

P53 Gene: Mutation and Immunohistochemical Analysis in Patients with Invasive Ductal Carcinoma of Breast

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ABSTRACT

The p53 tumor suppressor gene is the most commonly mutated gene in cancer. In breast cancer, the presence of p53 gene alterations has been associated with worse prognosis. This study was attempted to associate p53 gene mutations with its protein expression in North Eastern Indian population. We used single-stranded conformation polymorphism to screen samples for mutations in five conserved regions, exons 4, 5, 6, 7 and 8, of the p53 gene. Mutations were confirmed by direct DNA sequencing. Samples were also analyzed for expression of p53 immunohistochemically. We found two critical mutations in the exon 4. A well known missense mutation at codon 72 (pro to arg) with a frequency of 47% was found which was significantly correlated with the immunohistochemical analysis of p53 protein in such patients. A novel nonsense mutation at codon 107 which leads to stop codon was also found. Although the occurrence of this mutation was very less, we did not find expression of p53 protein immunohistochemically. We support that mutation in p53 gene can be exploited as a prognostic marker for the early diagnosis of breast cancer, although more clinical and epidemiological data is required to establish this claim.

Keywords: Breast Cancer, p53, ER, PR, HER2

1. INTRODUCTION

In the light of the existing literature breast cancer is the most prevalent cancer among women and affects approximately 1 million women worldwide each year comprising 18% of all female cancers (Baum, 2002). Several epidemiological studies have documented extensively for the incidence of breast cancer in Indian scenario (Badwe *et al.*, 1990; Jussawalla *et al.*, 1980; 1985; 1998; Kapoor *et al.*, 1993). One in fifty eight women are affected by breast cancer in the age group of

30-70 years and are mainly seen in the urban areas. The well established epidemiological risk factors for the development of the breast cancer are age at diagnosis, family history, parity, age at the menarche and menopause, diet, socioeconomic status, history of exposure to radiation and use of oral contraceptive pills (Helmrich *et al.*, 2009; Dupont and Page, 1985).

In the last decade several lines of evidence have correlated a significant association between the polymorphism of p53 gene and development of breast cancer (Lacroix *et al.*, 2006). p53 plays a key role in

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mediating cell response to various stresses, mainly by inducing or repressing a number of genes involved in cell cycle arrest, senescence, apoptosis, DNA repair and angiogenesis. The qualitative and quantitative activity of p53 depends on its integrity (mutation status), its amount and its specific post-translational modifications induced by the activation of the different stress-induced signaling pathways (Lacroix *et al.*, 2006). This leads to variable patterns of association between p53 and a number of other co-regulatory proteins, of which some may be tissue- or cell type-specific. Several association studies between the p53 expression, polymorphism and development of breast cancer have been carried out in various geographical regions of India like Bombay, Eastern Rajasthan, Aligarh, Bangalore, Jammu, Surat and Chennai (Jussawalla *et al.*, 1998; Kapoor *et al.*, 1993; Sharma *et al.*, 1994; Hussain *et al.*, 1994; Nandakumar *et al.*, 1995; Gadhia *et al.*, 1995; Juneja *et al.*, 1990; Lin *et al.*, 2001; Nayak and Das, 1997; 1999a; 1999b; Nayak *et al.*, 1996; 1998; Kannan *et al.*, 2000; Keasri *et al.*, 1997). In continuation, the present study was designed to explore three specific issues of p53 in the subjects suffering from Invasive Ductal Carcinoma (IDC) of breast among middle North-Eastern Indian population: (a) Does the exonic region of p53 contain any sequence aberration? (b) Is there any correlation between the mutational analysis of p53 with clinico-pathological data of the subjects? (c) Is there any correlation between the p53 mutation (if any) with expression status of p53 protein?

2. MATERIALS AND METHODS

2.1. Subject Selection

A total of 62 (58 females and 4 males) fresh excision biopsies were obtained from the patients of Invasive Ductal Carcinoma with the mean age of 53.35 ± 9.29 years (aged between 33 to 75). All the patients included in the present study were recruited from in patient department of Cancer Research Institute, North Eastern Railways, Lehartara, Varanasi, India, between January 2009 to March 2010 with their prior informed consent and their detailed history was obtained. Patients receiving chemotherapy and radiotherapy were excluded from the study. Study was approved by the institutional ethical committee.

