and 14/50 (28%) of IDC showing expression of this protein, as illustrated in *Figure 2d* and tabulated in *Table 2.*

p53 expression correlated positively to increasing histological grades of both DCIS and IDC (p=0.001 and p=0.006 respectively), but no significant difference was observed between DCIS and IDC as whole groups.

p53 correlated positively with Ki67 and cerbB2 (p=0.001 and p=0.0011 respectively) and negatively with ER (p=0.018) but no correlation with Bcl2 or Bax was observed (p=0.45 and p=0.8 respectively). Similar correlations were observed within DCIS groups but within IDC, p53 did not correlate as well with ER.

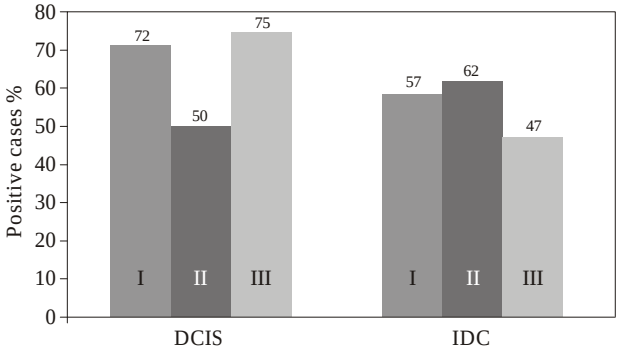


*Figure 2a. Bax incidence in breast carcinoma*

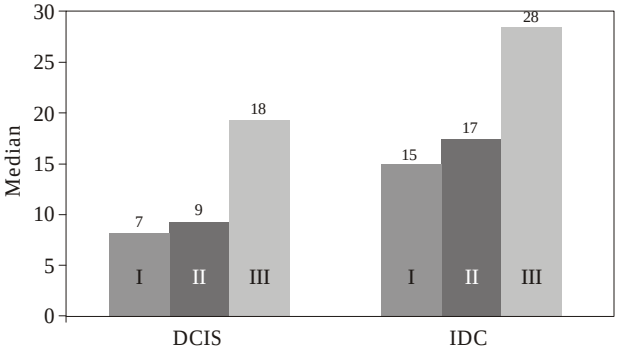


*Figure 2b. Ki67 incidence in breast carcinoma*

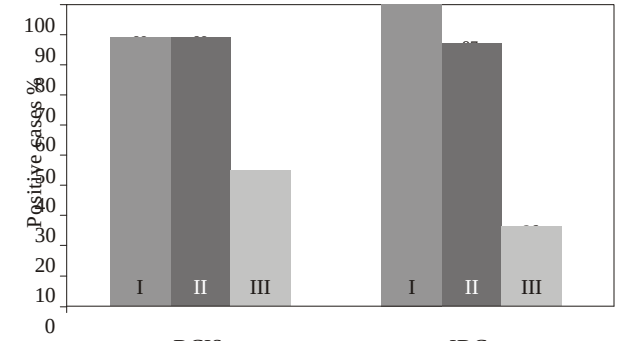


*Figure 2c. ER incidence in breast carcinoma*

*Relationship of Bax Expression to cErbB2*

Overall positivity for cerbB2 was 44/106 (42%) with 25/56 (45%) of DCIS and 19/50 (38%) of IDC expressed this oncoprotein, illustrated in *Figure 2e* and tabulated in *Table 2.*

cErbB2 expression correlated positively to increasing histological grades of both DCIS (p=0.001) and IDC (p=0.01), but no significant difference was observed between DCIS and IDC as whole groups.

cErbB2 correlated positively with Ki67 (p=0.004) and p53 (p=0.0011), and negatively with ER (p=0.02) but no correlation with Bcl2 or Bax was observed (p=0.672 and p=0.069 respectively). These correlations were similar within DCIS and IDC groups.

*Relationship of Bax Expression to Bcl2*

Overall positivity was 45/106 (42%), with 25/56 (45%) of DCIS and 20/50 (40%) of IDC showing Bcl2 expression, illustrated in *Figure 2f* and tabulated in *Table 2.*

Bcl2 expression displayed a negative correlation with increasing histologic grades of DCIS and IDC (p=0.02 and p=0.04 respectively) but no significant difference was observed between DCIS and IDC as whole groups.

