

RESEARCH COMMUNICATION

***p53* Exon 4 (codon 72) Polymorphism and Exon 7 (codon 249) Mutation in Breast Cancer Patients in Southern Region (Madurai) of Tamil Nadu**

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Abstract

Background: We investigated the association between polymorphisms in the *p53* tumor suppressor gene and breast cancer risk in women especially in the Southern part of India. **Methods:** Genotyping was performed for 50 breast cancer women and 50 controls to determine the status of *p53* exon 4 codon 72 polymorphism and exon 7 codon 249 mutation and their possible role in breast cancer risk. **Results:** Frequency of Arg/Arg at codon 72 was 18% in controls and 28% in patients, Arg/Pro frequency was 56% and 66% , Pro/Pro genotype was 8% in controls and 8% in patients. No significance was observed for breast cancer risk with either Arg/Arg or Pro/Pro genotype in codon 72 polymorphism. Similarly, mutation analysis of exon 7 codon 249 revealed that 72% of breast cancer patients have mutation, which is not statistically significant. However, there is a strong association between increase in exon 7 codon 249 mutation and exposure to pollution. **Conclusion:** The results suggested that there is no risk for exon 4 with Arg/Arg or Pro/Pro polymorphisms in the *p53* gene and there is no strong correlation between breast cancer patients and mutation in exon 7 codon 249 in South Indian women.

Keywords: Breast cancer - *p53* - exon 4 codon 72 - exon 7 codon 249 - South India

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Introduction

Breast cancer is the most common invasive malignancy affecting 1.3 million women worldwide each year (Ganjewala, 2009; Pugalendhi et al., 2010). The incidence of breast cancer in women is rising in the world especially in developing countries such as India. According to Indian Council of Medical Research (ICMR), incidence of breast cancer in India is on the rise and is rapidly becoming the number one cancer in females pushing the cervical cancer to the second spot (Abdaheer et al., 2009). It is reported that one in 22 women in India is likely to suffer from breast cancer during her lifetime (Pushkala et al., 2009; Pugalendhi et al., 2010). The incidence varies between urban and rural women; the incidence in Mumbai is about 27 new cases per 100,000 women per year while in rural Maharashtra it is only 8 per 100,000 (Chopra, 2001). According to the National Cancer Registry Programme report on time trends in cancer incidence rates (1982-2005) of the ICMR, the estimated number of breast cancer cases in India in 2010 is 90,659 (Takiar et al., 2010). This is because more and more women in India are beginning to work outside their home which allows the various risk factors of breast cancer to come into play. These include exposure to pollution, radiation and other factors. There

are a couple of factors which collectively or individually lead to the disease. Because of Westernization of Indian culture, high stress at a young age, lack of vitamin D, early puberty, excess consumption of alcohol, rise in pollution levels, post menopausal obesity, intake of oral contraceptive, dietary factors among others are the main reason behind the disease (Weir et al., 2007).

The tumor suppressor gene *p53* is crucial for host defence against genomic mutations that might give rise to many types of tumors. Somatic mutations of *p53* that alter the DNA binding and transactivation function of the *p53* protein are known to be involved in diverse types of cancers (Chosdol et al., 2002). Mutations in the TP53 gene are the most common genetic alterations in human cancer and they can be found in 20-30% of the sporadic breast carcinomas (Damin et al., 2006). The wild type of *p53* protein exists as two variants: Arg and Pro at codon 72. This Arg/Pro polymorphism at codon 72 of the wild type *p53* gene is shown to be associated with various cancers (Chosdol et al., 2002).

The association between codon 72 polymorphisms and risk of cancer has been reported in different populations, although results with regard to most cancers remain controversial. There are expanded bodies of literature studies regarding *p53* gene at codon 72 polymorphism

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in different ethnic group which showed a wide range of variation (Chosdol et al., 2002). Beckman et al. (1994) have also revealed striking ethnic differences and correlation between the arginine allele frequency and latitude suggesting that the polymorphism may be maintained by natural selection related to climatic factors. Over the last two decades, a number of studies were conducted to investigate the association between the *p53* gene at codon 72 polymorphism and breast cancer risk in several human populations but no reports are available to address their roles in women affected with breast cancer especially in Southern part of India.

