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ORIGINAL ARTICLE

Histopathological Grading of Breast Cancers and its Correlation with ER, PR and C-erb B_2 Receptor status

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ABSTRACT
Breast cancer is the leading cause of cancer and cancer related mortality in the females. The presence of hormone receptors and C-erbB_2 has prognostic and therapeutic implications in breast cancer. The aim of the present study is to assess the ER, PR and C-erbB_2 status and to evaluate their relationship with histopathological grading. The study was 1 year retrospective and 1 year prospective comprising of 100 cases of invasive breast carcinomas. Histopathological grading was done by the Elston and Ellis modification of Scarff Bloom Richardson system. Indirect technique of immunohistochemistry using the standard ABC (Avidin Biotin technique) was employed. Descriptive statistics was carried out with respect to type and grade of tumor, Chi square was used as a test of significance. Statistical analysis was carried using SPSS version-17. Out of 100 cases, 30 (30%) cases were grade I, 45 (45%) cases were grade II and 25 (25%) were grade III tumours. ER positivity was seen in 63 (63%) cases, PR positivity in 53 (53%) cases and C-erb B_2 positivity in 24 (24%) cases. A statistically significant correlation was found between receptor status and histological grade (ER vs. grade $p < 0.00001$ and PR vs. grade $p < 0.00001$) and between C-erb B_2 and histological grade ($p < 0.00001$). Both ER and PR reactivity significantly decreased with the increase of tumor grade while as C-erb B_2 reactivity increased with increase in tumor grade ($p < 0.00001$). This study concludes that a strong correlation exists between histopathological grading and ER, PR and C-erb B_2 status. An inverse correlation was found between ER / PR and C-erb B_2 expression with higher grades of breast cancers being associated with C-erb B_2 positivity and ER/PR negativity. It is recommended to include immunohistochemical analysis along with histopathological grade in diagnosis so as to help in the management of the patients.

***Keywords**: Breast cancer, histopathological grade, ER, PR, C-erb B_2.*

***Abbreviations:** ER = Estrogen receptor; PR = Progesterone receptor; PBS = Phosphate buffer saline; RTU = Ready to use*

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INTRODUCTION

Breast cancer is the most common cause of cancer among women worldwide [1]. It is the most common cause of cancer among women in both developed and developing countries and also the most common cause of cancer related mortality among women globally [2]. However, the mortality rate of breast cancer has decreased significantly during the past two decades, the reasons being detection of early stage tumors because of increased screening and early use of adjuvant systemic therapy [3, 4]. More reliable methods for evaluation of factors predicting tumor progress are necessary in order to develop adequate therapy regimens for different types of breast cancer [5].

There are many variables which affect the prognosis and management of breast cancer such as histological type and grade, tumor size, lymph node status, status of hormone receptors -Estrogen receptor (ER) and Progesterone receptor (PR) of the tumor and HER-2/neu status [6]. Histopathological grade is a part of the pathology report since the work of Broder, 1921 and his predecessors [7]. Grade of carcinomas is an estimate of differentiation and if used in conjunction with other variables it provides significant aid in predicting the outcome and the progress of disease. Histopathological grading is now widely accepted as a robust prognostic factor in invasive breast carcinoma. A fundamental aspect of

histopathology has been the recognition of morphological appearances of tumor that can be co-related with degree of malignancy [8]. The histological appearance of breast cancer is heterogeneous. A number of variables are included in microscopic diagnosis of these lesions in order to characterize the histologic and prognostic factors in a particular tumor.

A remarkable number of tumor and host related features have been suggested to carry out the prognostic significance [9]. Several molecular markers are gaining importance in breast cancer. They can be used not only as prognostic markers but also as predictors of response to therapy. The important ones include the steroid receptors {(estrogen receptor (ER), progesterone receptor (PR)} [10, 11] Her2/neu [12], Ki67 [13] and p53 [14].

ER is expressed in 70% to 80% of invasive ductal carcinomas and 70% to 95% of invasive lobular carcinomas and PR is expressed in 60% to 70% of invasive breast carcinomas [15, 16]. The prognosis and survival outcome is better in case of ER and/or PR positive breast cancers while it is least favorable for ER/PR negative tumours [17, 18]. HER2/neu amplification has been found in 10% and 35% of invasive breast carcinomas [19]. HER2/neu overexpression is associated with a number of adverse outcomes, including decreased overall and disease-free survival [20, 21 and 22].

