

## Relationship of p53 expression with clinicopathological variables and disease outcome: a prospective study on 315 consecutive breast carcinoma patients

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### Abstract

Breast cancer is an increasingly important cause of illness and death among women. In recent years several novel prognostic determinants of breast cancer have been identified which includes p53. Alterations of p53 are one of the most common abnormalities detected in primary breast cancer. In this study alteration of p53 in primary carcinoma breast was **correlated** with other pathological variables and disease outcome. In this prospective study the expression of p53 oncoprotein was analyzed immunohistochemically on 315 patient's tumour specimens of infiltrating ductal carcinoma of breast from 1992 to 1997. These patients also had axillary lymph nodes sampling. Both univariate and multivariate statistical analysis was performed to analyze results including disease outcome. Overexpression of p53 was observed in 55.23% tumours. **Axillary** lymph node metastasis had significant correlation with positivity of p53 ( $p < 0.05$ ). A significant number of p53 patients developed local recurrence and distant metastases to brain, liver, lung and bone ( $p < 0.05$ ). At a median follow-up of 48 months (4 years) in p53 positive patients, the median overall survival (OS) was 3.0 years and disease free survival (DFS) was 2.5 years. p53 negative tumour patients showed a better survival. In this group the median OS was 3.8 years and the DFS was 3.3 years. The above findings have reinforced the view that p53 immunohistochemical detection is of help in detecting a subgroup of breast carcinoma patients who are at high risk. This may also be of particular relevance in decisions regarding adjuvant chemotherapy to these patients.

**Key words:** Carcinoma breast, p53, nodal metastases, disease outcome

### INTRODUCTION

In recent years, there has been increasing interest in the spectrum of genetic alterations that occur in human malignancies, as well as on the utility of their detection for the purpose of evaluating tumour behavior. Nuclear accumulation of p53 protein due to p53 gene mutation emerges as a common molecular event in most human neoplasias that is detectable by immunohistochemistry because of the abnormal p53 protein prolonged half-life. The functionally normal wild-type p53 is very unstable and therefore is not identified in routinely processed histopathological material. In contrast, **most** mutant forms of p53 gene are associated with relatively stable proteins amenable to immunohistochemical study<sup>1</sup>.

p53 is a critical controller of normal growth and homeostasis of cells and tissues. It is a tumour suppresser gene, which is located on the short arm of chromosome 17<sup>2</sup>, the inappropriate function of which can lead to disordered growth

and malignancy. Normal p53 acts as a "guardian of the genome" by preventing the proliferation of cells with damaged DNA. It does this by the production of normal or wild type p53 protein (a 53kDa nuclear phosphoprotein), which acts on downstream genes to arrest the cell cycle until the DNA damage is repaired, or to cause apoptosis.

p53 is the most commonly mutated gene in human cancers with probable involvement in the development of at least 50% of clinical tumours. Mutations of this gene results in progression of cells with damaged DNA through the cycle. p53 loss is believed to lead to widespread karyotypic instability resulting in aneuploidy with multiple genetic amplifications and deletions such as loss of heterozygosity. Inactivation of p53 can occur through several mechanisms, including loss of p53 alleles or by deletion, insertions, or point mutations. Most mutations in the DNA are misense base substitutions in the p53 coding

sequence that changes a single amino acid in the core domain, resulting in conformational change and stabilization of the translated protein in the central region of the molecule. One aberrant allele can be sufficient to compromise tumour suppressor function and will thus be selected for.

The main objective of this study was to assess the independent and interdependent prognostic value of p53 in carcinoma of breast in our female population. P53 role as a prognostic marker in breast carcinoma has been widely studied in recent years<sup>3,4</sup>, however results are varied and a consensus has not been achieved about its utility in clinical practice. It is increasingly appreciated in recent years that ethnic differences may result in a different biological behavior of the same disease in different patient populations<sup>5</sup>. Breast cancer is no exception to that. Studies have indicated that breast cancer may have a different outcome among different ethnic groups in a country such as United States. These ethnic groups include the black, Hispanic, white and other population groups<sup>6</sup>. Data of such a nature is sparse from our part of the world. In general in Pakistan mean age of breast cancer patients at the time of diagnosis is ten years earlier than the west. Most of our patients are still diagnosed at an advanced stage while modified radical mastectomy with axillary clearance followed by medical/radio therapy is the standard mode of treatment in most cases.