TP53 mutations are distributed in all coding exons, with a strong predominance in exons 4-9, encoding the DNA-binding domain of the protein. About 30% of all mutations fall within six 'hotspot' codons and are detectable in almost all types of cancer (codons 175, 245, 248, 249, 273, 282). The results of several studies has shown that codon 249 is one of the most important sites introduced as a hot-spot for *p53* gene. Specific base substitutions particularly G->T transversion at third position of codon 249 (AGG to AGT) in exon 7 of the *p53* gene results in replacement of arginine with serine. Plenty of literature has been published regarding the mutation at codon 249 in hepatocellular carcinoma (HCC) and lung cancer. But there are no reports in concern to the relation between mutation at codon 249 and breast cancer from South India.

In the present study, we screened for the presence of mutation and polymorphism in *p53* gene codon 249 and codon 72 respectively in breast cancer patients from South Indian population. We also analyzed the allelic distribution for the correlation with the other risk factors for disease outcome.

Materials and Methods

Chemicals

The restriction enzyme *BstU1* was obtained from New England Biolabs, UK. *HaeIII*, Primers, *Taq* polymerase, dNTPs, and the chemicals used for PCR amplification was obtained from Genie (Bangalore, India). Other chemicals used were of analytical grade.

Collection of blood samples

Blood samples were collected from cancer patients of Guru Cancer Hospital, Devaki Multispeciality Hospital, Dr. G. K. Mohana Prasad Laboratories, Madurai, Tamil Nadu, India using EDTA coated vacutainers. 50 samples were collected from various types of cancer patients and 50 samples were collected from non-cancer individuals as controls.

DNA extraction from blood

Blood samples anticoagulated with EDTA were stored at 4°C until use. DNA was extracted from 200µl of whole blood by the method of Jeffreys et al., 1987.

PCR amplification of exon 4 (codon 72) and exon 7 (codon 249)

The expected size of the PCR product for exon 7 was

286 base pairs (bps), and this fragment which is located in the exon 7 of the *p53* gene was amplified using 20 pmol each of the primers (5' GGCGACAGAGCGAGATTCCA 3' and 5' GGGTCAGCGGCAAGCAGAGG 3') in a 25µl reaction volume containing 200µM of dNTPs, 1X concentration of standard PCR buffer, 1U *Taq* polymerase, 2mM MgCl₂, and 50ng DNA template. The thermocycling conditions were 95°C for 3 min, amplification was carried out by 35 cycles of 95°C for 30 sec, 66°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 5 min in a Autorisierter Thermocycler (Eppendorf India Limited, Chennai). The amplification product (286 bp) was visualized by staining with ethidium bromide, after electrophoresis on 1% agarose gels.

The expected size of the PCR product for codon 72 was 309 bp, and this fragment located in the exon 4 of the *p53* gene was amplified using 25 pmol of each primer (5'TTCACCCATCTACAGTCC3' and 5'CTCAGGGCAACTGACCGT 3') in a 25µl reaction volume containing, 200µM of dNTPs, 1X concentration of standard PCR buffer, 1U *Taq* polymerase, 1.5mM MgCl₂ and 50ng DNA template. The thermocycling conditions were 94°C for 5 min, amplification was carried out by 35 cycles of 94°C for 1 min, 57°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 7 min in a Autorisierter Thermocycler(Eppendorf India Limited). The amplification product (309 bp) was visualized by staining with ethidium bromide, after electrophoresis on 1% agarose gels.