The present study was undertaken to study the reactivity of breast cancers to ER, PR and C-erbB_2 and to evaluate the relationship of histopathological grading with the ER, PR and C-erbB_2 status.

MATERIALS AND METHODS

The study was carried out on 100 cases of breast cancer, which were reported in the Histopathology section of department of Pathology, Government Medical College, Jammu from December 2006 to November 2008. It was a retrospective (1 year) as well as a prospective study (1 year).

Archival material was retrieved. A pre-structural proforma was prepared which captured detailed data with special reference to age and presenting symptoms from the histopathology records. The summary of the FNAC/excisional biopsy was also recorded. A detailed analysis of the H&E stained slides was carried out with special reference to the histological type of carcinoma, grade of carcinoma, presence or absence of necrosis, vascular and lymphatic invasion, edge of the tumour and inflammatory infiltrate.

Prospective study material consisted of breast biopsy specimen which were fixed in 10% buffered formalin, dehydrated in ascending grades of alcohol, cleared in xylene and finally embedded in paraffin. 2-5 micron thick paraffin sections of uniform thickness were cut on a rotary microtome, dewaxed and stained with hematoxylin and eosin. Hematoxylin and eosin staining was done as described by Bancroft and Stevens (1996) [23]. Histopathological grading of breast carcinoma was done using Elston and Ellis modification of Scarff Bloom Richardson system [8]. Carcinomas were graded by adding up scores of three criteria i.e., tubule formation, nuclear pleomorphism and mitotic count each of which is given 1 to 3 points.

Criteria		Score
% Tubule formation	75% of the tumour	1
	10-75% of tumour	2
	<10% of the tumour	3
Nuclear Pleomorphism	Mild pleomorphism	1
	Moderate pleomorphism	2
	Severe pleomorphism	3
Mitotic count	0-9/10 HPF	1
	10-19/ HPF	2
	≥20/ HPF	3

Grade 1 (Score: 3-5): Well-differentiated.

Grade 2 (Score: 6-7): Moderately differentiated.

Grade 3 (Score: 8-9): Poorly differentiated.

Immunohistochemical staining for Estrogen and Progesterone receptors and C-erb B_2 protein:

Immunostaining was done on routinely processed tissue using paraffin fixed blocks. Three sections from each block were taken one for each mentioned receptor. Poly-L-lysine was used as adhesive. Positive and negative controls were used with each batch. The kits were provided by Novocastra laboratories. Mouse monoclonal antibodies were used.

Primary antibodies: Estrogen receptor Mouse monoclonal antibodies (NCL-ER-6F11), Progesterone receptor Mouse monoclonal antibodies (NCL-PGR-312), C-erb B_2 oncoprotein Mouse monoclonal antibodies (NCL-CB11).

Secondary antibody: Novostatin Universal detection kit (Ready to use) (NCL-RTU-D). The kit had three reagents in ready to use form

1. Pre diluted normal horse serum
2. Pre diluted biotinylated secondary antibody which recognizes IgG, mouse IgM.
3. A pre diluted streptavidin peroxidase conjugate

Technique

Indirect technique of immunohistochemistry using the standard ABC (Avidin Biotin technique) was used. Higher temperature antigen unmasking technique for immunohistochemical demonstration on paraffin sections was used.

Sections were deparaffinized in xylene and rehydrated to water by passing through descending grades of alcohol. The sections were blocked using 0.5% hydrogen peroxide/methanol for 10 minutes and then washed in tap water. Pressure cooker antigen retrieval method was used. After removing the slides from pressure cooker, they were rinsed in phosphate buffer saline (PBS) for 5 min. followed by incubation with horse normal serum for 10 min.

Sections were incubated for estrogen receptor, progesterone receptor and C-erbB_2 separately for 1 hour, then washed twice in PBS for 5 min each. Sections were incubated with a biotinylated RTU secondary antibody for 30-60 min. followed by two PBS washings for 5 min. each. This was followed by incubation with RTU streptavidin / peroxidase complex. Again the sections were washed with PBS 4 times for 5 min. each. Slides were incubated in DAB (Diamino benzidine) solution for 10 min. and washed in distilled water. Staining was done in Harris hematoxylin for 30 sec. Slides were dehydrated by passing through ascending grades of alcohol, cleared in xylene and mounted in DPX. Control positives and negative slides were used in each run of staining.

