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Prognostic Correlation of Tumor Growth Fraction and Hormone Receptor Status in Breast Carcinoma

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Abstract

Objective: The aim was to assess the status of estrogen and progesterone receptors, p53 and MIB-1 labeling in invasive duct carcinomas in our institute. **Study design:** A series of 63 invasive duct carcinomas occurring in women for consecutive five years were studied at our institute. **Subjects and methods:** In this study 63 cases of invasive duct carcinoma, including all the histological subtypes were reviewed with original archival data including histological type of tumour and tumour grade. Tumour blocks of each case were retrieved for immunohistochemical staining of estrogen and progesterone receptors, MIB-1 and p53. The expression was studied using established protocols. The majority of the cases were above 30 years of age and postmenopausal. In total 77.8% had node positive disease with 71% having nodes greater than 2 cm. Estrogen and progesterone (ER and PR) receptor positivity was seen in 32.5% and 28.5% respectively. According to the percentage of nuclear staining, 60.3% of the cases were p53 positive and 77.7% showed proliferation activity as seen by MIB-1 labeling. The cases were analysed after following up with survival data. **Conclusion:** P^{53} and MIB-1 are independent indicators of aggressiveness and grade of the tumors. They have the potential to replace SBR grading of breast cancer.

Keywords: Immunohistochemistry; Breast carcinoma; MIB-1 labeling index.

Introduction

Globally more than a million women are diagnosed with breast cancer every year. Incidence rates vary considerably, with the highest rates in the developed world and the lowest rates in Africa and Asia. Over 50% of breast cancer incidence occurs in the developed world [1]. However, it still ranks as the commonest cancer among women in these regions. Breast cancer is the second leading cause of cancer death in women, exceeded only by lung cancer. It is responsible for 19% of all cancer related deaths in women in the world [2-5].

The population based cancer registry data from the various parts of the country, has revealed breast cancer as the commonest cancer among women in Delhi, Mumbai, Calcutta and Trivandrum. Cancer of the breast has replaced cancer of the cervix as the leading site of cancer in all urban Population Based Cancer Registry (PBCR), except Chennai and

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the Age Adjusted Annual Incidence Rates (AAR) of this site of cancer have also been on the rise. In the rest of the other Indian registries, breast cancer is listed as the second leading site among women [6,7]. This malignancy accounts for 19–34% of all cancer cases among women nationally [8].

Histological types of tumor, grade, axillary lymph node involvement, bilateralism etc. have been established as the predictors of tumor behavior. These prognostic factors are indicators of the inherent aggressiveness and the extent of the disease. Based on these factors, treatment decisions are being taken.

The aim of the study was to review, grade and stage all cases of infiltrating duct carcinoma for five consecutive years. Immunohistochemistry for presence of p53 and MIB-1 in the above said tumors was carried out and correlation with proven biomarkers was attempted. A correlation of the studied biomarkers with disease prognostication was carried out. The numerous questions regarding the new molecular markers in the background of prognostic factors and indicators of inherent aggressiveness have lead to the effort presented here.

Materials and Methods

Sixty-three consecutive cases of infiltrating duct carcinoma breast over a period of five years at the oncology department were studied. Regularly followed up, female patients of infiltrating duct carcinoma, whose paraffin blocks were available in the archives, were included in the study. Follow-up data was obtained from case notes and the malignant diseases treatment centre registry. Duration of follow-up was documented as time from diagnosis to the last recorded outpatient visit or inpatient hospital stay. Thus the maximum possible follow up period was 54 months and the minimum was 6 months in a patient currently alive at the time of analysis.

The cohort of 63 Indian women was aged between 27 to 80 years. Information regarding the surgery, tumor dimensions, surgical margins' status, and number of retrieved axillary lymph nodes was collected. The clinical records were reviewed for data regarding adjuvant treatment and outcome parameters. For each case, representative H&E stained slides were reviewed to assess tumor grade, histological type. The status of estrogen and progesterone receptors (ER & PR), MIB-1 labeling index (MIB-1 LI) and p53 score was examined by IHC on paraffin blocks. Scarff's modification of Bloom and Richardson's grading system was used to determine the malignancy grade of tumor samples.