Detection of polymorphism by restriction analysis

The 286 bp DNA fragments, which is derived from exon 7 of *p53* gene, was subjected to restriction enzyme digestion as follows: 0.5 µl (5 units) of enzyme *HaeIII*, 2µl of buffer, 15µl of DNA fragment, 2.5 µl sterile milliQ water (20 µL total reaction volume). These reactions were kept for 2h at 37°C in a water bath. The 309bp of the DNA fragment, which is derived from exon 4 of *p53* gene, was subjected to restriction enzyme *BstU1* digestion as follows: 0.5 µl (10units) of *BstU1* enzyme, 2 µl of 1X buffer, 15µl of DNA fragment, 2.5 µl sterile MilliQ water (20 µL total reaction volume). These reactions were kept for 2h at 60 C in a water bath. The restriction products were staining with ethidium bromide, after electrophoresis on 15% non-denaturing polyacrylamide gels in 1xTBE buffer at 100 V for 8 hours. Enzyme *Hae III* cleaves a GG/CC sequence at codon 249, generating 92 bp, 66 bp and several small fragments from the 286 bp DNA product of the PCR reaction. If there is a polymorphism at codon 249, it results in an uncleaved 158 bp fragment and this feature will be distinguished from that of normal samples on 15% non-denaturing polyacrylamide gels stained with ethidium bromide. Absence of the band at 286bp (full-length PCR products) provides a control for complete digestion of the PCR product.

Enzyme *BstU1* cleaves a CG/CG sequence at codons 72, generating 174 bp and 135 bp from the 309 bp DNA product of the PCR reaction. Electrophoresis was carried out on non-denaturing 15% polyacrylamide gels in 1xTBE buffer at 100 V for 8 hours. The genotype was determined under UV. Illumination using a gel documentation system

after staining with ethidium bromide.

Statistical analysis

The statistical analysis was performed using the SPSS software version 17. χ^2 was used to analyze categorical variables and the Student t-test was used to compare the continuous variable age. The association between the TP53 polymorphism and breast cancer was determined using the logistic regression method (adjusted by age) to assess odds ratio (ORs) and 95% confidence intervals (95% CI). The association between the TP53 polymorphism and risk factors (both epidemiological and environmental) of the breast carcinomas was assessed using the X2 test (categorical variables). Similarly the association between the codon 249 mutation and breast cancer was also determined. Comparisons were made between two genotype Ser/Ser Vs Arg/Arg. P-values less than 0.05 were considered statistically significant.

Results

The amplified PCR product of exon 4 and exon 7 are 309 bp and 286 bp respectively as shown in the Figure 1. The polymorphism at codon 72 and mutation at codon 249 as identified by restriction digestion is shown in Figure 2a.2b. Detection of TP53 gene codon 72 polymorphism by PCR-RFLP was successfully conducted in all cases

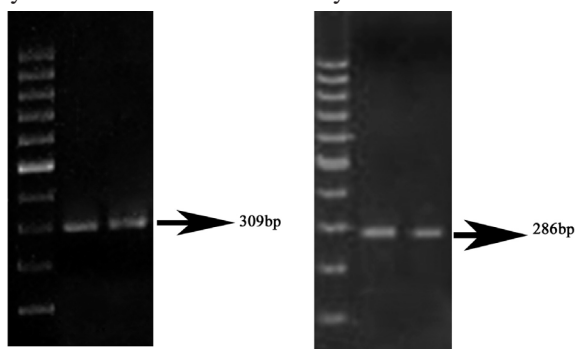


Figure 1. Electrophoresis Map of PCR Products. Lane 1 left: DNA molecular weight markers (50bp); Lane 2: PCR product of exon 4 (309bp); Lane 1 right: DNA molecular weight markers (100bp); Lane 2: PCR product of exon 7 (286bp)

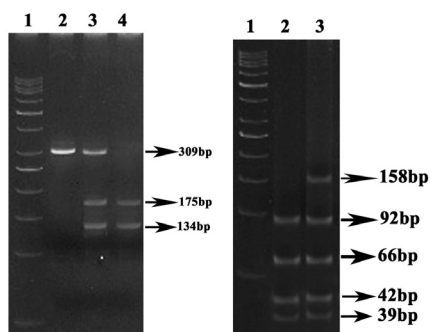


Figure 2. PCR-RFLP Analysis of the p53 Gene Exon 4 (codon 72) Exon 7 (codon 249). Lane 1 left: DNA molecular weight markers (50bp); Lane 2: The Pro allele is not cleaved by *Bst*UI at codon 72 and has a single band with 309bp; Lane 3: heterozygotic pattern of Arg/Pro; Lane 4: The Arg allele when cleaved by *Bst*UI yields two small fragments (175 and 134 bp). Lane 1 right: DNA molecular weight marker (50bp); Lane 2: Arg allele 158bp which is cleaved by *Hae* III, Lane 3: Ser allele 158bp which is not cleaved by III

Table 1. Distribution of TP53 Exon 7 Codon 249 Mutations in Patients and Controls.

