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Apoptotic regulators (Bcl2 & Bag1) association with the molecular classification of breast cancer: an immunohistochemical study.

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ABSTRACT

The WHO classification of breast cancer into different types failed to predict the prognosis & treatment possibilities, therefore recent research focused on finding new markers of prognosis using gene profile, furthermore, application of immunohistchemistry to detect protein expression have been used to identified immunophentypes of breast carcinoma . This study focused on immunohistochemical expression of bcl2 & bag-1 in breast cancers & their association with different molecular types of breast carcinoma. We analyzed the expression of Bcl2 & Bag 1 in 60 cases of breast cancer , this expression was assessed in relation to different clinicopathological data & molecular types of breast carcinoma cases. The results found that 44 % & 51% of breast cancer cases were positive for Bcl2 & bag1 respectively, and both markers are significantly associated with the molecular subtypes of breast cancer, in conclusion , the association of bcl2 & bag 1 immunohistochemical expression with molecular subtypes of breast cancer is promising factor to the determine the prognosis of patient and reassessed the type of treatment accordingly.

Keywords: breast carcinoma, bcl2, Bag 1, molecular classification, immunohisitochemical expression.

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INTRODUCTION

Breast cancer is a heterogeneous tumor with different clinical outcome, it's the most prevalent malignancy among women, with an incidence of 178.480 new diagnosed cases in USA in 2007. [1] Several prognostic &predictive factors have been estimated for breast cancer like tumor size, lymph node status, receptors for estrogen & progesterone , HER2 /neu status, histological grades & stages. [2,3] Unfortunately these factors sometimes fail to predicts the prognosis of breast cancer & treatment possibilities, so another method is mandatory required & another classification have been adopted recently which based on gene profile of breast carcinoma & more practically breast cancer have been classified according to immunohistochemical expression of protein derived from gene expression.[4,5] according to this protein expression breast carcinoma classified into four types (luminal A, luminal B, her 2 & triple negative or basal like).[6,7]

Bcl2 is a member of cytoplasmic proteins family, the transcription of these protein is modulated by P53while their activities are regulated by tyrosine kinases. [8,9]

Bcl2 exerts tumoregenic effects by inhibition of apoptosis through the inhibition of cytochrome –c and apoptosis inducing signals from the mitochondria to the cytoplasm [10-11] and also through activation of caspase 3 (one of the major proteins involved in apoptosis) by inhibition of its activator protein. [12,13]

The Bcl2 gene are over expressed in B cell lymphocytes in the germinal centers in cases of follicular No – Hodgkin's lymphoma. Its over expression as a result of t(14;18)chromosomal translocation, however Bcl2 over expression can occur in absence of this translocation in different solid tumors.[13,14,15]

BAG-1 (bcl2- associated athanogene 1) co- chaperone protein, which exist into three major isoforms BAG –Is, BAG- IM, & BAG –IL, and one minor isoform. [16] it's a multifunctional protein, it enhanced the anti apoptotic activity of Bcl2, however BAG 1 has its own anti apoptotic activities that are independent of BCL2. [17] BAG 1 inter acts with the heat shock proteins HSC70 and HSP70, in which it acts as nucleotide exchange for HSP 70. [18, 19]

The importance of BAG -1 in breast cancer have been demonstrated in different studies [16,20,21], the BAG 1 protein is primarily cystolic, where as BAG- IM & BAG –I are nuclear in their locations. [22]

Several studies focus on the BAG1 immunohistochemical expression & clinical significance. In breast cancer a consistent finding is that cystolic BAG-1 expression is detected in 2/3 of cases of breast cancer, while BAG 1 nuclear expression varied in different studies ranging from 20% to 70%. [23,24,25,26]

The association between BAG 1 , Bcl2 , ER, and PR are variable, some studies report positive correlation(16,27), while other studies report no significant relationship. [23, 24] These differences could be addressed due to technical variability , small sample size, & different methods for assessment of immunohistochemical reports.