Immunohistochemistry

IHC was performed on paraffin sections (5ì thick) placed on poly-L-lysine coated glass slides. Monoclonal mouse antibody to human ki-67 antigen (clone MIB-1), p53 (clone DO7), ER (clone 1D5) and PR (clone PgR 636) were obtained from DAKO (Dako, Carpinteria, California). Antigen retrieval was done in citrate buffer (0.01 mol/l, pH6.0) with a microwave oven at 700 watts. The slides were then cooled to room temperature for 1 hr. Endogenous peroxidase activity was blocked by immersing sections in 3% hydrogen peroxide in methanol for 30min. Sections were then incubated with primary mouse anti human ki-67 antibody (1/100 dilution) for 60 minutes at room temperature. Biotinylated anti mouse secondary antibody was applied for 60 min at room temperature. Bound antibody was visualized with Universal Dakocytomation Labeled Strepavidin-Biotin 2 system, Horseradish Peroxidase (LSAB-2 System-HRP code K0675, Carpinteria, California) for 60 minutes at room temperature. The colour was developed by 3,3' diaminobenzidine tetrahydrochloride (DAB). Between the steps, the slides were rinsed three times for 10 min in Tris buffered saline (pH7.6). The slides were counterstained lightly in Harris' Haematoxylin and were dehydrated and mounted. Appropriate positive and negative controls were put up. Antigen retrieval for ER and PR was also carried out using Tris EDTA buffer pH 9.0.

The details of the procedure are given elsewhere [9].

IHC evaluation and scoring

MIB-1 expression was quantified using visual grading system. 500 cells were counted at 40x magnification, in hot spots, among them the cells with positivity for MIB-1 were counted and percentage positivity for MIB-1 staining was calculated. Three ordered categories of MIB-1 were created based on percentage positivity as follows - d"9.5%, >9.5-d"15.5%, and >15.5%. The selection of these cut off values was based on the proportion of tumors that scored 1, 2, and 3 points of mitotic activity using criteria of Elston and Ellis [10].

Immunoreactivity for p53 was classified as 0, 1, 2 and 3 if 0–9%, 10–25%, 26–50%, >50% of the tumour cell nuclei, respectively, were positive. A carcinoma was classified as p53 positive when at least 10% of the nuclei were immunoreactive. Immunostaining patterns for ER and PR in breast carcinomas are normally heterogeneous and a total score of e" 3 was considered positive as per the method used by Allred et al [11].

Results

Disease free survival (DFS) is defined as the time from randomization to recurrence, metastasis, appearance of second primary tumour, or death from any cause whichever occurred first. Overall survival (OS) is defined as time from randomization to death from any other cause. The statistical analysis was carried out on SPSS (Statistical Package for Social Sciences) for windows version 14.5. Confidence interval was set to 95% i.e. with the p values < 0.05, the difference between two groups could have occurred by chance alone in less than 1 in 20.

We studied 63 patients in all, out of which 30 (47.6%) were £ 50 years and 33 (52.4%) were >50 yrs of age. 30 (47.6%) women were premenopausal and 33 (52.4%) postmenopausal. 14 (22.5%) women had node negative (N0) disease and 49 (77.8%) had nodal involvement (N1, N2 or N3). 18 (28.5%) patients had tumor d"2cm in diameter, and 45 (71%) had >2cm. ER status was positive for 20 (32.7%) patients. PR was expressed in 18 (28.5%). 38 (60.3%) patients were positive for p53 and 49 (77.7%) were positive for MIB-1. The follow up was analyzed in three patient groups viz. no evidence of active disease (NED); Alive with disease (AWD), irrespective of whether the disease was residual, recurrent, or metastatic; Dead.

The number of patients in the follow up groups was 36, 19 and 8 respectively. The following parameters were studied for statistical significance independently in patients with p53 positive tumors viz. size, Scarff Bloom Richardson (SBR) grade, ER, PR, and follow up in both node negative as well as node positive group. Similar parameters were studied for MIB-1 positive tumors. Spearman correlation co-efficient was studied for correlation between p53 and MIB-1 positivity. Kaplan Meier Survival curves were plotted for both p53 and MIB-1 groups.