Total	p53 codon 249 ve- (%) Arg	p53 codon 249 ve+ (%) Ser	OR ^a	CI	P
Breast cancer	50	36 (72%)	0.389	0.170-0.892	0.024
Control	50	7(14%)			

* ^aOdds ratio (adjusted for age). Ser/Ser vs. Arg/Arg and Arg/Ser

and controls. The Arg was cleaved by *Bst* UI, yielding two smaller fragments (175 and 134 bp). The Pro allele was not cleaved by *Bst* UI, having a single 309 bp band. Heterozygotes contained three bands, corresponding to 309,175 and 134 bp (Figure 2a).

The distribution of the codon 72 genotypes in patients and control did not deviate from the Hardy-Weinberg equilibrium. The genotype frequencies in cases and controls are presented in Table 1, with a no significant statistical association of the Arg/Arg (OR 0.564; 95% CI 0.218-1.459; P<0.235) and Arg/Pro heterozygous variant (OR 1.525; 95% CI 0.679; P<0.305) with breast cancer risk. The relative frequency of each allele was 0.6 for Arg and 0.4 for Pro in patients with cancer, and 0.51 for Arg and 0.49 for Pro in normal controls. The frequency of the Arg allele was 28% in patients (n=50) and 18% in controls (n=50). The Pro allele was detected 8% each in patients (n=50) and controls (n=50). The studied characteristics such as exposure to pollution and radiation of cancer patients and controls were analyzed according to codon 72 genotype. There was no significant difference between the p53 codon 72 polymorphic genotype and patients characteristics.

Detection of TP53 codon 249 mutations by PCR-RFLP was successfully performed in all cases and controls. The wildtype 249 genotype was cleaved by *Hae*III, yielding 4 fragments (92,66,42 and 39 bp). The 249Ser genotype lost the *Hae*III restriction site and it was not cleaved by *Hae*III, having 158, 92, 66, 42 and 39 bp. The distribution of the codon 249 mutation in patients and control did not deviate from the Hardy-Weinberg equilibrium. The genotype frequencies in cases and controls demonstrated no statistically significant association of the 249Ser genotype (OR 0.389; 95% CI 0.170-1.892; P<0.024) with breast cancer risk. The relative frequency of each allele was 3.7 for Arg and 6 for Ser in patients with cancer, and 6.56 for Arg and 2.6 for Ser in normal controls. The frequency of the Arg allele was 28% in patients (n=50) and 43% in controls (n=50). The 249Ser was detected in 36% (n=50) in patients and in 7% (n=50) in controls.

When risk factors such as exposure to pollution and radiation of cancer patients and control were analyzed according to 249Ser genotype, statistically significant correlations with tobacco intake, living in urban areas, exposure to indoor pollution (P<0.004) and radiation and also intake of non vegetarian foods (P<0.001) were observed with increasing risk of breast cancer with 249Ser genotype. Statistically, there was no significance observed between other risk factors and the 249Ser genotype (see

Table 2. Characteristics and Exposure to Environmental Pollution of Cases and Controls for the Exon 7 Codon 249 Mutation