ER staining - Nuclear
PR staining - Nuclear
C-erb B_2 - Membrane

A case was considered ER or PR +ve when carcinoma in situ or invasive carcinoma showed any number of nuclei (regardless of percentage) containing immunoperoxidase reaction product. Staining was considered negative, when no positive tumour nuclei could be obtained. The nuclei within the counting field were assigned and intensity score of 0 was given when no staining was detectable, 1+ when weak staining was detectable and 2+ when strong staining was present [24].

Positivity for evaluation of C-erb B_2 staining was scored according to the criteria recommended by Dako for Hercep test. The score of 0 was given when no discernable staining or background staining was discovered, 1+ for equivocal discontinuous membrane staining, 2+ for unequivocal membrane staining with moderate intensity and 3+ for strong and complete plasma staining.

Statistical analysis

Data was entered in SPSS version-17 and frequencies were calculated. Chi square test was used as a test of significance for comparing various grades, ER/PR and C-erb reactivity status. Chi square was also used to compare ER/PR and C-erb B_2 reactivity across various grades.

RESULTS

In the present study, maximum no. of patients was between 30-60 years of age (80%) and median age was 47 years. Only 2 (2%) were male patients. Histological typing of breast carcinomas revealed 92 (92%) cases of infiltrating duct cancer followed by colloid carcinoma 2 (2%), medullary carcinoma 2 (2%), papillary carcinoma 2 (2%), lobular carcinoma 1 (1%) and metaplastic carcinoma 1 (1%) case (Table - 1).

Out of 100 cases, 30 (30%) cases were grade I, 45 (45%) cases were grade II and 25 (25%) were grade III tumours. Estrogen receptor positivity was seen in 63 (63%) cases. Grade I tumours showed positivity for ER receptor in 93% cases, Grade II in 73% and grade III in 8% of the cases (Table-2). Progesterone receptor positivity was seen in 53 (53%) cases. Grade I tumours showed positivity for PR receptor in 83% cases, Grade II in 57% and grade III in 8% of the cases (Table-3). Out of 100 cases, 24 (24%) cases showed positivity for C-erb B_2. Grade III tumours showed strong staining for C-erb B_2 in 14 (56%) cases, grade II in 10 (22%) cases whereas all grade I cancers were negative for C-erb B_2 (Table-4).

A statistically significant correlation was found between receptor status and grade (ER vs. grade $p < 0.00001$ and PR vs. grade $p < 0.00001$) (Table-2 and 3 respectively). Relationship of c-erb B_2 and histological grade was found to be statistically significant ($p < 0.00001$) (Table-4).

Out of 24 grade III tumours, 14 (56%) showed strong staining for c-erb B_2 which were negative for ER and PR. Out of 45 grade II tumours 10 (22%) were positive for c-erb B_2; however 3 of them were also positive for ER and PR. (Table-5) C-erb B_2 reactivity increased significantly when compared to ER/PR status across different grades of tumor increased ($P<.00001$) (Table-5). C-erb B_2 reactivity increased

significantly ($p < .0001$) from grade I to grade III and showed an inverse relationship with ER/PR reactivity (Table-6).

Table-1: Age, gender and clinic-pathological features of Breast cancer patients.

Age in years	**N (%)**
21-30	6 (6%)
31-40	24 (24%)
41-50	32 (16%)
51-60	24 (24%)
61-70	10 (10%)
71-80	4 (4%)
Median age	47
Gender	
Females	98 (98%)
Males	2 (2%)
Histopathological Types	
Histopathological type	**Number of cases**
Inflitrating ductal carcinoma	92
Medullary	2
Colloid	2
Papillary	2
Lobular	1
Metaplastic	1

Table-2: Histological grading and ER receptor status of 100 cases of infiltrating ductal carcinoma. ER reactivity decreased significantly as the tumor grade increases.

Total cases	100		
ER status	100		
Positive	63		
Negative	37		
Histological grade	ER + ve	ER – ve	X^2 value
I	28 (93%)	2(7%)	46.3463.
II	33(73%)	12 (27%)	P< .00001
III	2(8%)	23(92%)	

Table-3: Histological grading and PR receptor status of 100 cases of infiltrating ductal carcinoma. PR reactivity decreased significantly as the tumor grade increases.