Since the new molecular classification of breast carcinoma have prognostic importance in breast cancer & also can target the type of drugs that are used for treatment . in this study we determine the association between this classification & antiapoptotic factors (Bcl2 & BAG1)with the aim to characterized subsets of patients based on this association.

MATERIALS & METHODS

Sixty paraffin- embedded breast carcinoma tissue blocks were retrieved for this study. All the patient were female residence at Hilla city. Data base were obtained from the archive of the teaching hospital & from different private labs in Hilla city, study were carried out at the teaching laboratory of the department of pathology & forensic medicine at the college of medicine in Babylon university from the period of May 2016 – to December 2016.

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Clinical & histopathological features:

Informations regarding age, sex, tumor size, and lymph nodes status were reviewed.

The slides for tissues tumors were reevaluated for the histopathological diagnosis, grading of the tumors which was carried out according to the modified Scarff- Bloom Richardson into three grades.

The hormonal , Her 2/neu status Ki67 proliferating index of the tumor tissues were reassessed by reviewing the slides , scoring was done based on Allred scoring system& Dako Herceps test protocol . (28-29). So classification of the tumors into four subtypes : Luminal A, Luminal B, Her2/neu & triple negative were done accordingly.

Ethical clearance for this study was obtained from the scientific committee, college of medicine, Babylon university.

Immunohistochemical study:

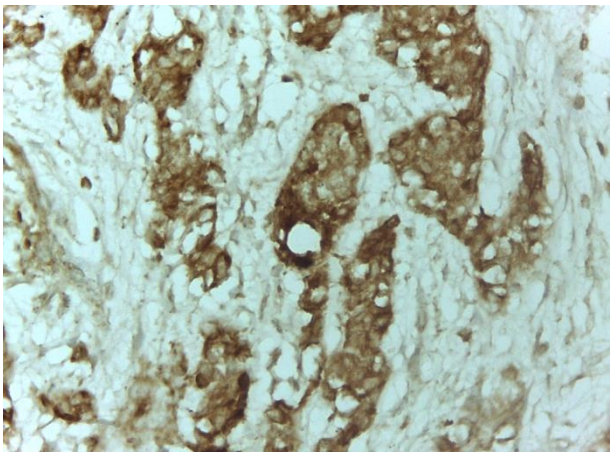
This study carried out by using four micrometer thickness, formalin fixed, paraffin –embedded tissue sections , deparaffinization & rehydration were done then sections were subjected to antigen retrieval by immersing in citrate buffer using water bath for 30 min at 92C.

Endogenous hydrogen peroxidase activity were blocked by hydrogen peroxide for 10min, the Streptavidine biotin peroxidase technique was used for immunohistochemical evaluation of the tissue sections.(L SAB kit. Dako, Denmark), primary antibodies were applied for 30 min to tissues sections at room temperature, the antibodies were Bcl2 (mouse monoclonal Ab. Clone124 Dako at dilution 1:200), BAG 1 (human Bag1 protein fragment ab85158 Abcam at dilution 1:250), then incubation with secondary biotinylated antibody for 30 min at room temperature followed by incubation for 20 min with the peroxidase labeled sterptavidin, visualization of Ag- Ab reaction was done by Diaminobenzidine as a chromogen(DAB). Counter staining done by Myers hematoxylin application followed by dehydration in graded alcohol & xylem then DPX mounting. With each run positive & negative control slides were submitted.

Evaluation of immunohistochemistry:

The scoring was done by single pathologist, foe Bcl2, both the percentage& intensity of cytoplasmic staining positive cells were recorded & a cutoff value of 10% was used.

Bag 1staining either cytoplasmic and /or nuclear & also 10 %of positive cells were used as a cutoff value in the assessment of Bag1 immunohistochemical study. Fig 1.