Bcl2 correlated positively with ER (p<0.0001) and Bax (p=0.003), but no correlation with p53 (p=0.45) and cerbB2 (p=0.672) was seen. It showed a trend of negative correlation with Ki67 (p=0.06), correlations were similar within IDC cases. However, within DCIS, Bcl2 did not correlate significantly with Bax.

## *Discussion*

Apoptosis (programmed cell death) is an actively regulated cellular process that leads to the destruction of individual cells.[44-47] It can be triggered by several stimuli, such as radiation, drugs and toxins or by deprivation of hormones or growth factors.[48-49] The apoptotic process is controlled by several genes, including inducers (p53, $bclX_S$, Bax, Bak) and repressers (Bcl2, $BclX_L$, Mcl-1). The balance between expression of these genes regulates the cell cycle and apoptosis. The balance between these proteins is also regulated by other stimuli such as p53 protein or oestrogen receptors in breast carcinomas.

Heterodimerization of Bcl2 and Bax proteins plays a pivotal role in regulating the fate of individual cells co-expressing these proteins.[50,51] Excess of Bcl2 promotes cell survival by inhibiting apoptosis, whereas excess of Bax accelerates cell death.[1,2]

Bax is normally expressed in several epithelia including those in breast, small intestine, colon, prostate, respiratory tract and skin.[42] Reduced expression is associated with poor response rates to chemotherapy and shorter survival in metastatic breast adenocarcinoma.[52] We investigated Bax protein expression in DCIS and its different grades, in comparison to IDC and its different grades. In this study, the predominant intracellular distribution of Bcl2 was in the cytoplasm and/or cell membranes including the nuclear envelope, but Bax immunostaining was cytoplasmic.

Kapucuoglu et al[53] reported on Bax protein expression in DCIS and its histological grades, which showed Bax expression to be 67% in DCIS, similar to the results reported in this study. However, no significant correlation of Bax with poorly differentiated DCIS was seen, which is in contrast to the results reported by Kapucuoglu et al,[53] although it did seem to be highest in poorly differentiated DCIS. Furthermore, the cases of DCIS did not exhibit inverse correlation of Bax and Bcl2, but in accordance with the results reported by Kapucuoglu et al[53] the cases of IDC showed a positive correlation between Bax and Bcl2. Similar results have also been reported by Krajewski et al.[52]

*Table 2.* **Bax, Ki67, ER, p53, cerbB2 and Bcl2 Incidence in Breast Carcinoma**

| | *Bax (%)* | *Ki67 (Median)* | *ER (%)* | *p53 (%)* | *cerbB2 (%)* | *Bcl2 (%)* |
|---|---|---|---|---|---|---|
| DCIS GI | 72 | 7 | 89 | 0 | 17 | 61 |
| DCIS GII | 50 | 9 | 89 | 22 | 44 | 56 |
| DCIS GIII | 75 | 18 | 45 | 50 | 70 | 20 |
| **Total** | **66** | **10** | **73** | **25** | **45** | **45** |
| IDC GI | 57 | 15 | 100 | 0 | 29 | 57 |
| IDC GII | 63 | 17 | 87 | 21 | 33 | 50 |
| IDC GIII | 47 | 28 | 26 | 47 | 47 | 21 |
| **Total** | **56** | **24** | **66** | **28** | **38** | **40** |
| **Grand total** | **61** | **22** | **70** | **26** | **42** | **42** |