## MATERIALS AND METHODS

A sample of 315 patients with histologically proven diagnosis of invasive ductal carcinomas (IDC) of breast with lymph nodes sampling from January 1992 to December 1997 were included in the study. Based on available information we assumed a difference of 1.5 years in survival time of patients with p53 positive and p53 negative expressions. Our sample size of 315 was expected to detect this difference with a power of at least 90% at 5% level of significance.

Age and morphological variables like grade, lymphovascular invasion, lymph node status and tumour size were recorded. Tumours were graded according to the modified Bloom and Richardson grading system taking into account three parameters i.e., degree of tubule formation, nuclear pleomorphism and number of mitosis. Other variables like menopausal status, parity, distant metastasis, treatment protocol and

survival details were retrieved from their medical records. For staging, the TNM classification was used.

Fixation and processing was by routine methodology. After processing, the tissues were embedded in paraffin using the HistoCenter 2 from Shandon. 5mm thick sectioning was done by Microtome AS 325 from Shandon. The same breast tumour paraffin blocks were used to prepare further sections for immunohistochemistry (IHC). The sections were cut and picked on poly-L-Lysine coated slides. Expression of p53 protein was evaluated using mouse anti-human p53 oncoprotein monoclonal antibody p53 D07 (Dako, Denmark), diluted at 1:25, following pre-treatment of sections in a microwave oven (5x3 min. at 630 W) using PAP technique. A breast carcinoma section expressing p53 was used as a positive control. The same case, with omission of the primary antibody, served as a negative control with each staining procedure. Oestrogen and progesterone receptor (ER/PR) status was also determined by IHC using monoclonal antibodies against ER (clone 1D5) and PR (clone 1 A6, Dako). Only nuclear staining was taken into account and any cytoplasmic staining was discarded. A semiquantitative evaluation as described by McCarthy was adopted.<sup>7,8</sup> Evaluations were recorded as percentages of positively stained tumour cells in each of five intensity categories denoted as 0 (no staining), 1+ (weak but detectable), 2+ (mildly distinct), 3+ (moderately distinct) and 4+ (strong). For each tissue a value designated as HSCORE was derived by summing the percentages of cells staining at each intensity multiplied by the weighted intensity of staining. An HSCORE of <74 was established as negative, between 75-99 as weak positive, 100-119 as intermediate positive, and 120 and more as strong positive.

The percentage of p53 positive tumour cells was estimated semi-quantitatively and only when brown nuclear staining was seen in tumour cells in up to 10% of tumour cells. They were graded on a scale of 0-3, 0% (negative), <10% (mild i.e., any detectable staining in up to 10% of tumour cells), 10-50% (moderate) and >50% (strong). In addition to the number of cells the intensity of reaction was also taken into account and scored on a scale of I-IV (-, +, ++, +++ or ++++). However in further analysis (Tables 1 & 2) only the former grading based on the number of tumour cells showing nuclear staining was taken into account. This is in consensus with the approach used by IHC external quality surveys

of the College of American Pathologists (CAP) programmes. To minimise subjectivity, slides were scored on a double-headed microscope (Olympus **BX50**) separately by two senior histopathologists. Immunostaining was repeated on equivocal cases and consensus was achieved between the two pathologists in all cases.

### *Statistical analysis*

Our main interest was to estimate the survival time for breast cancer patients and look into the relationship between survival time and their prognostic variables. The death of the patients was considered as an event. The data was examined carefully, and decisions were made on how a variable was to be analyzed. Either a continuous variable could be analyzed as such, or categorized according to cut off levels, which are biologically plausible.

The Kaplan Meier estimator is an important tool for analyzing censored data. The survival curves, the mean (Standard error for mean), median survival time (standard error for median) along with the 25th and 75th percentiles were estimated for each prognostic variable using this method.

*Univariate analysis* was done to examine the relationship of each prognostic factor with the survival time using the Cox proportional hazard model or Log rank test. For qualitative variables, if more than two categories existed, then dummy variables were introduced. Hazard ratios along with their 95% **CI** were used to describe the relationship between each prognostic variable and the outcome variable.