Total cases	100		
PR status	100		
Positive	53		
Negative	47		
Histological grade	PR + ve	PR – ve	X^2 value
I	25(83%)	5 (17%)	31.8168.
II	26(57%)	19 (43%)	P< .00001
III	2(8%)	23 (92%)	

Table-4: Histological grading and C erbB2 status of 100 cases of infiltrating ductal carcinoma.

Total cases	100		
C-erbB2 status	100		
Positive	24		
Negative	76		
Histological grade	C-erbB2 + ve	C-erbB2 - ve	X^2 value
I	0 (0%)	30 (100%)	23.5867
II	10(22%)	35(78%)	P< 00001
III	14 (56%)	11(40%)	

Table-5: Histological grading with receptor ER and PR negative and C-erbB2 positive status (Percentages in parentheses out of total in that grade)

Histological grade	ER - ve	PR - ve	C-erbB2 + ve	X^2 value
I	2(7%)	5 (17%)	0 (0%)	58.027
II	12 (27%)	19 (43%)	10(22%)¶	P<. 00001
III	23(92%)	23 (92%)	14 (56%)	

¶ Three tumors were ER/PR positive as well.

Table-6: Histological grading with receptor reactivity all types.

Marker	Positive		Negative		X^2 value
Cases (100)	Frequency	%	Frequency	%	
ER	63	63	37	37	32.9732
PR	53	53	47	47	p<.00001
Cerb2	24	24	76	76	

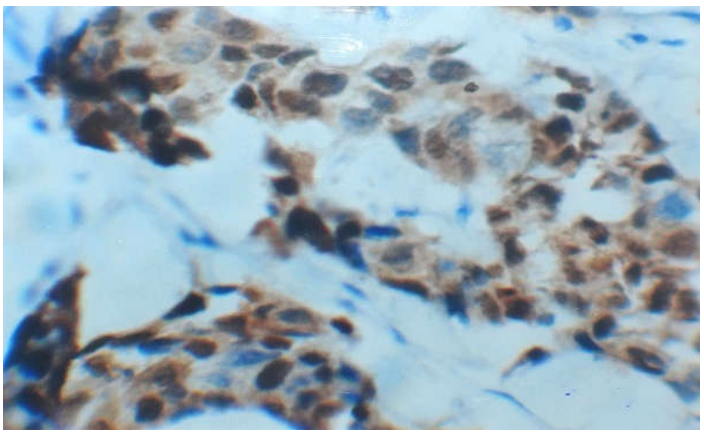

Figure-1: Estrogen receptor immunostaining showing strong nuclear positivity. (400X)

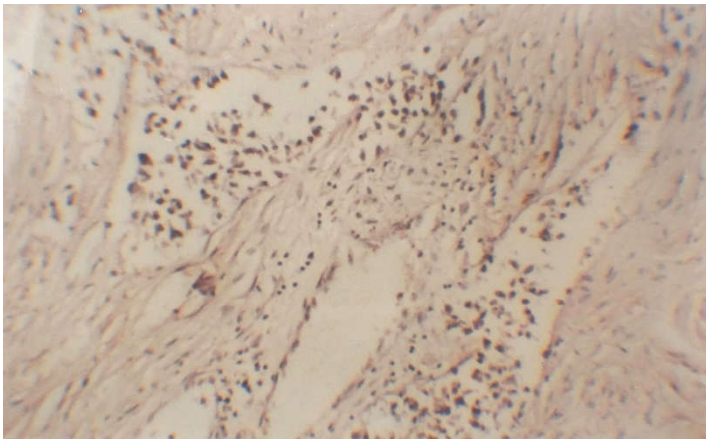

Figure-2: Immunohistochemical staining for progesterone receptor showing nuclear positivity. (40X)

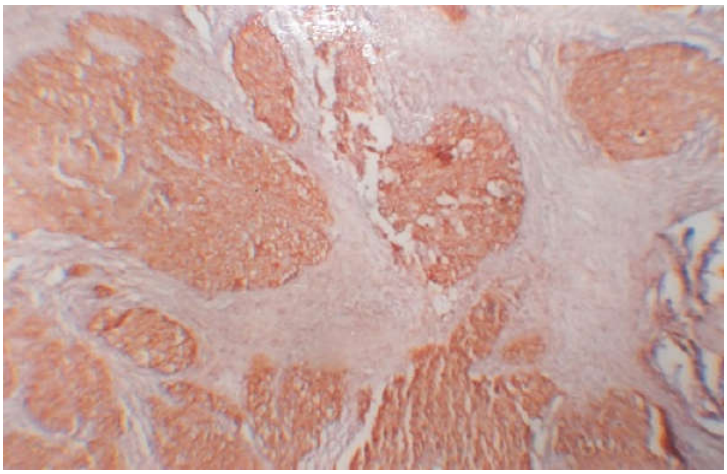

Figure 3: Immunohistochemical staining for C-erb B_2 showing diffuse positivity in tumour cells (40X)