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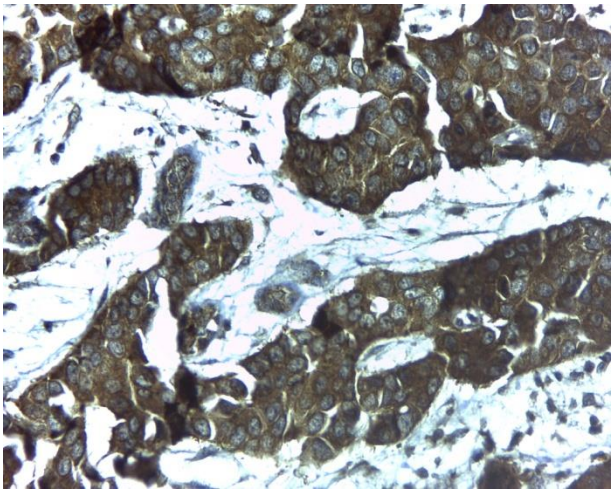

B

Figure 1. overview of bcl2 and bag 1 in breast carcinoma tissues , cytoplasmic immunostaining of bcl2 in moderately differentiated ductal carcinoma (A: x200); very strong cytoplasmic immunoreactivity of bag 1 in invasive ductal carcinoma (B: x 200).

Statistical analysis:

In his study Pearson chi- square test was used for statistical analysis & P value of < or equal to 0.05 was considered significant in the correlation to different parameter involved in this study.

RESULTS

Clinical & histopathological features of the tumor:

Patients characteristic & tumor features are listed in **Table 1**, the mean age of patents at diagnosis was 57 years, more than three quarters of the patients (88.3%) had invasive ductal carcinoma type, the majority of them lied with in grade I & II (86.7%).

The estrogen & progesterone receptor status for each case were reviewed, 73.3% of patients were ER positive, while 39 patients were PR positive, while Her2/neu positivity were observed in only 17 cases.

Table 1. The Distribution of Patients with Breast Cancer According to Study Variables

Age (years)	(57.56 ± 12.01)	(4-68)
Tumor size		
< 2 cm	11	18.3%
≥ 2 cm	49	81.7%
Total	60	100.0%
Grade		
Grade I and II	52	86.7%
Grade III	8	13.3%
Total	60	100.0%
Tumor type		
IDC	53	88.3%
Med C	2	3.3%
ILC	5	8.4%
Total	60	100.0%
Lymph node		
No	6	10.0%
(1-3)	16	26.7%
(3 or more)	38	63.3%
Total	60	100.0%
Estrogen receptor		

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Positive	44	73.3%
Negative	16	26.7%
Total	60	100.0%
Progesterone receptor		
Positive	39	65.0%
Negative	21	35.0%
Total	60	100.0%
Her2 receptor		
Positive	17	28.3%
Negative	43	71.7%
Total	**60**	**100.0%**

Expression of Bag 1& Bcl2 proteins

The immunohistochemical protein expression of Bag1& Bcl2 were positive in 85% and 73.3% of the cases respectively **Table 2**

Table 2 Distribution of patients according to study markers

Study markers	N	%
Bag 1		
Positive	51	85.0%
Negative	9	5.0%
Total	60	100.0%
Bcl 2		
Positive	44	73.3%
Negative	16	26.7%
Total	60	100.0%

No significant statistical association were observed between IHC expression of Bcl2 & Bag 1with tumor size, lymph nodes status, & tumor types. (P>0.05). While Bcl2 expression was strongly associated with tumor grade, ER, PR & Her2/neu status (p< 0.05) **Table 3**. Bag 1 IHC show significant correlation only with PR (P< 0.05) **Table 4**.

Table 3 Association between Bcl2 immunohistochemical expression and Study Variables

Study variables	Bcl 2		P-value
	Positive (%)	Negative (%)	
Tumor size			
< 2 cm	9 (20.5)	2 (12.5)	0.71
≥ 2 cm	35 (79.5)	14 (87.5)	
Total	44 (100.0)	16 (100.0)	
Grade			
Grade I and II	42 (95.5)	10 (62.5)	**0.003***
Grade III	2 (4.5)	6 (37.5)	
Total	44 (100.0)	16 (100.0)	
Lymph node			
No	5 (11.4)	1 (6.2)	
(1-3)	13 (29.5)	3 (18.8)	0.604
(3 or more)	26 (59.1)	12 (75.0)	
Total	44 (100.0)	16 (100.0)	
Tumor type			
IDC	38 (86.4)	15 (93.8)	
Med C	2 (4.5)	0 (0.0)	1.000
ILC	4 (9.1)	1 (6.2)	
Total	44 (100.0)	16 (100.0)	
Estrogen receptor			**0.006***
Positive	37 (84.1)	7 (43.8)	