*Multivariate analysis* was done to identify a subset of prognostic variables that relate significantly to the hazard, and consequently the survival of the patient. The model fitting was aimed to fit the most parsimonious model, which was biologically able to explain the data. The **multivariate** analysis also helped us to control for the confounding and study effect modification. An adjusted hazard ratio along with their 95% **CI** was used to describe the relationship between the set of prognostic variables and the **outcome** variable.

## **RESULTS**

### *Descriptive analysis*

Table 1 provides the descriptive statistics about the samples. Analysis was done on a total number of 315 observations, with 36.2% survivals until the end of this study i.e. May 1999. There were four censored observations due to deaths from

causes other than breast cancer. The mean and median survival times were calculated using the Kaplan Meier technique. Since in our country breast carcinomas occur at a relatively younger age (approximately 10 years younger than the Western world) with the incidence more common in the reproductive age group, we dichotomized the age at a cut off level of 49 years. Thus 51% of the subjects were in the reproductive age group, with a mean survival time of 3.35 years (standard error {**SE**}=0.13) in contrast to the 49% in the post-menopausal age with a mean survival time of 3.17 years (**SE**=0.14). On an average, 25% of the subjects in the **pre**-menopausal group were surviving more than 4.67 years, in contrast to 4.16 years in the postmenopausal group. The median survival time was also better among the pre-menopausal group, with 50% of the subjects surviving more than 3.58 years, in contrast to the **3.00** years median survival time for the post-menopausal group.

Histological grading showed a median survival time of 3.33 years and 3.17 years for the subjects with grade I and grade **II** tumours, with corresponding mean survival times of 3.41 years and 3.11 years, while those with grade **III** tumours had median survival times of 2.67 years with a mean of 2.91 years. Similarly, with increasing tumour size, the prognosis appeared to worsen, with better mean and median survival times among subjects with smaller lesions. When the prognostic marker p53 was absent, the mean and median survival times were found to be significantly better. Mean overall survival in p53 negative patients was much better compared to when this marker was positive. This correlation with mild, moderate and strong p53 positivity is also shown in Table 1. For staging we used the TNM (tumour, node, metastasis) classification. A better survival for the subjects in an early stage is seen, with a **mean** survival of 3.45 years and a median survival time of 3.75 years for stage I in comparison to the mean survival of 2.86 years and a median survival time of 2.46 years for stage IV.

Chemotherapy and hormonal therapies both appeared to improve the prognosis by improving survival, since better mean and median survival times in subjects are observed among patients who were on these therapies in comparison to those who did not receive the intervention. The presence of metastatic lesions in any organ of the body was negatively associated with prognosis as mean survival time for subjects with distant metastasis was 2.86 years (median 2.42) when compared to the median survival

**TABLE 1: Descriptive analysis showing the summary of survival data for prognostic factors associated with survival in patients with breast carcinoma**

Variables		Total cases n (%)	Median survival time (SE)	Mean survival . time (SE)
Age	< 49 years	161 (51.11)	3.58(.18)	3.35(.13)
	≥ 49 years	154 (48.99)	3.00(.17)	3.00(.14)
Tumour Grades	I	45 (14.28)	3.33(.16)	3.41(.13)
	II	214 (67.93)	3.17(.25)	3.11(.22)
	III	56 (17.77)	2.67(.27)	2.91(.23)
Tumour size	≤ 2 cms.	68 (21.58)	3.50(.18)	3.32(.13)
	2-5 cms.	175 (55.55)	3.33(.16)	3.26(.17)
	> 5 cms.	72 (22.85)	3.00(.17)	3.06(.21)
Lymphovascular invasion	Negative	165 (52.3)	3.33(.16)	3.27(.13)
	Positive	150 (47.7)	3.00(.17)	3.24(.14)
P53	Negative	141(45)	4.00(.18)	4.44(.13)
	Mild +ve	43(25)	3.00(.16)	3.52(.34)
	Moderate +ve	93(53)	2.89(.17)	3.11(.18)
	Strong +ve	38(22)	2.5(.25)	3.06(.39)
Tumour Stages	Stage I	38(12.00)	3.75(.39)	3.45(.24)
	Stage II	114(36.20)	3.58(.27)	3.65(.16)
	Stage III	128(40.63)	2.50(.12)	2.89(.14)
	Stage IV	35(11.11)	2.46(.36)	2.86(.28)
Family history	No	267 (84.7)	3.25(.13)	3.22(.11)
	Yes	48 (15.3)	3.50(.28)	3.43(.26)
Hormonal therapy	None	114(36%)	2.92(.19)	2.88(.16)
	Yes	201(64%)	3.58(.27)	3.47(.12)
Chemotherapy	None	80(25.4)	3.00(.17)	3.11(.15)
	Yes	235(74.6)	3.33(.16)	3.32(.12)
Radiotherapy	None	231(73.0)	2.60(.10)	2.4(.09)
	Yes	84 (27.0)	3.05(.16)	2.9(.15)
Distant metastasis	None	211(67%)	3.58(.14)	3.50(.13)
	Yes	104(33%)	2.42(.13)	2.86(.15)