Pearson Chi square value for relationship between patients' age and menopausal state when comparing with MIB-1 score was 0.041 ($P<0.05$), which is statistically significant. Pearson Chi square values for relationship between tumor size and MIB-1 score in node negative and node positive patients is 0.018 and 0.860. Thus tumor size and MIB-1 score correlate in patients with tumor size d" 2 cm ($P=0.018$). Pearson Chi square values for Comparison between SBR grade & MIB-1 score in node negative group was 0.23 and in node positive group 0.0001(strongly significant) (Table 1).

Table 2 shows cross tabulation between ER, PR expression, p53 score and follow up with MIB-1 labeling index score. Pearson Chi square values for relationship between ER status and MIB-1 in node negative and positive patients are 0.758 and 0.031 respectively. Thus MIB-1 score and ER positivity correlate only in node positive patients ($P=0.031$). Pearson

Chi square values for relationship between PR status and MIB-1 score in node negative and node positive patients is 0.152 and 0.136 respectively. Thus progesterone receptor status does not statistically correlate with MIB-1 score in both groups. Table 2 also shows that Pearson chi square test for co-relationship between p53 score and MIB-1 labeling index in node negative and node positive patients were 0.370 and 0.001. Thus p53 and MIB-1 scores have significant correlation in node positive cases.

Pearson's chi square test values for relationship between patient survival and MIB-1 score in node negative and node positive patients were 0.719 and 0.021 respectively. Thus a statistically significant correlation has been found between patient's follow up and MIB-1 score in node positive patients (p=0.021) only.

Pearson chi square value for relationship between age, menstrual status and p53 was <0.001, which is highly significant. Pearson chi square value for relationship between tumor size and p53 score in node negative and positive patients is 0.044 and 0.081 respectively. Thus tumor size and p53 correlate in patients with tumor size £2cm in node negative patients. Pearson Chi square values for Comparison between SBR grade and p53 score in node negative and positive patients were 0.060 and 0.108 which is not significant. Table-4 shows that Pearson Chi square values for relationship between ER status and p53 score in node negative and node positive patients were 0.226 and 0.027(significant) respectively.

Table 5 shows that Pearson Chi square values for relationship between PR status and p53 score in node negative and node positive patients were 0.078 and 0.009 (significant). Table-6 shows that Pearson Chi square values for relationship between patients follow up and p53 score in node negative and positive patients were 0.417 and 0.099 (not significant).

Table 1. Relationship between patients' parameters (age, menopause, size of tumor and SBR grade) and MIB-1 Score

S.No.	Parameters			MIB-1 Score				Tota	p value
				0	1	2	3		
1.	*Age*	*=50*		7	3	11	9	30	0.041
		>50		7	11	122	3	33	
2.	*Premenopausal*			7	3	11	9	30	0.041
	Postmenopausal			7	12	21 11	3	33	
3.	*T size*	*LN -ve*	*=2cm*			4		4	
			>2cm	3	4	1	2	10	0.018
		LN+ve	*=2cm*	3	3	6	2	14	
			>2cm	8	8	11	8	35	0.860
4.	*SBR*	*LN-ve*							
	Grade-1			2	3			5	0.23
	Grade-2			1	1	4	1	7	
	Grade-3					1	1	2	
		LN+ve							
	Grade-1			9	6	1	4	20	0.0001
	Grade-2			1	4	12	2	19	
	Grade-3			1	1	4	4	10	

LN- lymph node status; SBR – Scarff Bloom Richardson Grading

Table-2. Relationship between patients' parameters (ER and PR status, p53 score and follow up) and MIB-1 score