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Negative	7 (15.9)	9 (56.2)	
Total	44 (100.0)	16 (100.0)	
Progesterone receptor			
Positive	32 (72.7)	7 (43.8)	**0.037***
Negative	12 (27.3)	9 (56.2)	
Total	44 (100.0)	16 (100.0)	
Her2 receptor			
Positive	7 (15.9)	10 (62.5)	**0.001***
Negative	37 (84.1)	6 (37.5)	
Total	44 (100.0)	16 (100.0)	
Molecular type			
Luminl A	36 (81.8)	4 (25.0)	
Luminal B	1 (2.3)	2 (12.6)	**< 0.001***
Her 2neu	5 (11.4)	5 (31.2)	
Triple neagtive	2 (4.5)	5 (31.2)	
Total	44 (100.0)	16 (100.0)	

*p value ≤ 0.05 was significant. Fisher-exact test.

Table 4 Association between Bag 1 immunohistochemical expression and Study Variables

Study variables	**Bag 1**		**P-value**
	Positive (%)	**Negative (%)**	
Tumor size			
< 2 cm	8 (15.7)	3 (33.3)	0.593
≥ 2 cm	43 (84.3)	6 (66.7)	
Total	51 (100.0)	9 (100.0)	
Grade			
Grade I and II	45 (88.2)	7 (77.8)	0.345
Grade III	6 (11.8)	2 (22.2)	
Total	51 (100.0)	9 (100.0)	
Lymph node			
No	5 (9.8)	1 (11.1)	
(1-3)	14 (27.5)	2 (22.2)	1.000
(3 or more)	32 (62.7)	6 (66.7)	
Total	51 (100.0)	9 (100.0)	
Tumor type			
IDC	46 (90.2)	7 (77.8)	
Med C	2 (3.9)	0 (0.0)	0.281
ILC	3 (5.9)	2 (22.2)	
Total	51 (100.0)	9 (100.0)	
Estrogen receptor			
Positive	39 (76.5)	5 (55.6)	0.23
Negative	12 (23.5)	4 (44.4)	
Total	51 (100.0)	9 (100.0)	
Progesterone receptor			
Positive	36 (70.6)	3 (33.3)	**0.05***
Negative	15 (29.4)	6 (66.7)	
Total	51 (100.0)	9 (100.0)	
Her2 receptor			
Positive	13 (25.5)	4 (44.4)	0.256
Negative	38 (74.5)	5 (55.6)	
Total	51 (100.0)	9 (100.0)	
Molecular type			
Luminal A	37 (72.5)	3 (33.3)	
Luminal B	1 (2.0)	2 (22.2)	**0.018***
Her 2 neu	7 (13.7)	3 (33.3)	
Triple negative	6 (11.8)	1 (11.2)	
Total	51 (100.0)	9 (100.0)	

*p value ≤ 0.05 was significant. Fisher-exact test

Correlation between Bag1,Bcl2 immunohistochemical expression & molecular classification of breast cancer:

According to ER, PR, Her2/neu& Ki67 status the patient were sub classified into luminal type A (40 cases), luminal type B (3 cases), Her2/neu type (10 cases) & triple negative tumors (7 cases). Both antiapoptotic markers involved in this study show strong positive relationship with the molecular classification of the breast carcinoma (P<0.05) tables 3, 4.

DISCUSSION

Different & variable clinical & histopathological factors are used to categorized patient with breast carcinoma in order to determine prognosis & describe the appropriate management.

