Indian Journal of Science and Technology, *Vol 7(4), 472–479, April 2014*

ISSN (Print): 0974-6846
ISSN (Online) : 0974-5645

Immunohistochemical Assessment of p53 Protein and its Correlation with Clinicopathological Characteristics in Breast Cancer Patients

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Abstract

Breast cancer is the most common cancer in women, containing approximately one third of all illness in women. Changes in p53 genes exist in 20–40% of aggressive breast cancer. Mutant protein of p53 has greater stability because longer half time than the wild type protein that can be detected by Immunohistochemistry (IHC) technique. The aim of this study was to detect expression of p53 protein in tissue samples of breast cancer patients and correlate it with other Clinicopathological characteristics of breast cancer patients. The study comprised 104 tumor samples of breast cancer patients. Immunohistochemistry technique was used for detecting the expression of p53 protein in breast tissues. Positive staining of p53 was found in thirty patients (28.84%), and negative staining of p53 was found in seventy-four (71.15%) patients. There was no significant correlation between p53 immunostaining with clinicopathological parameters like grade, stage, tumor size, age of menarche, histological type, family history, and age of first pregnancy, but there was significant correlation between p53 staining with age (p-value=0.000). Spearman's rho was used for assessment of statistical dependence between age and p53 (Correlation Coefficient=0.417, p-value=0.002). Also, there was significant difference between age in p53 positive and negative group (p-value <0.05), but there was no significant difference between other clinicopathological characteristics in breast cancer patients. In conclusion, immunohistochemical method proves to be reliable in determining the status of p53 protein. Besides, the result of this study showed that p53 nuclear accumulation can increase with aging in breast cancer patients.

Keywords: Breast Cancer, Immunohistochemistry, p53, Tumour-suppressor Gene

1. Introduction

Breast cancer is the most common cancer in women[1], containing approximately one third of all illness in women[2]. It affects one of every 8 women in the United States[2]. Also, it is one of the most frequent malignancies among Iranian women[3]. Interventions of genetic changes in breast cancer have been well documented. Among the probable changes, mutation and alteration in the products of several genes such as p53 gene have been considered very important[4,5]. Changes in p53 gene are prevalent in many cancers[6,7], so that more than 50% of all cancers including breast cancer contain changes in the p53 gene[8]. p53 is known tumor suppressor gene[9–15] placed on chromosome 17 [8,16–19]. The p53 gene codes a 53 KDa[20,21] nuclear phosphoprotein[22] that plays an important role in many critical cellular events,

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related to human aging and cancer[23] including DNA damage[24], telomere shortening, and oxidative stress[23]. This apart, it regulates expression of at least two-gene p21 and bax that code products regulate growth arrest and apoptosis[25–27]. Studies have shown that mutant protein of p53 has longer half time and greater stability than wild type protein[16]. In the nucleus, p53 binds to MDM2 protein and MDM-p53 complex is exported to cytoplasm and is degraded by proteosome[28,29]. This process causes low concentration of p53 protein in cell[28–30]. In response to oncogenic stresses, ARF activity induces accumulation of p53 protein[31]. Specifically, ARF binds to the RING finger domain of MDM2 or MDM2-P53. Major consequence of this interaction is MDM2 inactivation and stabilization of nuclear p53 levels[32,33]. Activation of p53 is also mediated by multitude of covalent post translational in p53 protein. DNA damage may activate protein kinase (such as ATM, DNA-PK, or CHK2) to phosphorylate p53, but MDM2 has no effect on phosphorylated p53 [32,33], therefore, expression of p53 protein increases[32,33]. These processes result in accumulation of p53 protein in nucleus[31] that can be detected by immunohistochemical technique[28,34,35]. Results of immunohistochemical studies of p53 protein in breast cancer patients are contradictory. Many studies showed that overexpression of p53 protein in breast tumors can be associated with high cell proliferation[25,36,37] and increased risk of progression. Another study showed that overexpression of p53 proteins is associated with high histologic grade, clinical aggressiveness and poor survival. Therefore, it can be considered as an index for increased malignancy and worse anticipation in breast cancer patients[38]. Accumulation of p53 was significantly associated with increased local relapse of breast cancer following mastectomy with, or without, but another study showed that p53 was not a significant risk factor for local recurrence after breast-conserving therapy and radiation therapy[39]. Moreover, p53+ and p53− breast tumors are not associated with very distinct risk profiles[40]. In another study, Khaliq reported that p53 mutation was present in breast cancer patients but there was no significant correlation between p53 mutation and tumor aggressiveness[41]. The purpose of this study was to evaluate the expression of p53 protein in tissue samples of 104 breast cancer patients in central Iran and determine the correlation between p53 protein expression with other clinicopathological factors, such as malignancy grade, age, histopathological type etc in breast cancer patients.