2.2. Sample Collection

Tumor biopsies were collected directly from the surgical operation theater of the department of oncology in chilled phosphate-buffered saline from the patients who

underwent mastectomy. Each freshly microdissected tumor was divided into two equal portions by use of a disposable scalpel. Slices were taken from the outer, viable, cellular region of one half for later DNA analysis and sequencing. Corresponding slices from the other half taken in 10% formalin were prepared for histopathological examination. The part of the tumor that was frozen was stored at -80°C until analysis. The adjacent tissue biopsy from each individual was taken as control.

2.3. DNA Isolation

Cellular DNA from collected breast tumor biopsies was isolated using standard proteinase K digestion, phenol-chloroform extraction and ethanol precipitation method routinely being used in our laboratory (Sambrook and Russell, 2001). The quality and concentration of DNA was measured on an ethidium bromide-stained 1% agarose gel and by standard spectrophotometric methods.

2.4. Polymerase Chain Reaction

Approximately, 100-200 ng DNA was utilized for conventional PCR using the protocol routinely followed in our laboratory. Briefly, the method involved a 50 μL reaction mix containing 100-200 ng DNA, 5.0 μL of 10X PCR buffer, 3.0 μL of 25 Mm MgCl_2 , 0.5 μL of 10 Mm dNTPs, 0.5 μL of 10 pmol forward primer, 0.5 μL of 10 pmol reverse primer, 0.5 μL of Taq DNA polymerase ($1 \text{ U } \mu\text{L}^{-1}$). The PCR primers for exonic regions (4 to 8) of p53 used for amplification have been mentioned in Table 1.

2.5. Single-Strand Conformation Polymorphism Analysis

Single-Strand Conformation Polymorphism (SSCP) analysis was performed as described by Michaelides *et al.* (1995). Depending on the PCR product concentration, 4 ± 1.2 mL of the PCR product was added to the same volume of denaturing solution containing 800 μL formamide, 100 μL of 1% Bromophenol Blue, 100 μL of 1% xylene cyanol, 2 μL of 0.5 M EDTA and 1 μL of 10 M NaOH per 1 mL solution. Samples were denatured at 93°C for 5 min and transferred immediately to ice to prevent renaturation of DNA. Each sample was loaded onto a 10% polyacrylamide gel with 0.5 mm thickness. Electrophoresis was performed in 1X TBE at 4°C and at 19 mA for 5 to 7 h depending on the size of the PCR product. Each PCR product was electrophoresed at least three times to increase precision and to prevent false positive results. Bands were visualized after silver staining of gel, as described (Bourn *et al.*, 1994).

Table 1. Primers used in amplification of exons 4 to 8 of p53 gene

p53 Exon	Primer sequence
	F = Forward Primer, R = Reverse primer
4	F TGACTGCTCTTTTCACCCAT R GGAAGCCAGCCCCTCAGGGC
5	F TGTTCACTTGTGCCCTGACT R CAGCCCTGTCGTCTCTCCAG
6	F GCCTCTGATTCCTCACTGAT R TTAACCCCTCCTCCCAGAGA
7	F ACTGGCCTCATCTTGGGCCT R TGTGCAGGGTGGCAAGTGCC
8	F TAAATGGGACAGGTAGGACC R TCCACCGCTTCTTGTCCTGC

2.6. Sequencing

Purified PCR products of the samples showing mobility shift on SSCP analysis and randomly chosen samples were sent for sequencing. Direct DNA sequencing using an automated ABI 3100 Genetic Analyzer (Bangalore Genei) using ABI's AmpliTaq FS dye terminator cycle sanger's sequencing chemistry.