DISCUSSION

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer deaths in women worldwide, with an estimated 1.4 million new breast cancer cases and 458,000 deaths in 2008 [25].

Breast cancer is the second most prevalent cancer among Indian women, the first being cervical cancer [26]. In India, breast cancer is the commonest cancer in urban women and second common among rural women [27]. The present study consisted of analysis of 100 cases of infiltrating breast carcinoma with histological grading and immunohistochemical staining for estrogen/progesterone (ER/PR) and c-erb-B_2 protein.

The age of the patients ranged from 21 to 80 years with a median age of 47 years. Maximum number of patients was between 30-60 years of age (80%). Out of 100 patients, only 2 were male (2%) (Table-1).

In the present study, histological typing of breast carcinomas revealed 92 (92%) cases of infiltrating duct cancer followed by colloid carcinoma 2 (2%), medullary carcinoma 2 (2%), papillary carcinoma 2 (2%), lobular carcinoma 1 (1%) and metaplastic carcinoma 1 (1%) case. The findings in our study are similar to results obtained by Patel et al (2002) who found 93% of their cases to be ductal carcinoma [5]. A predominance of infiltrating ductal carcinoma was also reported by Niveditha and Bajaj (2003) who found 80% of cases to be of infiltrating ductal type [28].

Histological grading was done by Elston and Ellis modification of Bloom and Richardson method [8]. Out of 100 cases, 30 (30%) cases were grade I (Well differentiated), 45 (45%) cases were grade II (moderately differentiated) and 25 (25%) were grade III tumours (poorly differentiated). The predominance of grade II tumours in the present series is in corroboration with that of the previous studies. Patel et al 2002 reported maximum no. of cases in grade II (41%), followed by grade I (37%) and grade III (22%) [5]. Niveditha and Bajaj 2003 reported 55% of the cases to be grade II tumours [28]. However, some studies have found grade III to be the highest grade like Elston and Ellis 1991 who graded 1830 tumours and found grade III (47%) comprised the largest group followed by grade II (34%) and grade I (19%) [8].

Immunohistochemical staining for estrogen receptor, progesterone receptor and c-erb B_2 protein was done in 100 cases of breast carcinoma. Estrogen receptor positivity was seen in 63 (63%) cases (Table-2). Progesterone receptor positivity was seen in 53 (53%) cases (Table-3). The estrogen and progesterone receptor positivity was graded according to the intensity of staining of tumour nuclei [24]. The findings of the present study are similar to that of Lesser et al 1980 who reported 64% positivity for ER receptors but PR positivity was lower (22%) than our study [29]. Bonneterre 1987 and Anderson & Poulsen 1989 showed a higher percentage of ER positive tumours, 81% and 70% respectively [30, 31]. However PR positivity in Bonneterre study was 57% which is in close corroboration with the present study [30]. Nadji et al 2005 observed positive nuclear staining for ER and PR in 75% and 55% of invasive carcinomas, respectively [32]. Our study showed combined reactivity of ER & PR in 53% cases which was quite similar to results obtained by Fatima et al (2005) who showed 55% ER and PR reactivity [33].

Immunohistochemical staining for C-erb B_2 was done on 100 cases. 24 (24%) cases showed positivity (Table-4). The positivity was either membrane or diffuse cytoplasmic. The results in the present study correlate well with many studies like that of Singleton et al (1992) who showed strong cytoplasmic membrane reactivity in 23% cases [34]. Kim et al (2006) found 25% positivity for c-erb B_2 [35]. Sjogren et al (1998) showed c-erb B_2 overexpression in 19% of cases [36]. However, some studies have found high positivity for C-erb B_2 like that of Shin et al (2006) and Shahrun et al (2008) who found positivity of 51.5% and 44.4% respectively [37, 38].