2. Materials and Methods

2.1 Study Population

A total of 104 breast cancer patients were chosen from Shaheed Sadoghi and Mortaz hospital (2010–2013) in central Iran and studied in Yazd research and Clinical Center for Infertility after taking their consent. In addition, the study was approved by the Ethics Committee and Research Committee of Yazd Research and Clinical Center for Infertility.

2.2 Histopathological Analysis

Tumor tissues of breast from patients were taken fresh in the Department of Pathology. The specimens were fixed in 10% neutral buffer formalin, then they are placed in graded of concentration alcohol 70%, 80%, 90% and 100%, then immersed in xylene and afterwards put into paraffin in automatic tissue processor. Following fixation, the specimens were embedded on wax paraffin and sliced to 4 μm in thickness for staining. The haematoxylin and eosin (H & E) as histological method was used to stain and analyze tissue sections. The histological grade of tumor is determined by Bloom and Richardson[42] modified by Elston[43].

2.3 Immunohistochemical Method

Immunohistochemistry technique was done on specimens that was embedded on wax paraffin from the main tumors. In summary, poly-L-lysin coated slides were chosen and 4 μm thick histological sections were mounted on them. Then, slides were dewaxed with xylene and rehydrated with decreasing intensity of alcohol. For blocking endogenous peroxidase activity, sections were treated with 3% hydrogen peroxide for 15 min. Subsequently, slides were transferred to citrate buffer and boiled for 15 minutes in a microwave oven for antigen retrieval. Then, sections were washed 3 times with phosphate buffered saline. For blocking non-specific binding sites, the slides were incubated in 1% BSA in Phosphate Buffered Saline (PBS) for 20 min. Further, the sections were exposed with mouse monoclonal anti-p53 antibody (DO-7, Leica, England) at a dilution 1:50 in PBS/1% BSA overnight at 4°C. The section were washed with PBS, and exposed with Horseradish peroxidas conjugated anti mouse Ig (Ebnesina, Iran) used at a dilution of 1:200 in PBS/1% BSA for 60 min. After washing with PBS, the sections were incubated

with 3,3-diamino-benzidine tetrahydrochloride (Sigma). Afterwards, sections were counterstained with hematoxylin and rinsed in tap water, followed by immersing in graded alcohol, xylene and finally mount. Negative control was performed by replacement the primary antibody with fetal bovine serum in each series.

2.4 Scoring

The percentage of tumor staining was scored following +3 = strong staining (more than 50 %stained), +2= moderate staining (between 25% and 50% stained), +1= weak staining (between 5 and 25% stained), 0= negative (less than 5% stained). Tumors with 2 and 3 points were considered positive for p53 staining.

2.5 Statistical Analysis

Statistical analysis was performed using SPSS software16 version. For comparing p53 positive and negative group with respect to characteristics, Independent Samples T-test and Fisher exact test were used. And for relation between parameters, Analysis of Variance (one-way Anova), and Fisher exact test were used. For measuring the statistical dependence between two variables, such as age and p53 protein, Spearman's rank correlation coefficient (Spearman's rho) was used. Statistical significance was considered as $P<0.05$.