2.7. Sample Processing for Histology and Analysis

Paraffin-embedded tumor sections on poly-L-lysine coated slides were dewaxed in xylene and rehydrated in ethanol and distilled water. Antigen retrieval in a microwave oven at 750W (two cycles for 10 minutes each) enhanced p53 antigen accessibility to antibodies. The expression of Estrogen Receptor (ER), Progesterone Receptor (PR) and HER2 (Human Epidermal growth factor Receptor-2) were also checked using specific antibodies. Positive p53 IHC staining was seen in the nucleus of cancer cells. IHC-positive samples were sub-classified with regard to immunostaining intensity and extent according to graded scales that ranged from 1 to 3. Allred's method was used for the scoring of p53, ER and PR and for HER2 expression, Hercep Test; DAKO was used (Allred *et al.*, 1990; Dako and Hoffman, 2002). Super sensitive polymer-HRP detection system and primary antibodies p53, ER, PR and HER2 from Biogenex, CA, USA were used. All slides were viewed and judged microscopically by the pathologist.

2.8. Statistical Analysis

Analysis was performed with the Statistical Package for Social Science (SPSS) windows version 16. Chi-square test was used to compare the frequencies. Statistical significance was considered when $p \leq 0.05$.

3. RESULTS

Clinico-pathological features of the recruited patients are presented in **Table 2**. Most of the patients at

diagnosis were either in tumor stage II (75.1%) or in stage III (24.1%). All patients underwent mastectomy.

Sequencing of amplified p53 exons (4 to 8) from all the recruited subjects were carried out to confirm the mobility shift bands detected on SSCP gel. Out of 62 patients, we found 29 subjects with p53 mutations. **Table 3** summarizes the age wise frequency of patients with and without p53 mutations. We found 2 critical mutations in the exon 4 at codon positions 72 and 107 resulting in the missense and nonsense mutations, respectively (**Fig. 1**) as compared to the corresponding controls. **Table 4** represents a summary of p53 gene mutations in breast cancer patients. No sequence aberration was found in exons 5, 6, 7 and 8 in the patients with respect to their corresponding control biopsies. The sequencing results were further confirmed by Single-strand conformation polymorphism analysis also (**Fig. 2**). The nonsense mutation at codon 107 occurred along with the missense one at codon 72. To search for suitable mutations of p53 gene PCR-SSCP analysis was used as a screening technique.

All the mutated and wild type sequences were submitted to NCBI and the accession numbers obtained were as follows: exon 4 (wild type): BankIt1451832 Seq1 JF923567, exon 4 (mutation A): BankIt1451832 Seq2 JF923568, exon 4 (mutation B): BankIt1451832 Seq3 JF923569, exon 5: BankIt1451832 Seq4 JF923570, exon 6: BankIt1451832 Seq5 JF923571, exon 7: BankIt1451832 Seq6 JF923572 and exon 8: BankIt1451832 Seq7 JF923573.

Table 5 summarizes the frequency of p53 mutation positive patients and their clinicopathological features. Menopausal status ($p = 0.013$), histological grade ($p = 0.048$) and axillary lymph node involvement ($p = 0.018$) were found to be significantly associated with the p53 mutation positive cases.

Out of 62 biopsies from patients, 27 were found to be p53 positive, further immunohistochemical analysis revealed a significant higher expression of p53 protein in the breast cancer biopsies as compared to their corresponding control biopsies (**Fig. 3**). A total of 51 (82.0%) tumors were found negative for ER, 54 (87.0%) tumors were found negative for PR and 44 (71.0%) were found positive for HER2 membrane staining. Interestingly we did not find the expression of p53 in the 2 patients having nonsense mutation. **Table 6** summarizes the age wise frequency of patients with p53 expression. Correlation of menstrual status with p53 expression and HER2 expression was found to be significant, their p values being 0.031 and <0.001 , respectively.