In the present study, Grade III tumours showed strong staining for C-erb B_2 in 14 (56%) cases, grade II in 10 (22%) cases whereas all grade I cancers were negative for C-erb B_2 (Table-4). Looi et al (1997) in their study on 112 cases showed c-erbB-2 immunopositivity rates of 0%, 20.6% and 51.4% in grade I, II and III tumours respectively [39]. Tsuda et al (1990) found HER-2 amplification in 0% of grade 1, 10% of grade 2 and 33% of grade 3 ductal carcinomas [40]. Rilke et al (1991) studied a large series of 1,210 cases and found HER-2 overexpression rates of 3.9%, 20.4%, and 38.9% in tumors of grades 1, 2, and 3, respectively [41]. Hoff et al (2002) in a study of 388 cases found that HER-2 was amplified in less than 1% of grade 1, 17% of grade 2, and 23% of grade 3 tumors [42].

The relationship of histological grade and other biomarkers have been studied by many previous studies and a strong correlation was found in the present study. In the present series, 93% of grade I tumours were positive for ER and 83% for PR. Grade II tumours showed 73% positivity for ER and 57% for PR. 8% of grade III tumours were positive for ER or PR. A statistically significant correlation was found between receptor status and grade (ER vs. grade $p < 0.00001$ and PR vs. grade $p < 0.00001$) (Table-2 and 3 respectively). Fatima et al (2005) showed 55% ER and PR reactivity and found that ER positivity decreased with increasing tumor size and grade, however, no significant correlation was seen with lymph node metastasis [33]. Lal et al (2005) et al in their study found that the rate of ER and PR expression in nuclear grade 2 HER-2+ tumors was significantly higher than that in nuclear grade 3 tumors (ER and PR, $p < .0001$) [43].

A statistically significant relationship of c-erb B_2 and histological grade was present ($p < 0.00001$) (Table-4). Hoff et al (2002) found a statistically significant correlation between amplification of HER-2/neu in SBR grade 1 invasive ductal carcinomas when compared with SBR grade 2 and grade 3 invasive ductal carcinomas ($P < .005$ and $P < .001$, respectively) [42]. Ferrero-Poüs et al (2000) observed an association between c-erbB-2 overexpression and histoprognostic grade (SBR) [44]. Most previous studies like that of Rilke et al [41] have suggested that c-erbB-2 positivity is related to higher SBR grade. However the findings in the study of Heintz et al (1990) showed no relation between tumour membrane immunoreactivity and histological grade [45].

C-erb B_2 immunoreactivity was associated with a high tumour grade and negative ER and PR receptors in most of the cases. Out of 24 grade III tumours, 14 (56%) showed strong staining for C-erb B_2 which were negative for ER and PR. Out of 45 grade II tumours 10 (22%) were positive for C-erb B_2; however 3 of them were also positive for ER and PR. These observations were made in several previous studies including study by Tandon et al (1984) who showed HER-2 overexpression with steroid receptor negativity as well as with shortened disease free survival [20]. Ferrero-Poüs et al (2000) observed a strong correlation between c-erbB-2 protein overexpression and steroid receptor negativity [44]. A significant negative association of c-erb B_2 with estrogen and progesterone presence was shown by Heintz et al (1990) in 50 cases [45] and also by Keshgegian (1995) who showed inverse correlation in 320 cases [46].

Cianga et al (2003) showed 40.91% of positivity for c-erb B_2 protein along with a positive nodal status and absence of hormonal expression ($p<0.05$) [47]. Hlupic et al (2004) also in a retrospective study identified the presence of aggressive biological behavior and metastatic potential in breast carcinoma among a number of invasive biomarkers such as steroid receptors and oncogenes and tumour suppressor gene products [48]. Yang et al (2004) also found significant correlation between c-erb B_2 and receptor negative status. Also the c-erb B_2 expression was correlated with advanced clinical stage, high histological grade and axillary node involvement [49].

CONCLUSION

This study concludes that a strong correlation exists between histopathological grading and ER, PR and C-erb B_2 status. C-erb B_2 overexpression was limited to high grade cancers. An inverse relation was found between ER / PR and C-erb B_2 expression with higher grade cancers being associated with C-erb B_2 positivity and ER/PR negativity.

Hence, it is recommended to include the immunohistochemical assessment of ER, PR and C-erb B_2 status along with histopathological grading in the diagnosis of breast cancers so as to guide the management of the patients.

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