JK SCIENCE pISSN: 0972-1177

ORIGINAL ARTICLE

Significance of Bcl-2 and Ki-67 Expression in Breast Carcinoma and their Correlation with Histologic Grade

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Abstract

Background: ER, PR, HER2, and Ki-67 are established prognostic markers in breast carcinoma. The anti-apoptotic factor B-cell lymphoma 2 (Bcl-2) is emerging as an additional prognostic indicator. This study aimed to assess the correlation of Bcl-2 expression with Ki-67 scores and histological grading. **Material and Methods:** A hospital-based, prospective observational study was conducted on 52 mastectomy specimens after applying exclusion criteria. Gross examination and routine histopathology with Hematoxylin and Eosin staining were followed by immunohistochemistry for ER, PR, HER2, Ki-67, and Bcl-2. Cases were graded using the Nottingham Histologic Score. Bcl-2 expression was correlated with Ki-67 scores and histological grades, and statistical analysis was performed. **Results:** Out of 52 cases, 50 (98.1%) were invasive ductal carcinoma and 2 (1.9%) were lobular carcinoma. Histologic Grade 3 tumors constituted 57.7% of cases. A Ki-67 score e$\geq$20% was significantly associated with higher grades (p = 0.0285770). Bcl-2 expression was inversely correlated with histological grade (p = 0.004302). Low Bcl-2 expression was significantly associated with a high Ki-67 score (p = 0.03708). **Conclusion:** Strong Bcl-2 expression correlates with lower-grade, less aggressive tumors and is inversely related to high Ki-67 expression. Bcl-2 combined with Ki-67 may enhance prognostic assessment in breast carcinoma.

Key Words: Breast carcinoma, Biomarkers, Histologic Grading, Bcl-2, Ki-67.

Introduction

Breast carcinoma is one of the most common cancers in women throughout the world as well as in India. The estimated number of breast cancer cases in India during 2012 was 145,000 cases with age standardized incidence rate of 25.8 per 100,000 women.[1,2] Several studies have reported that breast carcinoma is often triggered by mutations that lead to an over-expression of biomarkers, namely, estrogen-receptor (ER), progesterone receptor (PR), Human Epidermal growth factor receptor 2 (HER2) and Ki-67.[3] These markers have been demonstrated to be important determining factorsfor endocrine therapy andanti-HER2 therapy.[3,4]

A novel molecular marker, B-cell lymphoma 2 (Bcl-2) is currently emerging as a tool for prognostication of breast carcinoma, guiding therapy, and predicting treatment response. The present study will focus on the correlation of a newly emerging biomarker, Bcl-2, with proliferation marker (Ki-67 score) and histological grading. Bcl-2 is an apoptotic regulator that has a role in maintaining the balance between cell proliferation and apoptosis. Hence its mutation can lead to cell immortalization and tumor progression.[5,6]

The Bcl-2 family protein, Bcl-2, is upregulated by estrogens in breast cancer, through a direct consequence of transcriptional induction. Some authors reported that Bcl-2 constitutes a strong protein marker in breast cancer and is a favorable prognostic factor in ER-positive breast cancer.[7] Recent studies have also suggested that Bcl-2 is a reliable prognostic marker, particularly for hormone

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Manuscript Received: 01.03.2025; **Revision Accepted**: 08.05.2025;
Published Online First: 10th Oct, 2025
Open Access at: https://journal.jkscience.org

Cite this article as: Mukherjee S, Sarkar S, Mistry S, Chakrabarti S. Significance of Bcl-2 and Ki-67 Expression in Breast Carcinoma and their Correlation with Histologic Grade. JK Science 2025; 27(4):225-229

receptor (HR)-positive breast cancer.[8] Bcl-2-positive expression is associated with better outcomes of metastatic and early breast cancer treated with either hormone therapy or chemotherapy.[9] The mechanism for the differences in prognosis remains unclear, but Bcl-2 expression is dependent on the molecular subtype,especially the ER status.[10]

Proliferative index, Ki-67 has a substantial role in molecular classification of breast cancers.[11] A cut-off Ki-67 score of 14% is used in the classification ofbreast carcinoma at the molecular level as Luminal A and Luminal B subtypes.[12] High Ki-67 expression was found to correlate with increased rates of clinical response and pathological complete response following neoadjuvant chemotherapy (NAC).[13,14] Some studies have shown that its expression has a strong correlation with different tumor grades of breast cancer.[15,16]