Factors like patient's age, tumor size, lymph node numbers, histopathological features and of course hormonal receptors expression & Her2/neu status are used to subdivide patients into different risk categories. [30]

Recently , using of global gene expression profile (GEP) which subdivide invasive breast carcinoma into 5 intrinsic subtypes have increasingly provide help to refine breast cancer & to assess prognosis & response to therapy.[31] Immunohistochemical surrogates by applying ER, PR, Her2/neu & Ki67 tumor biomarkers have been shown to be useful for understanding the predictive & prognostic values of molecular classification.[32]

Several studies have pointed to the correlation of anti apoptotic factors & breast cancer hormonal status , but few if non study the correlation of these markers to molecular classification of breast cancer & their significance in modifying the management accordingly.

The present study were designed to assess the expression of Bag 1& Bcl2 in breast carcinoma specimens with the hormonal status of the breast cancers & in turn molecular classification of breast carcinoma.

We demonstrate significant bag1 expression with PR, while Bcl2 was significantly associated with ER, PR immunohistochemical expression, such correlation could be consider one of the important prognostic factors, in which application of Bcl2 targeting chemotherapy for those patients with ER, PR positive tumors & chemotherapy resistance have reversed the status.[33, 34]

The association between Bag 1, Bcl2, ER & PR are discussed in different researches & variable results were observed some have reported positive & strong relationship [23, 24, 25] while others are not. [16,27]

In this study we observed that over expression of bag1 & bcl2 in breast cancer were not significantly associated with other prognostic factors like tumor size, lymph node status & tumor type, and in turn it can be estimated as predictive factors for good prognosis independently of these factors. One of the largest Cohort study which was conducted by Callagy & colleagues reveled that Bcl2 over expression in breast carcinoma is an independent predictor of outcome and seems to be useful as a prognostic adjunct to the Nottingham prognostic index. [35]

This article represent one of the few studies carried worldwide which focus on the IHC expression of Bcl2, Bag 1 and molecular classification of breast cancer.

We observed strong association between molecular classification of breast cancer and immunohistochemical over expression of bag 1 & bcl2, to our knowledge no other similar study have been carried out focusing on such association, except for few studies which determine the association between hormonal status of breast cancer and anti apoptotic markers separately, other study carried out by Abd El Majeed et al who investigate the correlation between Bcl2 among triple negative & Non triple negative breast cancers which revealed no significant differences between two groups. [36] in this study most of the cases with positive Bag1 & Bcl2 were luminal A type(72.5%) and (81.8%) respectively, while Her 2/ neu and triple negative types show the lowest positive percentage for bag1 & bcl2 respectively. Table 2, 3.

This association of different types of breast cancer molecular classification are parallel to the prognostic value of each type and in turn Bag1 & bcl2 can determine the survival rate in patients with breast carcinoma.

Several modifications for breast carcinoma management have been introduced since the introduction of MC of breast carcinoma & several studies determine the association of Bag1 & bcl2 over expression and the type of treatment, Millar et al have demonstrated that Bag1 over recurrences & distant metastasis and improved out come in tamoxifen treated patients, they noted that bag1 over expression augment the antiestrogen induced growth arrest. [34] While Papadakis et al had noted for first time that elevated bag 1 protein expression correlate with that of Her2/neu and its important for growth inhibitory effects of trastuzumab, in turn he concluded that targeting Bag1 function in combination with anti- Her2 therapy might prove beneficial .[37]

Similarly several studies have been carried out to determine the relationship between BCl2 over expression in breast cancer & different modalities of treatment, Vernnese et al and Kobayashi et al found that over expression of Bcl2 is an indicator of favorable outcome following endocrine treatment [38, 39], while Van Slooten et al did not demonstrate any predictive value for Bcl2 for a single cycle of perioperative chemotherapy in a study of 423 patients. [40]

In conclusion, over expression of apoptosis regulators (Bag 1 & Bcl2) were significantly associated with different breast cancer molecular subtypes & this correlation can be applied in prognostic stratification of patients and their potential therapeutic implications in selecting patients for treatment.

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