S No.				MIB-1 Score				Total
				0	**1**	**2**	**3**	
1.	ER Status	LN-ve	ER-ve	2	3	3	2	10
			ER+ve	1	1	2		4
		LN+ve	ER-ve	5	5	14	9	33
			ER+ve	6	6	3	1	16
2.	PR status	LN-ve	PR-ve	1	4	4	2	11
			PR+ve	2		1		
		LN+ve	PR-ve	5	7	13	9	34
			PR+ve	6	4	4	1	15
3.	P53 score	LN-ve	P53-1	2	2	1	1	6
			P53-2	1	2	1		4
			P53-3			3	1	
		LN+ve	P53-1	7	11	6		24
			P53-2	4		5	1	10
			P53-3			6	9	15
4.	Follow up	LN-ve	NED	3	3	4	2	12
			AWD		1	1		2
		LN+ve	NED	9	8	5	2	24
			AWD	2	1	9	5	17
			Dead		2	3	3	8

NED – No evidence of disease; AWD – Alive with disease; LN – Lymph node

Table 3. Relationship between patients' parameters(age, menopause state, tumor size and SBR grade) and p53 score cross tabulation

S No				p53 Score				Total	p value
				0	**1**	**2**	**3**		
1.	Age	=50	years	4	2	9	15	30	<0.001
		>50		21	3	5	4	33	
2.	menopause		Pre-	4	2	9	15	30	
	status		Post-	21	3	5	4	33	
3.	Tumor	LN-ve	=2cm	1			3	4	0.040
	Size		>2cm	5		4	1	10	
		LN+ve	=2cm	3	3	5	3	14	0.081
			>2cm	16	2	5	12	35	
4.	SBR	LN-ve	Gd-1	4		1		5	0.06
	Grade		Gd-2			3	4	7	
			Gd-3	2				2	
		LN+ve	Gd-1	9	3	2	6	20	0.108
			Gd-2	9		5	5	19	
			Gd-3	1	2	1	6	10	

Table.4. ER, p53 and Node status Cross tabulation

Node Status			p53 Score				Total
			0	*1*	*2*	*3*	
Negative	*ER*	*Negative*	3		4	3	10
		Positive	3			1	4
	Total		6		4	4	14
Positive	*ER*	*Negative*	8	4	8	13	33
		Positive	11	1	2	2	17
	Total		**19**	**5**	**10**	**15**	**49**

Table 5. PR, p53 and Node status Cross tabulation

Node Status			p53 Score				Total
			0	*1*	*2*	*3*	
Negative	*PR*	*Negative*	3		4	4	11
		Positive	3				3
	Total		6		4	4	14
Positive	*PR*	*Negative*	8	4	8	14	34
		Positive	11	1	2	1	15
	Total		**19**	**5**	**10**	**15**	**49**

Table.6.Follow up, p53 and Node status Cross tabulation

Node Status			p53 Score				Total
			0	*1*	*2*	*3*	
Negative	*Follow up*	*NED*	6		3	3	12
		AWD			1	1	2
	Total		6		4	4	14
Positive	*Follow up*	*NED*	13	3	4	4	24
		AWD	3	1	6	7	17
		Dead	3	1		4	8
	Total		19	5	10	15	49

NED= No evidence of active disease; AWD= Alive with disease

Table 7. Spearman's correlation coefficient between MIB-1 and p53

			MIB-1	p53
Spearman rho	MIB-1	Correlation coefficient	1.000	.608**
		Sig. (2 tailed)	.	.000
		N	63	63
	p53	Correlation coefficient	.608**	1.000
		Sig. (2 tailed)	.000	.
		N	63	63

**Correlation is significant at the .01 level (2-tailed)

Discussion

In India, breast cancer is the most common cancer among women in many regions and has overtaken cervix cancer, which was the commonest cancer a decade ago. The continuing rise in breast cancer incidence and mortality has made it a necessity to aggressively manage the disease with the help of upcoming diagnostic molecules which may predict tumor aggressiveness and tumor response to various treatment modalities. Breast cancer appears to have a complex molecular biology, possibly with interplay of many causal factors including genes like p53, PTEN, BRCA-1, BRCA-2, etc and expression of proliferative antigens like ki-67, c-erb1, c-erb-2 (HER-2-neu), c-erb3, etc. Analysis of newer parameters like p53 and MIB-1 could be helpful in rapidly labeling the patient as high grade or low-grade disease, followed by planning of future treatment modalities.