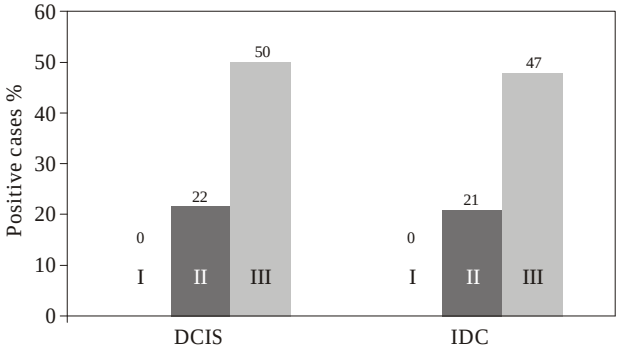


*Figure 2d. p53 incidence in breast carcinoma*

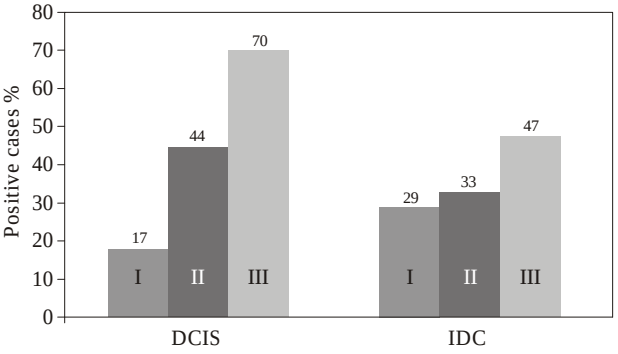


*Figure 2e. cerbB2 incidence in breast carcinoma*

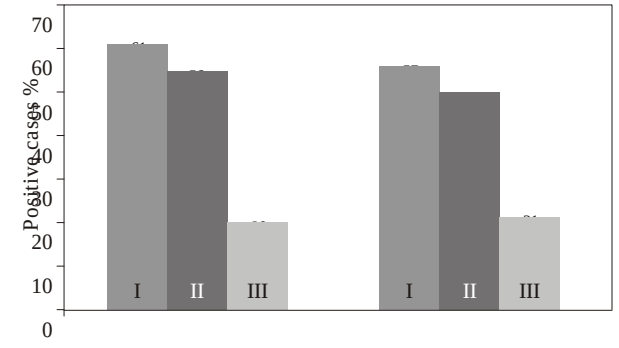


*Figure 2f. Bcl2 incidence in breast carcinoma*

In this study, Bax posistivity did not correlate with the histological grades of either DCIS or IDC. Similar findings for Bax correlation with histological grades of IDC have also been reported by Yang et al,[54] who studied Bax, Bcl2, p53, MIB-1 and ER in 177 invasive cancers and did not find any correlation of Bax with Bcl2 or ER. However, in this study a positive correlation of Bax and Bcl2 in IDC was observed but no correlation in the DCIS cases could be established. Similar to Yang et al[54], no correlation between Bax and ER in either of the DCIS or IDC cases was found, although there was a trend towards positive correlation but it was not significant.

In another recent study by Rochaix et al,[55] Bax was studied in 110 IDC and found to be expressed in 75% of cases. It did not correlate to tumour grade, ER, p53 or Bcl2. Bcl-X was positively correlated to ER, Bcl2 and Bak emerged as critical determinants of regulating apoptosis in breast carcinoma.

In accordance with the above two studies[54,55] no correlation between p53 and Bax was found, which could be explained by the mutation or inactivation of p53, the latter being unable to promote Bax gene expression.

In this study, 42% Bcl2 positivity was observed and it correlated significantly with well differentiated DCIS, well differentiated IDC and also with ER. The correlation of bcl2 with well-differentiated IDC is similar to the findings reported in literature.[54,56,57] The relationship between Bcl2

and differentiation grade has also been reported in DCIS.[58,59]. A positive correlation between Bcl2 and ER has been reported by Yang et al[54] as well. Their study and our present study support the hypothesis that oestrogen regulates the expression of Bcl2 but not Bax in breast cancer. Our study evaluated this relationship within DCIS cases in addition to IDC cases. Bcl2 did not correlate significantly to cerbB2 or p53, which has been reported by Quinn et al.[59]

A strong inverse correlation between Bcl2 and proliferative activity has been reported to exist in breast cancer.[54] Our data also showed a trend towards this correlation. This supports the suggestion that apoptosis and proliferation are mechanistically linked.[54] No correlation between Bax staining and MIB-1 expression was found.

We have shown in our study that out of these markers (Bax, Ki67, ER, p53, cerbB2 and Bcl2) only Ki67 was the one that differentiated between DCIS and IDC as groups. The others were not significantly different between the two groups. Ki67, p53 and cerbB2 correlated positively, whereas ER and Bcl2 correlated negatively with increasing histological grades of both DCIS and IDC. However, Bax did not correlate to histological grades of either.

### *Conclusions*

The results obtained in this study suggest that the regulation of apoptosis is important in ductal carcinoma in situ of the breast as well as invasive ductal carcinomas. Bcl2 is associated with good prognostic markers in both DCIS and IDC, whereas the regulation of Bax is complex and dose not necessarily correlate with mutant p53.

### *Acknowledgement*

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