	N (%)	Arg/Arg (%)	Ser/Ser (%)	P
Age (n=50)				
≥49	34 (68)	9 (64.2)	25 (69.4)	0.017
50-59	8 (16)	3 (21.4)	5 (13.8)	
≤60	8 (16)	2 (14.2)	6 (16.6)	
Body mass index (n=50)				
<18.5	5 (10)	1 (7.14)	4 (11.1)	0.279
18.5-24.9	21 (42)	4 (28.5)	17 (47.2)	
25-29.9	18 (36)	7 (50)	11 (30.5)	
≥30	6 (12)	2 (14.2)	4 (11.1)	
Tobacco (n=50)				
Yes	29 (58)	8 (57.1)	21 (58.3)	0.001
No	21 (42)	6 (42.8)	15 (41.6)	
Residence (n=50)				
Urban	20 (40)	4 (28.5)	16 (44.4)	0.009
Rural	30 (60)	10 (71.4)	20 (55.5)	
Diet (n=50)				
Veg	5 (10)	2(85.7)	3 (91.6)	0.001
Non Veg	45 (90)	12 (14.2)	33 (8.3)	
Indoor pollution (n=50)				
Yes	36 (72)	6 (42.8)	30 (83.3)	0.001
No	14 (28)	8 (57.1)	6 (16.6)	
Radiation (n=50)				
Yes	35 (70)	9 (64.2)	26 (72.2)	0.001
No	15 (30)	5 (35.7)	1 (27.7)	
Exposure to asbestos (n=50)				
Yes	26 (52)	6 (42.8)	20 (55.5)	0.227
No	24 (48)	8 (57.1)	16 (44.4)	

Table 2.

Discussion

Polymorphisms in the p53 tumor suppressor gene are potential molecular markers for inherited predisposition for breast cancer (Alawadi et al., 2010). The frequency, timing, and mutation spectrum of the p53 can provide clues to the etiology and pathogenesis of human cancer (Hussain et al., 2001). Studies have been conducted to evaluate the potential role of codon 72 polymorphism as a risk factor for different types of cancer, such as gastric, lung and bladder carcinomas. This polymorphism has also been investigated in patients with breast cancer. So far, the results have been inconclusive. In the present study, we have demonstrated that there is no significance for the presence of Arg/Arg or Arg/Pro genotype at codon 72 of the TP53 gene with increased risk of breast cancer. Our findings are not in line with those that detected a higher prevalence of homozygosity for Arg in patients with breast cancer, as reported in women from Greece (Kalemi et al., 2005; Papadakis et al., 2000), Turkey (Buyru et al., 2003) and Southern Brazil (Damin et al., 2006). Keshava et al., 2002 were able to find a higher prevalence of the Arg allele in Caucasian women with breast cancer from New York, but not in Latin or African-American patients. Ohayon et al., 2005 studied Jewish women with breast carcinoma, finding a higher prevalence of the Arg allele among Ashkenazi Jewish and Arab women (Alwadi et al., 2010), a high-risk population for breast cancer (Damin et al., 2006). Contradictory reports are also available in literature implicating that involvement of Arg/Pro

heterozygous variant increases the risk of breast cancer from North Indian population. In this study also 28% of populations from breast cancer patients were Arg/Pro genotype with no risk. A polymorphism at codon 72 in p53 gene significantly affects its function. Arg 72 is more susceptible to degradation by human papilloma virus (HPV) E6 type-18 protein and suppresses cellular transformation more effectively than Pro72. On the other hand Arg 72 is more efficient than Pro72 at inducing apoptosis (Singh et al., 2008).

There was no association between the Arg/Arg genotype and breast carcinomas in patients from Russia (Susptsin et al., 2003), Tunisia (Mabrouk et al., 2003) and Germany (Gohrke et al., 1998). Moreover, Pro/Pro genotype was more prevalent in Japanese (Noma et al., 2004) and in Swedish (Sjalander et al., 1996) women with breast cancer, as compared with normal controls (Damin et al., 2006). In Asia, which generally possesses low incidence of breast cancer, the mutation rate of p53 is diverse among different areas (Chen et al., 2004). Racial, ethnic, and environmental differences play a critical role in the pathogenesis of breast cancer and are probably responsible for differential findings. India is a country of diverse racial, ethnic and environmental exposures; therefore, assessment of contribution of germline mutations in p53 gene and their association with breast cancer risk was inevitable in Indian population (Singh et al., 2008).

This polymorphism also seems to be maintained by natural selection influenced by environmental factors, such as the degree of exposure to sunlight UV-B component, unique food habits and other life style factors in India. [Damin et al., 2006 & Singh et al., 2008]. The resulting North-South Arg-Pro gradient has been reported in different geographical regions. Population-based studies indicate that the Arg allele is most prevalent in individuals with light complexion and least prevalent in individuals with darker complexion, with a clear and consistent decline in the prevalence of Pro allele with increasing north latitude (Damin et al., 2006).