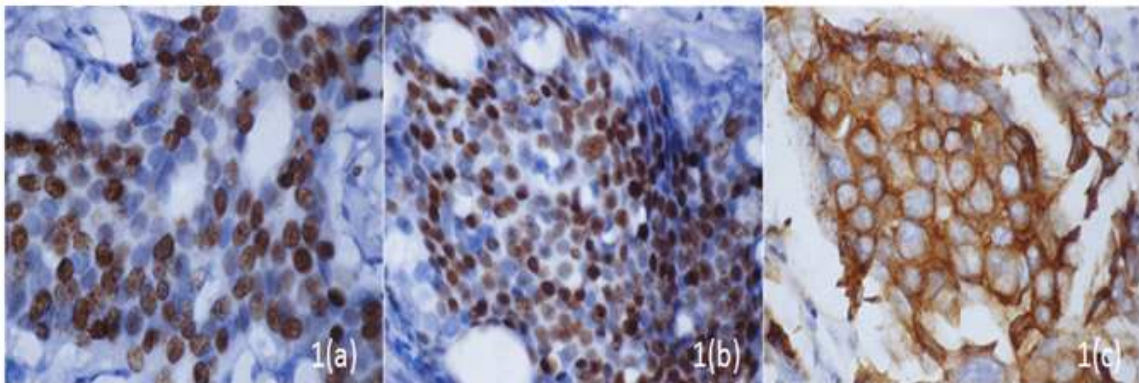


Fig. 1. Immunohistochemical staining of ER(1a), PR(1b) and HER2(1c) at 400X

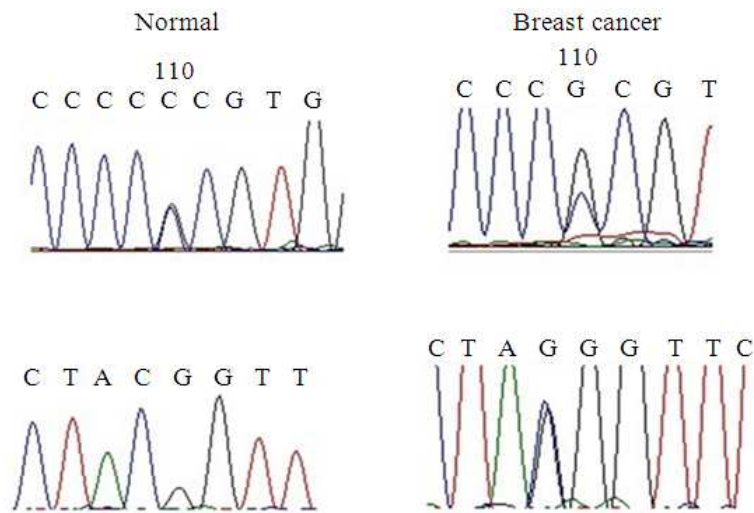


Fig. 2. Representative partial electropherograms of sequencing results showing two critical mutations in the exon 4 of p53 gene

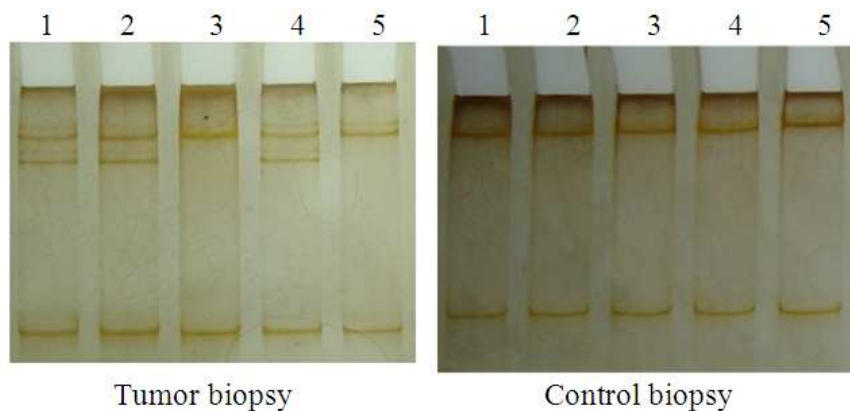


Fig. 3. Detection of mutations in exon 4 of p53 gene. 10% polyacrylamide gel picture showing the electrophoretic mobility shift of p53 exon 4 from various tumor biopsies as compared to their normal biopsies. Samples 1, 2 and 4 are showing the genetic aberration