3. Results

3.1 Patient Characteristics

In our study, the mean age of breast cancer patients was (44.75 ± 9.5) and mean diameter of tumor size was 3.37 ± 1.56 cm. Malignancy grade of patients was considered into three classes, containing low (17.3%), moderate (59.6%) and high risk (23.07%). The mean age of menarche was 12.72 ± 0.88 years and mean age of first pregnancy was23.0 ± 4.69 years. Also, tumor samples contain ductal (84.6%), Medulary (5.76%), Epidermal (5.76%) and Lobular (3.8%) breast cancer. In addition, clinicopathological characteristics of breast cancer patients were classified according to p53 expression. Age, Tumor Size, Age of first pregnancy, and menarche of breast cancer patients according to p53 expression are shown in Table 1. In the following, Histological type, Stage, Grade and history family of breast cancer patients according to p53 expression are shown in Table 2. The result of this Study showed that there was no significant difference between clinicopathological characteristics, such as age of first pregnancy, tumor size, age at menarche, histological type, grade, stage and familial history in p53 positive and p53 negative, but there was significant difference between age in p53 positive and negative (P-value < 0.05).

3.2 Immunohistochemical Analysis of p53 Protein

Immunohistochemical staining of breast cancer tissues showed that DO7 antibody specifically identified nuclear accumulation of changed p53 protein. Immunohistochemical staining of different expression of p53 protein in tissue samples are shown in Figure 1.

Number and percent of breast cancer patients according to p53 immunostaining are shown in Table 3.

Therefore in our study, negative staining of p53 protein according the score was found in seventy-four (71.15%), and positive staining of p53 protein was found in thirty patients (28.84%).

3.3 Correlation between p53 and other Breast Cancer Characteristics

The correlation between p53 expression with grade, stage, histological type, and family history of breast cancer are shown in Table 4. The results show that there is no significant correlation between p53 immunostaining with clinicopathological parameters, such as histological type, grade, stage, and family history of breast cancer patients. Also, the correlation between p53 with age, age of pregnancy, menarche and tumor size are shown in Table 5. The results of this study show that p53 immunostaining is significantly related to age (p-value=0.000). Besides, Spearman's rank correlation coefficient (Spearman's rho) was used to measure the statistical dependence between age and p53. The results show that there is a significant statistical dependence between p53 with age (Correlation coefficient = 0.417, p-value=0.002).

4. Discussion

p53 protein "guardian of the genome" is the product of TP53[44]. It delays or arrests cell cycling at DNA damage checkpoints preceding DNA replication (the G1/S checkpoint)[44,45] as well as inhibits damaged cells from entering mitosis (the G2/M checkpoint)[46].

Changes in p53 genes exist in 20–40% of aggressive breast cancer[47]. Mutant protein of p53 has greater stability

Table 1. Age, tumor size, age at first pregnancy and menarche of breast cancer patients according to p53 expression

Clinicopathological features	p53 positive		p53 Negative		p-value
					Independent sample T-test
	Mean ± SD	Number/percent	Mean ± SD	Number/percent	
Age	47.0 ± 6.3	6(40%)	43.08 ± 6.02	17(45.9%)	0.041
≤ 45		9(60%)		20(54.0%)	
> 45					
Tumor size (cm)	3.47 ± 1.63	5(33.3%)	3.45 ± 1.52	12(32.4%)	0.968
≤ 2 cm		8(53%)		18(48.6%)	
2 size ≤ 5 cm		2(13.3%)		7(18.9%)	
5 cm					
Age at menarche (years)	12.66 ± 0.95	5(33%)	12.73 ± 0.88	13(35%)	0.796
≤ 12		7(46%)		16(43.2%)	
12 Age ≤ 13		3(20%)		8(21%)	
13					
Age of first pregnancy (years)	22.2 ± 4.58	4(26.6%)	23.13 ± 4.69	7(18.9%)	0.515
≤ 18		8(53.3%)		18(48.6%)	
18 > Age ≤ 25		2(13.3%)		8(21%)	
25 > Age ≤ 30		1(6.67%)		4(10.8%)	
30					