Numerous studies of morphologic assessment of tumor differentiation have provided useful information in patients with breast carcinoma [11-23]. The concept that nuclear morphology of the tumor may have implications for their biologic behavior is due to von Hansemann, whose studies have been the starting point of many grading systems for carcinoma. Among the studies of Ki-67 staining in breast carci-noma, both the incidence of Ki-67 positivity and the per-centage of cells stained varied considerably. Bouzubar et al [24] found that high level of ki-67 expression was associated with early recurrence of breast cancer after mastectomy. However we did not have any recurrence during the study period. In a meta-analysis done by de Azambuja et al, it was found that High MIB-1 LI confers a high risk of relapse and worse survival in early breast cancer patients [25].

Walker and Camplejohn [26] reported ev-idence of staining in only 53 (56%) of 95 cases. In the former study, 58% of the cases showed staining in >15% of the cells and in the latter study, that degree of staining was, achieved in 30% of the cases. We observed Ki-67 or MIB -1 positivity in 49 (77.7%) of 63 cases, and 12 (24.48%) of those positive tumors had at least 15% of the nuclei, stained. The discrepancies between these studies in terms of per-centage of cells stained may be related to the number of tumor cells evaluated per section: the higher the number of cells, the more accurate the definition of percentage MIB-1 positivity. In our study, we minimized the counting bias by evaluating at least 10 HPF and counting 500 nuclei. Breast carcinomas are known to be heterogeneous in their expression of Ki-67 immuno-reactivity. The method of selecting areas for counting represents another possible source of variability.

In breast carcinoma patients, some authors have found no association between Ki-67 im-munoreactivity and other prognostic variables. Studies by Pinder et al [22] and Barbareschi et al [23] have reported an association with histological grade, lymph node status, age, tumor size, ER and PR status. An association between Ki-67 staining with both disease free interval and survival has been studied by Trihia et al (16) and it was observed that higher ki-67 percentages lead to decreased DFS as well as OS. Our study found a similar correlation, however we stratified the patients in node negative and node positive groups, and found that the association is significant only for node positive patients. In our study, all the 8 patients who died stained positively for MIB-1. Amongst them two had MIB-1 score of 1, three had a score of 2 and three had a score of 3. Statistically significant (P=0.021) relation was found between patient follow up and MIB-1 scoring in node positive patients. Bouzubar et al [24] found that high level of ki-67 expression was associated with early recurrence of breast cancer after mastectomy. However we did not have any recurrence during the study period. In a meta-analysis done by Azambuja et al, it was found that High MIB-1 LI

confers a high risk of relapse and worse survival in early breast cancer patients [25].

The relation ship between MIB-1 score and SBR grading in our study was significantly higher compared with chance alone (P=0.001) in node positive patients. Our study confirms the value of Ki-67 evaluation as an objective means for the prediction of histological grade and survival in patients with breast carcinoma. It is not suggested that measurement of Ki-67 alone can provide data of equivalent value to grading. What is demonstrated is that Ki-67 is a very reliable replacement for mitotic counts and would be easier to apply in FNAC and core biopsies, in which there are limited numbers of cells and tissue present.

As more conservative surgical and staging techniques increasingly are introduced into the management of patients with breast carcinoma, other use-ful prognostic information, including tumor size, tumor grading, vascular invasion, and lymph node involvement, will not be assessable. MIB-1 and p53 appear to be superior to others for assessing tumor proliferation on routinely fixed and processed material.

Direct correlation has been observed between p53 and MIB-1 and ER negativity. No association was found between p53 and age, Size, Grade, type of lymph node involvement and risk for both relapse and death from breast cancer by Shehsadri R et al [27]. We were also able to demonstrate a direct correlation between p53 and MIB-1 with Spearman's correlation coefficient of 0.608. We also got inverse correlation between ER and MIB-1 (Spearman's -0.398 in node positive group, and -0.102 in node negative group). We were further able to demonstrate negative correlation between PR and MIB-1 (Spearman's -0.382 in node positive and -0.333 in node negative).