Codon 249 exon 7 of the p53 gene is the most common and hot spot of mutation in HCC, almost 40% of TP53 mutation reported in this neoplasm (Nogueira et al., 2009). Array of literature described that incidence of 249Ser mutation was high in patients having HCC compare to other neoplasms (Liu et al., 2002). There is a relation between aflatoxin B1 exposure and the G-T transversion in codon 249 in 50% of Southeast Asian patients (Hsu et al., 1991) and 67% from Senegal (Coursaget et al., 1993). The substitution of arginine by serine in the p53 protein causing folding abnormality of the DNA binding domain on the protein (Igetei et al., 2008). Other work suggested that codon 249Ser mutation could increase p53 mRNA expression, and increased mRNA was an important factor in the over expression of mutant p53 proteins (Peng et al., 1998). These 249Ser mutations also affect cell cycle suppression function of p53 which results in loss of cell proliferation control (Mirmomeni et al., 2009).

Earlier report in North Indian population conducted by Katiyar et al. (2000) also observed a very low frequency (2 out of 21 cancer patients) of mutation in exon 7 (codon

p53 Exon 4 (codon 72) Polymorphism and Exon 7 (codon 249) Mutation in Breast Cancer in Tamil Nadu 249) showing GT transversions. Similarly, Makwane et al. (2009) from North Indian population suggests that the occurrence of p53 mutations relatively low in Indian women with breast cancer as there are only few other reported studies about the mutation frequency in p53 gene in the Indian population. In this study 249Ser mutation was found in 72% of breast cancer samples and it is significantly higher compare to controls. Still, there is no mutation registry with breast cancer from South India population association with 249Ser mutation.

A study by Andersen et al., 1993 also showed that the highest frequency of p53 mutations in breast carcinoma is in exon 7 per base pair, which were screened by constant denaturant gel electrophoresis and demonstrated a correlation of p53 mutations with adverse prognostic factors and decreased survival. Thorlacius et al. (1993) analysed only exons 5, 7 and 8 for p53 mutations and showed an association with low estrogen content and high mortality rates.

Hsu et al. (1991) stated that mutation at codon 249 was high in HCC patients from East Asia due to exposure to aflatoxin B1 and the other reports regarding mutation at codon 249 also deduced that people which reside in areas are highly exposed or contaminated with aflotoxin having high risk of cancer. There are several reports ascribed that the risk factors (tobacco, intake of alcohol and exposure to pollution) increases the risk of cancer. Hussain et al. (2001) revealed that exposure to pollution such as PAHs and tobacco smoke having mutation in codon 249 which increases the risk of lung cancer. In this study also, there is a correlation between the exposure to indoor pollution and radiation, tobacco intake and population lived in urban areas and 249Ser genotype from breast cancer. Results of the current study proved that there is a link between exposure to pollution and breast cancer. Igetei et al., 2008 also evidenced that 249Ser mutation from HCC patients also depends on the detoxifying effect of the enzymes responsible for its metabolism: epoxide hydrolase (EPHX) and glutathione S transferase M1 (GSTM1) and the efficacy of the DNA repairing process. Makwane and Saxena (2009) also hypothesized that there is a link between cancer causing carcinogens and sporadic breast cancer.

The main findings in this study in South Indian population are that combined analysis of p53 codon 72 polymorphism and codon 249 mutations showed evidence of polymorphism in 72 codon in cancer and controls and detecting mutation in the codon 249 for cancer and controls. We conclude from these data that p53 mutation frequency of these different groups provided the no strong relationship between Arg/Arg or Arg/Pro genotype with breast cancer risk. For codon 249Ser mutation there is no strong association between mutation and breast cancer from Southern part of India. A number of studies have been carried out to establish an eventual association between the polymorphism at codon 72 and codon 249 mutation and human carcinogenesis. However, the issue is still a matter of controversy. This initial study has a small sample size with limited power and additional studies including larger cohorts are warranted to confirm these results as well as to elucidate the biological effect of genetic

variation in breast cancer. This approach highlights the value of examining mutation and polymorphism in p53 genes as a common procedure to predict the risk estimates of cancer and further studies are to be conducted in breast cancer patients.

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