Table 2. Histologic type, Stage, grade and history family of breast cancer patients according to p53 expression

Clinicopathological features	p53 positive	p53 Negative	p-value Fisher exact test
	Number/percent	Number/percent	
Histological type			
Invasive Ductal Carcinoma	26(86.6%)	62(83.7%)	
Medulary Carcinoma	2(6.66%)	4(5.4%)	1.00
Epidermal Carcinoma	2(6.66%)	4(5.4%)	
Invasive Lobular Carcinoma	0	4(5.4%)	
Family history of breast cancer			
None	24 (80%)	62(83.7%)	0.829
First degree	4(13.3%)	6(8.1%)	
Second degree	2(6.67%)	6(8.1%)	
Stage			
0	2(6.66%)	4(5.4%)	
IIA	12(40%)	36(48.6%)	0.479
IB	2(6.66%)	2(2.7%)	
IIB	8(26%)	22(29.7%)	
III	6(20%)	10(13.5%)	
Malignancy grade			
1	4(13.3%)	14(18.9%)	
2	18(60%)	44(59.4%)	0.917
3	8(26.6%)	16(21%)	
Total	30(100%)	74(100%)	

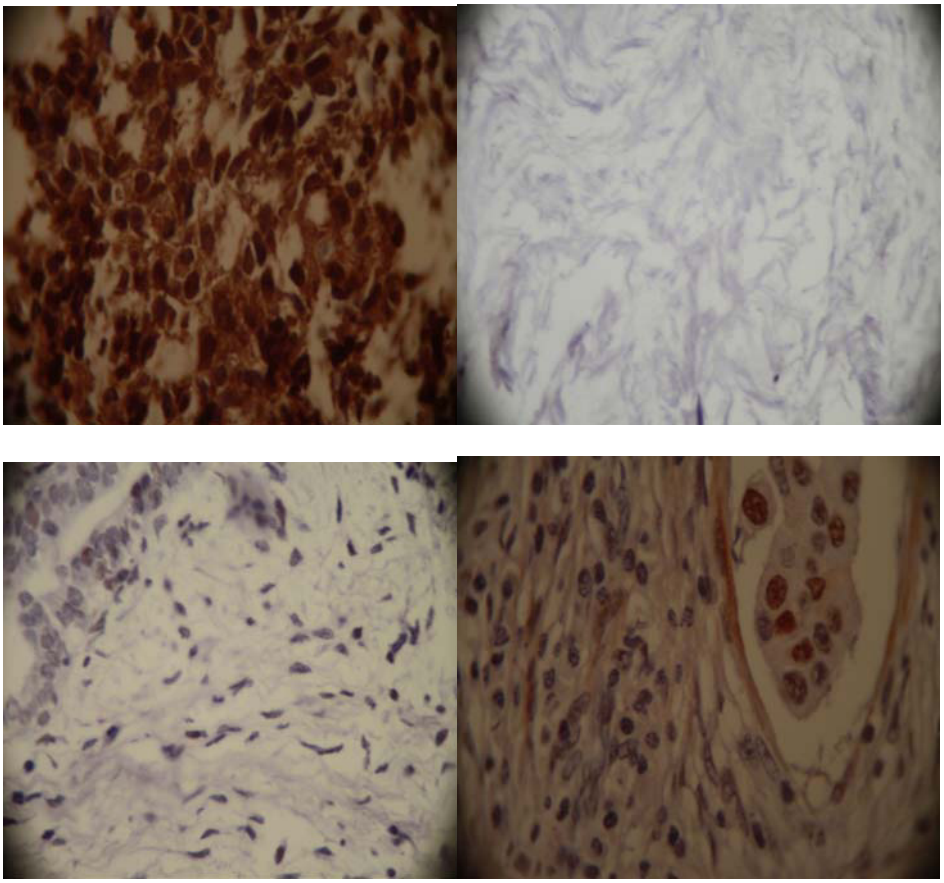

Figure 1. p53 staining in tumor cell of breast cancer (clockwise from top left): Strong p53 nuclei staining in tumor cells (100x); No Staining (100x); Weak Staining of p53 (100x); Moderate staining (100x).