We found that p53 was expressed more in patients less than 50 years of age premenopausal women (86.6%, p=0.041). Also p53 was found in all patients in follow up group 2 i.e. alive with disease, in node negative patients- p53 was positive in 82.3% cases. However no statistical significance was obtained for relation between patient follow up and p53 scoring. We found a statistically significant inverse correlation between p53 score and ER (p=0.027) and PR (p=0.009).

Therefore, MIB-1 and p53 can be applied to small samples and that may be of prognostic significance. There are many other possible parameters to assess, but there is a need for a large, controlled study to assess markers in small biopsies and FNACs, that can substitute for the parameters used in classic grading.

Conclusion

Both p53 and MIB-1 expression have been found out to be independent indicators of aggressiveness of the disease in our study. p53 as well as MIB-1 are independently inversely related to age and menopausal status. MIB-1 score is directly related to SBR grading, more so in node positive patients. MIB-1 is inversely related to ER expression in node positive patients. High p53 and/or MIB-1 indicates shorter DFS and hence an aggressive disease. MIB-1 and p53 have a potential to become a component of or replace the SBR grading of breast cancer. However multicentric studies with larger sample size and follow up need to be carried out to confirm this.

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Figure.1: Kaplan Meier plot for DFS according to MIB-1 LI. Observation- proportion of patients with a higher MIB-1 Labeling Index have a lower DFS vs. lower MIB-1 LI

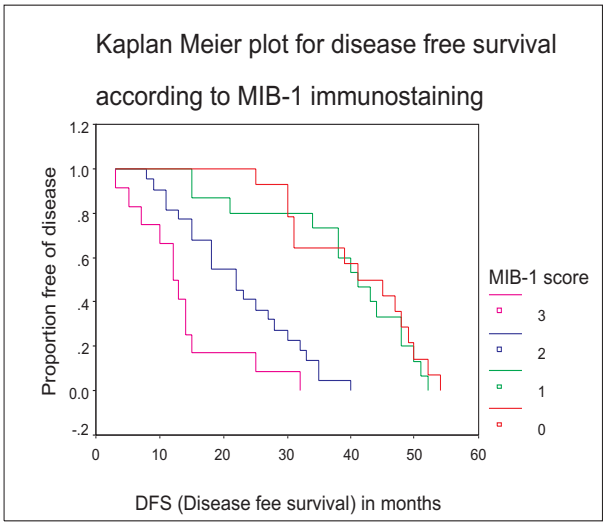

Figure.2: Kaplan Meier plot for DFS according to p53 score. Observation- proportions of patients with a higher p53 score have a lower DFS vs. lower p53 score

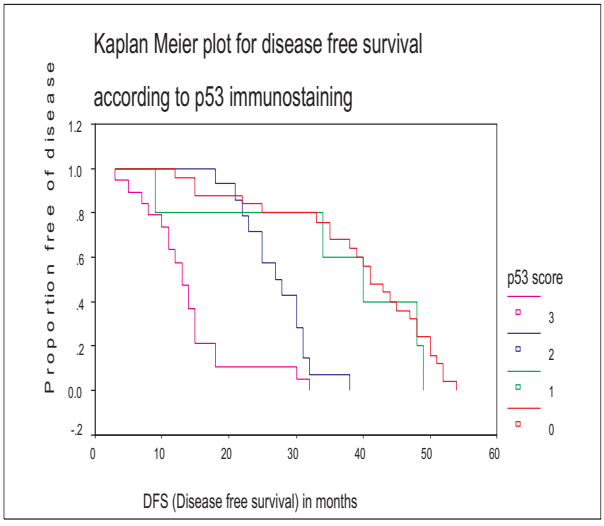

Figure 3 (A-H) : Photomicrograph of IDC showing SBR grade-1 (A : H&E x 40) and grade-3 (B : H&E x 40). Immunohistochemistry showing strong positivity for ER (C : ER x 40) and PR (D : PR x 40); low p53 (E : p53 x 40) and MIB-1 LI (F : MIB-1 x 100) in low grade IDC, and high p53 (G : p52 x 100) and MIB-1LI (H : MIB-1 x 100) in high-grade IDC