Bcl-2 is an ant apoptotic factor while Ki67 is a proliferative marker. Chemotherapeutic drug efficacy partially depends on apoptosis induction, thus making Bcl-2 and Ki-67 important targets in cancer therapy. Alterations in this pathway lead to tumorigenesis, tumor progression, overall tumor growth, and regression during chemotherapy. Only a few studies have been conducted worldwide to determine the correlation between molecular subtype, Ki-67 and recently emerging biomarker, Bcl-2. Moreover, no such study has been done in this geographic area. The primary objective of the present study was to determine the correlation among apoptotic marker Bcl-2 with tumor cell proliferation marker, (Ki-67 score) and histological grading in infiltrating duct carcinoma. The secondary objective of the study was to determine whether Bcl-2 can be append as a prognostic marker and possibly used as a therapeutic target in the future.

Material and Methods

The present study was conducted in the Department of Pathology taking all mastectomy specimens after obtaining ethical clearance from the Institutional ethical committee (No. ESI-PGIMSR/MKT/IEC/2020/04). It was a hospital-based observational prospective study conducted over a period of 20 months.

Inclusion and Exclusion criteria: The inclusion criteria were all primary breast carcinoma patients who underwent mastectomy irrespective of their age. The exclusion criteria were patients who previously received chemotherapy/radiotherapy and those who refused to give consent for the study. The Sample size is calculated by using the formula for determining the sample size of descriptive study N=4xPxQ/L2. According to the study of Eom et al, [7] P=0.8630, Q=1-P, L= 10% of P, the calculated sample size for the present study was at least 45 cases. Data was collected from hospital records and clinical history. Gross examination of the specimens was done along with routine histological study by hematoxylin and eosin (H&E) stain followed by immunohistochemistry (IHC) for ER, PR, HER2, Ki-67, and Bcl-2. The primary antibodies used for ER, PR, HER2, Ki-67, and Bcl-2 were Rabbit monoclonal antibodies of clone SP1, Y85, SP3, SP6, and Mouse monoclonal antibody respectively.

By H&E stain, the breast carcinomas were classified into their respective histological types and they were graded according to Nottingham Modification of Scarf Bloom-Richardson Criteria.

ER and PR status evaluation was done according to the Allred scoring system. Reporting of results of HER2 testing by immunohistochemistry was done as per ASCO and CAP recommendations.

Bcl-2 scoring was based on the numericalpercentage of Bcl-2 positive cells (nuclear membrane and cytoplasmic staining) as was done in the study of Ayadi *et al*.[17] Bcl-2 was graded according to the expression level ranging from low (positive cells d”30%), moderate (positive cells>30% but d”70%) and strong (positive cells>70%).

Ki67 was scored, based on the average numerical percentage of tumor cells showing nuclear positivity in a high power field (400X) out of 500 tumor cells. A score of 20 ormore was considered as high.

Statistical analysis was done namelythe significance test (Chi-square test) and test of correlation (Pearson Correlation Coefficient).using the Social Science Statistics (https://www.socscistatistics.com) and Statistics Kingdom (https://www.statskingdom.com/correlation-calculator.html) websites.

Results

A totalof 63 patients were initially evaluated for inclusion in the study. However, 7 patients had a history of previous chemotherapy and 4 patients underwent lumpectomy for which they were excluded from the study. Ultimately, 52 cases were selected for the present study.

The mean age of the patient population was 53.57 (± 10.5) years with an age range of 38 to 77 years. Only 2 cases in our study population were male breast carcinoma. The distribution of histological types, histological grades, and distribution of conventional biomarkers (ER, PR, and Her-2) are depicted in Table 1. Histological grade 3 was noted in 30 cases (57.7%), while grade 2 was noted in 13

Table 1. Distribution of the cases based on site, focality, presence/ absence of DCIS, status of margins, status of LVI, PNI, and histologic type.

Parameters	Frequency	Percentage (%)
Site		
Central	9	17.3
Lower inner	6	11.5
Lower outer	7	13.4
Retroareolar	4	7.6
Upper inner	8	15.3
Upper outer	18	34.6
Focality		
Multifocal	7	13.4
Unifocal	45	86.5
DCIS		
Absent	4	7.6
Not identified	25	48.1
Present	23	44.2
Margin		
Involved	7	13.5
Uninvolved	45	86.5
LVI		
Not identified	24	46.2
Present	28	53.8
PNI		
Not identified	37	71.2
Present	15	28.8
Histologic type		
IDC, IST	51	98.1
Tubular	1	1.9

cases (25.0%). (Table 2). Her 2 positive and triple negative subtypes were most commonly seen in the histologic grade 3 whereas grade 1 cases were most seen having luminal A or B molecular subtypes (Table 2).