Table 3. Number and percent of breast cancer patients without, with intermediate and with clear p53 over expression

Staining of p53 protein	Number	Percentage (%)
No staining	46	44.2
Weak staining	28	26.9
Moderate staining	16	15.3
Strong staining	14	13.4
Total	**104**	**100**

Table 4. Correlation between p53 immunostaining with grade, stage, histological type and family history in breast cancer patients

Clinipathological Parameters	P53 immunostaining
	p-value/ Fisher exact test
Stage	0.473
Histological type	0.702
Grade	0.052
History Family	0.484

P<0.05 was considered significant for statistical analysis

Table 5. Correlation between p53 immunostaining with age, age of first pregnancy, menarche and tumor size in breast cancer patients

Clinipathological Parameters	p53 immunostaining
	p-value/ one-way- Anova
Tumor Size	0.186
Age of first pregnancy	0.954
Age of menarche	0.875
Age	0.000

P<0.05 was considered significant for statistical analysis

than wild type protein, because of its longer half time. Longer half-life of mutated p53 protein[28] is related to change in its conformation and can be detected by immunohistochemical technique[48,49]. In this study, DO7 antibody was used to immunostaining of p53 protein in 104 tumor samples. Staining of tumor is classified to p53 positive staining and p53 negativestaining. p53 positive staining was found in 28.8% of breast cancer patients. Loss of p53

function increases cellular resistance to variety of drug in cancer-therapy. Therefore, high levels of over expression of p53 protein can help to predict cell responsiveness to anticancer drugs that require p53 protein to impel apoptosis[50]. In this study, the mean age of patients in p53 positive group is significantly more than patients in p53 negative group. Many studies have shown that mutations in p53 gene frequently occur in older patients than young ones. Also, other studies showed that in some families, lower age of onset of breast cancer is related to hereditary factors, and genes like BRCA1 and BRCA2 are responsible for increasing hereditary breast cancer even at younger (under below 45 years)[38]. The results of our study showed that there is a significant association between p53 staining with age. Zhang, consistent with our study reported that there is a significant association between advanced age and p53 nuclear accumulation[51]. Hasty reported that relation between p53 and aging is complicated and not well understood[52]. Another study showed that accumulation of p53 protein in response to DNA damage was dependent to age and accumulation of p53 protein was absent in young animal. These results show that the ability of cells to repair damaged DNA is reduced with age[53]. El-Domyati obtained same result and reported that persistent expression of wild-type p53 with age may be due to failure of the senescent cells to respond to physiologically produced p53 in response to DNA damage, thus it results to continuous expression of p53 [54]. Therefore, age-related accumulation of somatic DNA mutations is, likely, a major contributing factor for increased cancer incidence with age[23], but another study showed that higher incidence of p53 positive accumulation in younger patients than in the older ones, is probably related to the significantly higher incidence of grade III tumors in these patients[55]. In addition to all these, a number of other studies showed that there is an association between p53 overexpression and tumor grade[56,57]. Hong and etal reported that p53 immunostaining was correlated with high grade tumor, high mitotic frequency, and ductal type tumor. Therefore, they reported that p53 overexpression might be an indicator of more aggressive cancer and poor prognosis[25].

5. Conclusion

In our study, immunohistochemical method in determining the status of p53 protein proved reliable and valid. This apart, the result of this study showed that p53 nuclear accumulation could be increased with aging in breast cancer patients. However, further studies with more patients to assess broader role of p53 protein seem to be necessary.

6. Acknowledgement

This study was supported by a grant from Yazd Research and Clinical Center for Infertility.

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