time of 3.50 years (median 3.58) for subjects without metastasis. Controlling all potential contributors, the effect of p53 on survival time was still significant.

**Clinical, histopathological and immunohistochemical characteristics**

The histopathological characteristics of the tumours are listed in Table 2. All of them were infiltrating ductal carcinomas (IDC). Regarding histological grade, 45 (14%) were well-differentiated (grade I), 214 (68%) were moderately-differentiated (grade II), and 56 (18%) were poorly-differentiated (grade III) carcinomas. According to size, tumours were divided into three categories of <2 cm 68 (22%), 2-5 cm 175 (56%) and > 5 cm 72 (23%) in diameter. Positive axillary lymph nodes status was observed in 172 (54%), while negative axillary lymph node status was observed in 143 (46%) subjects.

p53 protein overexpression was observed in 174 (55.23%) out of 315 tumours. Its relationship

to histopathological and other immunohistochemical characteristics is shown in Table 2. Stain intensified positivity of p53 was dominated by ++ moderate positive 93 (53.44%) followed by + mild positive 43 (24.71%) and +++ or ++++ strong positive 38 (21.84%) (Table 1). The difference in p53 expression between patients aged <49 years and >49 years was not statistically significant (p= value 0.4368). By univariate analysis p53 expression was significantly correlated with histological differentiation (p 0.0361), tumour size (p 0.0158). and axillary lymph node metastases (p 0.0116). Brain, liver, lung and bone metastases were seen in strong p53 positive cases with p values of 0.0235, 0.0300, 0.0001 and 0.0262 respectively. 85 (46%) of 174 p53 positive cases showed ER or /and PR positivity. A significant but inverse relationship between p53 overexpression and ER content was observed (p 0.0575).

**TABLE 2: p53 expression and histological characteristics**

Parameters	Total no. of patients	p53 protein expression		P value
		Negative (n=141)	Positive (n=174)	
<b>Grade</b>				
I	45	24 (53%)	21 (47%)	0.0361
II	214	103 (48%)	111 (52%)	
III	56	14 (25%)	42 (75%)	
<b>Tumour size</b>				
≤ 2 cm.	68	50 (74%)	18 (26%)	0.0158
2-5 cm.	175	84 (48%)	91 (52%)	
> 5 cm.	72	29 (39%)	46 (61%)	
<b>Axillary lymph nodes</b>				
Negative	143	75 (52%)	68 (48%)	0.0116
Positive	172	68 (40%)	104 (60%)	
ER/PR negative	130	56 (43%)	74 (57%)	0.0575
ER and/or PR positive	185	100 (54%)	85 (46%)	
<b>Distant metastases</b>				
Brain	38	17 (45%)	21 (55%)	0.0235
Liver	18	08 (44%)	10 (56%)	0.0300
Lung	29	03 (10%)	26 (90%)	0.0001
Bone	73	29 (40%)	44 (60%)	0.0262

**TABLE 3: Independent variables related to prognosis (Cox multivariate analysis)**

Variables	Coefficient	Standard error	P value	Hazard ratio
Axillary lymph nodes <b>positive(0/1-3/4 +)</b>	0.6369	0.117	0.0001	1.891
Tumor size	0.5357	0.119	0.0001	1.709
Grade (I, II, III)	0.7352	0.242	0.0002	2.086
<b>P53(0/+/+/+/+++ or +++)</b>	0.2841	0.134	0.034	1.329
ER /PR <b>negative/ER/PR positive or ER positive</b>	0.2897	0.144	0.039	0.7418