Table 2. Clinicopathological features of the breast cancer patients

-----Parameters-----		N [Value (%)]
Breast Side	Left	32/62 (51.6)
	Right	30/62 (48.3)
Menstrual Status	Pre menopausal	18/58 (31.0)
	Post menopausal	40/58 (68.9)
Age at menarche	≤13 years	22/58 (37.9)
	>13 years	34/58 (58.6)
Parity	Nulliparous	9/58 (15.5)
	1-4 Children	35/58 (60.3)
	>4 children	14/58 (24.1)
Lifetime duration of breast feeding	<36 months	24/58 (41.3)
	36-54 months	12/58 (20.6)
	55-78 months	10/58 (17.2)
	>78 months	12/58 (20.6)
Pagets's Disease	Yes	4/62 (6.4)
	No	58/62 (93.5)
Tumor Stage	T2	0/62 (0)
	T3	36/62 (58.0)
	T4	26/62 (41.9)
	Grade I	0/62 (0)
Histological Grade	Grade II	47/62 (75.8)
	Grade III	15/62 (24.1)
	0	18/62 (12.9)
No. of Axillary Lymph Nodes	1-3	27/62 (43.5)
	>3	17/62 (27.4)

Table 3. Summary of p53 mutations in breast cancer patients

Age (years)	p53 Positive		p53 Negative	
	Number	Percentage	Number	Percentage
≤50	11/62	17.7	16/62	25.8
>50	18/62	29.0	17/62	27.4

Table 4. Nature of p53 mutations in sporadic breast cancer patients

No. of patients	Exon containing mutation	Codon Number	Nucleotide Number	Base Change	Amino Acid Change	Types of mutation
29	4	72	1138	CCC→CGC	Pro→Arg	Missense
2	4	107	1149	TAC→TAG	Tyr→Stop	Nonsense

Table 5. Patients' characteristics and clinic pathological features of p53 mutants.

-----Clinical features-----		p53 mutants n (%)
Age (years)	≤50	11/29 (37.9)
	>50	18/29 (62.0)
Residence	Rural	2/29 (6.8)
	Urban	27/29 (51.7)
Menopausal Status	Pre	11/25 (44.0)
	Post	18/25 (72.0)
Breast involved	Right	10/29 (34.4)
	Left	19/29 (65.5)
Provisional Diagnosis	IDC	29/29 (100)
Lymph Nodes involved	Paget's disease	2/29 (6.8)
	Yes	19/29 (65.5)
Histological Grade	No	10/29 (34.4)
	Grade I	0 (0)
	Grade II	22/29 (75.8)
Clinical Tumor Stage	Grade III	7/29 (24.1)
	T2	0 (0)
	T3	17/29 (58.6)
	T4	12/29 (41.3)

Table 6. Summary of the age wise frequency of patients with p53 expression

Age (years)	p53 IHC status			
	Negative [n (%)]	+1 [n (%)]	+2[n (%)]	+3[n (%)]
≤ 50	16/62 (25.8)	0/62 (0)	1/62 (1.6)	9/62 (14.5)
>50	3/62 (4.8)	9/62 (14.5)	1/62 (1.6)	18/62 (29.0)

Tumor size was found to be significantly associated with ER negativity ($p = 0.015$), histological grade was found to be significantly associated with p53 expression ($p = 0.018$) and ALN involvement was also found to be significantly associated with p53 expression (0.025).

4. DISCUSSION

p53, a well established tumor suppressor gene has the centre of attraction for the researchers in the field of cancer biology for its ability to regulate several fundamental cellular processes like cell cycle arrest, senescence, apoptosis, DNA repair and angiogenesis (Lacroix *et al.*, 2006). The status of p53 in human breast cancer has been the subject of intensive investigation (Gasco *et al.*, 2002; Anne-Lise, 2003). The presence of mutation, with or without accompanying Loss of Heterozygosity (LOH), was an early finding in cell lines and primary breast cancers, establishing p53 as a bona-fide tumor suppressor gene in the breast. Epidemiological studies have documented extensively the incidence of breast cancer in the western population (Ursin *et al.*, 1994) but the available information in India is limited. India is a vast sub-continent with a huge population of multiracial society with widely varying cultures and several ethnic groups. Different aspects of breast cancer involving epidemiology, risk factors and susceptibility have been dealt in various communities and regions. Some association studies between the p53 expression, polymorphism and development of breast cancer have been carried out in various geographical regions of India like Bombay, Eastern Rajasthan, Aligarh, Bangalore, Jammu, Surat, Chennai (Jussawalla *et al.*, 1998; Kapoor *et al.*, 1993; Sharma *et al.*, 1994; Hussain *et al.*, 1994; Nandakumar *et al.*, 1995; Gadhia *et al.*, 1995; Juneja *et al.*, 1990; Lin *et al.*, 2001; Nayak and Das, 1997; 1999a; 1999b; Nayak *et al.*, 1996; 1998; Kannan *et al.*, 2000; Keasri *et al.*, 1997). The present study is an extension of these Indian studies for the