An immunohistochemical score of nuclear expression for Ki-67 and cytoplasmic expression for Bcl-2 was evaluated according to the scoring system as described

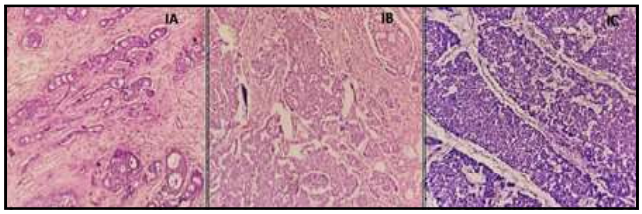

Figure 1: Different grades of invasive ductal carcinoma. Grade 1 (IA), Grade 2 (LB), Grade 3 (IC). [H&E; 100X]

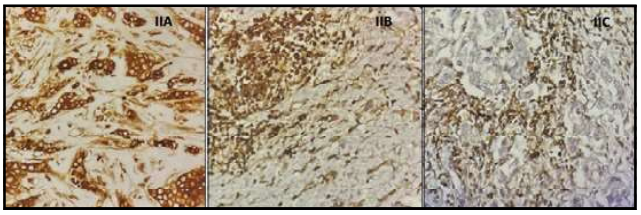

Figure 2: Corresponding immunohistochemistry with Bcl2 revealing progressively decreasing expression. Grade 1 (IIA), Grade 2 (IIB), Grade 3 (IIC). [DAB Chromogen; 400X]

in the "Materials and Methods" section. Immunomorphology of Bcl-2 expression in varying histologic grades has been delineated in Figures 1 and 2. Tumors with a Ki-67 score of 20 or more positively were associated with higher histological grades.In contrast, Bcl-2 expression was inversely correlated with the histological grade of tumors, as weak Bcl-2 scores were significantly associated with grade 3 (Table 3).

Statistical analysis showed highly significant differences between the histologic grading of breast carcinoma and Bcl-2 expression. Tumors having strong Bcl-2 expression were noted with Grade 1 ($p= 0.004302$). On the contrary strong Ki-67 score of 20 or more was associated with Grade 3 tumors ($p = 0.0285770$) (Table 3).

Pearson Correlation Coefficient was used to calculate the correlation between tumor grade withthe two biomarkers (Ki-67 and Bcl-2). Cases having a Ki-67 score of 20 or more are consistently and significantly correlated with cases having weak Bcl-2 scores in different histologic grades (Pearson correlation coefficient, $r = 0.9983$, $p = 0.03708$) (Table 4).

Table 2: Molecular subtyping of breast carcinoma and its occurrence in different histologic grades. Luminal A was the commonest, followed by the HER2 positivesubtype.

Molecular subtype	Number of cases (percent)			Total (percent)
	Grade 1	Grade 2	Grade 3	
Luminal A	5 (9.6)	4 (7.7)	8 (15.4)	17(32.7)
Luminal B	3 (5.8)	5 (9.6)	3 (5.8)	11 (21.2)
HER2positive	1 (1.9)	2(3.8)	10 (19.2)	13 (25.0)
Triple negative	0	2(3.8)	9 (17.3)	11 (21.2)
Total	9 (17.3)	13 (25.0)	30 (57.7)	52 (100)

Table 3. Distribution of Ki-67 and Bcl-2 score according to histologic grade. High Ki-67 scores and Weak Bcl-2 scores were more common in grade 3, which were significant by the chi-square test.

Score	Number of cases (percent)			Total	p value
	Grade 1	Grade 2	Grade 3		
Ki-67					
20 or more	2 (3.8)	9 (25.0)	21 (40.4)	32 (61.5)	0.0285770
Less than 20	7 (13.5)	4 (7.7)	9 (17.3)	20 (38.5)	
Total	9 (17.3)	13 (25.0)	30 (57.7)	52 (100)	
Bcl-2					
Weak	2 (3.8)	5 (9.6)	16 (30.8)	23 (44.2)	0.004302
Moderate	1 (1.9)	5 (9.6)	12 (23.1)	18 (34.6)	
Strong	6 (11.5)	3 (5.8)	2 (3.8)	11 (21.2)	
Total	9 (17.3)	13 (25.0)	30 (57.7)	52 (100)	

Table 4. Correlation between the Bcl-2 score with the Ki-67 score in the different histologic tumor grades. A weak Bcl-2 score in different grades of tumors (A) have a significant positive association with a Ki-67 score of 20 or more (D) in the Pearson correlation test (Pearson correlation coefficient, r = 0.9983, p = 0.03708).