**Survival analysis**

After a median follow-up of 48 months (range 3 to 73 months), the overall survival (OS) of breast cancer patients amounted to 65%. In univariate as well as in multivariate analyses p53 overexpression had a significant influence on survival. p53 mutation when correlated with OS, show significant correlation between p53 positivity and OS with a p value of 0.0523. At a median follow-up of 48 months, 65% of p53 positive patients died with an OS of 3.00 years and disease free survival (DFS) of 2.5 years. Among p53 negative patients OS was 3.8 years and DFS was 3.3 years.

In p53 positive patients regardless of axillary lymph node status, OS and DFS was poor compared to p53 negative patients.

In multivariate analysis, the independent prognostic factors for breast cancer patients were tumour size, axillary lymph nodes involvement, histological grade, p53 overexpression and ER / PR status (Table 3).

**DISCUSSION**

p53 gene mutations probably represent the most frequent molecular alteration occurring in human cancers. It is such a common event that it may be useful as a predictor of the biological behavior of certain neoplasms<sup>8</sup>. Several studies have demonstrated the association of p53 protein overexpression with various prognostic factors and in breast it has also been considered as an independent prognostic marker in patients with both node negative or node positive disease<sup>3, 10-12</sup>. In this study conducted on Pakistani women, we did find p53 as an independent prognostic factor as its association with several pathological variables and disease outcome was significantly correlated.

Among pathological variables the presence of axillary lymph node metastases is widely accepted as the single most important factor in breast cancer<sup>13</sup>. However, it is recognized that among both pN0 and pN1 tumours there are

subgroups with different prognosis<sup>14</sup>. The well-known heterogeneity in the biological behavior of breast cancer, particularly in the node negative carcinomas, has implications for decisions regarding **adjuvant** systemic therapy. This is the very reason that there has been a search for new prognostic markers. In this study, association between p53 oncoprotein and axillary lymph nodes metastases was significantly attributed to p53 positivity as significant number of p53 positive patients showed axillary lymph nodes metastasis compared to p53 negative tumours. Others like **Sirvant JJ et al 2001**<sup>4</sup> has reported similar findings. Likewise in our study, p53 expression was significantly but inversely correlated with **ER/PR** status, larger tumour size and higher histological **grade**<sup>3,15,16</sup>

In our study the correlation between p53 immune-expression, OS and DFS was also significant. This is in agreement with several other studies using univariate and multivariate analysis<sup>3,4,16,17</sup>. However in another study after 30 years of follow-up p53 did not appear to have an independent prognostic **value**<sup>10</sup>.

In summary, current data suggests that in spite of numerous studies examining p53 utility in breast cancer, the overall picture remains still somewhat unclear. This is largely due to lack of unanimity of results in various studies. These differences may be due to technique, study design, subjective interpretation of results and above all due to racial differences of the population under study.

Taking into account technical reasons, immunohistochemical techniques allow retrospective studies of large series of patients with long follow-up periods, which is critical to determine whether certain cell products correlate with disease outcome. Problems arise in interpretation of the results, because various studies use different methods and different monoclonal and polyclonal anti-p53 antibodies with distinct sensitivities and **specificities**<sup>11,18</sup>. Another cause of conflicting results is the criteria

for positivity and negativity. Some studies have considered that the majority of the cell nuclei should be **stained**<sup>19</sup>, whereas others have used a score considering not only the percentage of stained cells but also the nuclear staining **intensity**<sup>12,20</sup>. In other immunohistochemical studies, an arbitrary threshold has been set for the percentage of cell staining. The percentage of stained nuclei considered positive in a given neoplasm varies considerably, both in **paraffin**-embedded and frozen tissue; in some studies such a percentage is not even mentioned.

In conclusion the above findings have reinforced the view that p53 **immunohistochemical** detection will be of help in the clinical evaluation of breast carcinoma patients. This may be of particular relevance for the node negative patient with invasive ductal carcinoma, where p53 expression may help in decisions on adjuvant chemotherapy.

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