geographically distinct middle north eastern population in order to explore the sequence aberration in p53 gene and its protein expression as well as correlation with demographic and clinical findings.

Our study comprised of 62 subjects with confirmed diagnosis of IDC of breast. Association of the p53 mutation was found significant with gender postmenopausal status, histological grade and ALN involvement which was consistent with existing data in the literature (Giordano *et al.*, 2004; Cutler *et al.*, 2009; Madigan *et al.*, 1995). Our sequencing data revealed p53 gene mutations in 29 (47%) tumor biopsies from different individuals. We observed two critical mutations in the exonic region 4 of p53 (**Fig. 2 and 3**). The missense mutation at codon 72 (pro to Arg) has been shown to have varying ethnic and geographical distribution (Chosdol *et al.*, 2002; Ihsan *et al.*, 2011; Kalemi *et al.*, 2005). The frequency of this mutation in our breast cancer population was found to be around 50%. It has already been reported that the p53 Arg homozygous genotype could be a potential genetic risk factor for cancer (Buyru *et al.*, 2003; Papadakis *et al.*, 2000). Further our mutation data was correlated with the immunohistochemical analysis of significant high expression of p53 protein (**Fig. 4**). In healthy humans, the p53 protein is continually produced and degraded in the cell. The degradation of the p53 protein is associated with MDM2 binding. In a negative feedback loop, MDM2 is itself induced by the p53 protein (Haupt *et al.*, 1997). However, mutant p53 protein often does not induce MDM2 and is thus able to accumulate at very high concentrations (Peng *et al.*, 2001) and hence detectable by IHC. We reported another novel nonsense mutation at codon 107 which leads to the stop codon. Although the occurrence of this mutation is very less, but interestingly we did not find the expression the p53 protein by histological analysis which supports the translation of truncated p53 protein and ultimately leads to protein degradation (Heck *et al.*, 2010).

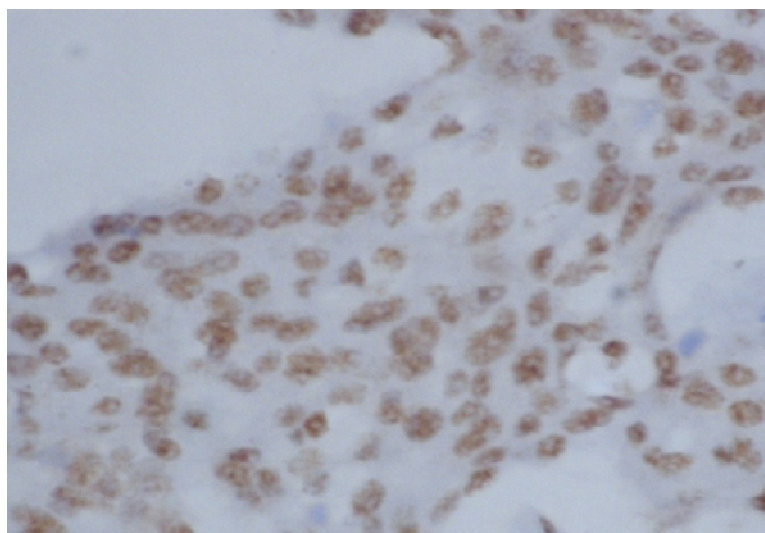


Fig. 4. Immunostaining showing p53 positivity at 400X

5. CONCLUSION

Our data thus indicate that p53 gene can be exploited as a prognostic marker for the early diagnosis of breast cancer, although more clinical and epidemiological data across the Indian and Asian geographical regions is required to establish this claim.

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