Histologic Grade	Bcl-2 score Number of cases		Ki-67 score Number of cases		Pearson correlation
	Weak (A)	Moderate and strong (B)	Less than 20 (C)	20 or more (D)	Pearson correlation coefficient r = 0.9983
Grade 1	2	7	7	2	
Grade 2	5	8	4	9	p = 0.03708
Grade 3	16	14	9	21	
Total	23	29	20	32	

Discussion

Normal breast development is controlled by a unique balance between cell proliferation and apoptosis. There is strong evidence that tumor growth is not merely a result of uncontrolled proliferation but it is due to reduced apoptosis. Thus, a balance between proliferation and apoptosis is necessary in determining the overall cell growth or regression of the tumor. The growth of breast cancer cells is dependent on various viability factors including hormones. The withdrawal of these factors stimulates apoptosis and accordingly, sex steroid receptor-negative tumors had higher apoptotic indices.[18] Apoptosis occurs spontaneously or may be induced by various anti-tumor agents and is regulated by several genes including Bcl-2. In the present study, the maximum number of cases belonged to Grade 3 (57.7%). Bcl-2 had a significant association with histological grade, showing weak Bcl-2 positivity mostly in the grade 3 tumors (16 cases, 30.8%). Conversely, strong Bcl-2 was mostly noted in grade 1 tumors, (6 of 9, p = 0.004302). Recent studies have shown that Bcl-2 protein is a promising prognostic and predictive marker in hormone receptor-positive, lymph node-negative breast carcinoma.[17] Moreover, several studies have demonstrated that Bcl-2 is a strong prognostic factor correlated with better survival.[15] In the current study, it was found that a Ki-67 score of e $\geq$ 20 is significantly associated with highly aggressive tumors (p = 0.0285770). Consequently, an inverse correlation was seen between the anti-apoptotic marker Bcl-2 and proliferation index Ki-67 (p = 0.03708). Such correlation was also seen in previously conducted studies.[17, 19] Ki-67 is an important biomarker that provides additional and independent predictive information regarding response to chemotherapy and prognosis in patients receiving neo-adjuvant treatment. Thus Bcl-2 is showing a significant role in further prognosis of breast carcinoma by co-expressing with lower proliferative index Ki-67. Bcl-2, an anti-apoptotic protein, blocks apoptosis both in vitro and in vivo. Therefore, an elevated level of Bcl-2 should often be correlated with lower apoptosis. Similar to the present study, Bcl-2 is associated with well-differentiated tumors and with lower proliferation index scores.[19]

In the present study, it was established that a higher Ki-67 score is associated with higher grades, and more

aggressive tumor morphology and is also inversely related to prognostic markers Bcl-2. Recently, in accordance to the present study, Ameh-Mensa *et al* [20] noted an inverse correlation between bcl-2 expression and histological grade. In a recent article on machine learning assisted analysis of breast cancer gene expression profiles, it was concluded that BCL11A can upregulate the expression of Bcl-2, BCL2-xL, and MDM2, which suppress p53 activities and would in future be used as additional prognostic marker.[21] Another recent study has noted that a low Bcl-2 expression is significantly associated with high BRCA1 expression.[22]

A weakness of this study is that the patients could not be followed up. Therefore, the studycould not be able to look at outcomes to determine whether Bcl-2 expression adds additional prognostic information above that provided by the standard biomarkers. A future study may be undertaken to evaluate the outcome in context with the expression of Bcl-2 and the outcome in cases of breast carcinomas.

Conclusion

The study established that strong Bcl-2 expression was statistically significantly correlated with lower histologic grades and lower Ki-67 scores while higher histological grades of breast carcinoma are associated with higher Ki-67 scores. Thus, it can be concluded that Bcl-2 expression is associated with less aggressive tumors and has an inverse relationship with proliferative index Ki-67. These two biomarkers, namely, Ki-67 and Bcl-2 can be used along with conventional biomarkers to predict the aggressiveness and prognosticate breast carcinoma.

Financial Support and Sponsorship : Nil
Conflict of Interest: Nil

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