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Frequency of Pro Allele on Codon 72 of *TP53* in Female Breast Cancer Patients of Pakistan: Molecular Stress or Geography

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Abstract.- This study was undertaken to understand the role of an important polymorphism at codon 72, exon 4 of tumor suppressor gene *TP53* which encodes a nuclear protein and prevents the cells from dividing before DNA damage is repaired. Presence of homozygous arginine allele at codon 72 considered is a risk factor for cancer. The objective of present study was to determine the frequency of codon 72 polymorphism of *TP53* gene in sporadic breast cancer patients and those with genetic lineage. One hundred and fifty female patients with sporadic breast cancer were included in this study, of these, one hundred female patients were recruited at Shaukat Khanum Memorial Cancer Hospital & Research Center, Lahore and fifty patients were recruited at Mayo Hospital Lahore Pakistan, from January 2005- December 2008. The median age of the patients was 40 years (range 18-65). From all the participant patients three type of samples *viz.* blood, normal and tumor tissue were collected. Besides the sporadic breast cancer patients, three families, two from Lahore and one from Multan were also included in the study. Out of these families, one family was detected for Li-Fraumeni syndrome like characteristics. Blood samples were collected from normal persons and families. Fifty normal females belonging to different areas of Pakistan, ranging from 18-65 years of age were also included in this study for comparison purpose. Genomic DNA was amplified in PCR reaction, which was then subjected to Restriction Fragment Length Polymorphism (RFLP) analysis. Proline allele was found to be more dominant compared to arginine allele. RFLP analysis showed that arg/pro (53%) and pro/pro (35%) genotypes were more significant in Pakistani breast cancer patients compared to arg/arg (12%) genotype. Similar type of genotypic prevalence was found in normal control samples. The arg/pro and pro/pro alleles were also prominent in familial breast cancer patients. Contrary to arg allele which is usually implicated with breast cancer development in western countries, the pro allele in the present study was more prominent in Pakistani sporadic breast cancer patients, normal subjects and those of genetic lineage. Frequency of pro allele codon 72 of *TP53* in female breast cancer patients of Pakistan may be due to specific geographical reasons.

Keywords: *TP53*, codon 72 polymorphism, breast cancer, Li-Fraumeni syndrome.

INTRODUCTION

TP53 is an important tumor suppressor protein which comprises 11 exons. The gene functions by preventing different type of cancers by responding to variety of stresses which can cause mutations and enhance tumor formation (IARC, 2011). About 20 polymorphisms have been described in this gene; the most studied polymormphism of *TP53* gene is of codon 72 which is located within the proline-rich region. Due to codon 72 polymorphism, three variants are observed in humans, which are arg/pro, pro/pro and arg/arg. It is reported that when the polymorphism resides on arg allele there is a selective growth advantage for development of cancer as compared to presence of pro allele. However, sharp ethnic differences in the arg allele frequency have been observed ranging from 0.60 to 0.83 (Ara *et al.*, 1990; Delacalle-Martin *et al.*, 1990).

The growth of breast carcinoma cells with the arginine 72 allele in Norwegian and Greek population is already reported (Langerod *et al.*, 2002). But some researchers reported that the *TP53* proline 72 variant was associated with increased risk of breast cancer due to a decreased ability to induce apoptosis of cell (Orsted *et al.*, 2007). At the cellular level, the evidence is that the arginine 72 allele favors apoptosis, while the pro allele favors a cell cycle arrest in response to stress (Van Heemst *et al.*, 2005). It has also been found that the ancestral allele is proline and not the arginine (Beckman *et al.*, 1994).

Pakistan represents the oldest civilization of the world in the form of the Indus civilization (Ratnagar, 2006). The rate of breast cancer in

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Pakistan is the highest in Asia, except for Jews in Israel (Bhurgri *et al*., 2006). It is reported that about one in every nine Pakistani women is likely to suffer from breast cancer (Sohail and Alam, 2007). Mamoon *et al.* (2009) have compared breast cancer status of three decades in Pakistan and observed that the age of presentation of cancer to physicians is younger compared to the western countries. It has also been reported that the incidence of breast cancer is higher in Indian/Pakistani women compared to Caucasians (Kakarala *et al.*, 2010).

Amongst all possible risk factors, the change in the genetic information is the most dominant factor. Prevalence of *BRCA*1 or *BRCA*2 mutations in breast cancer patients of Pakistan (Rashid *et al.*, 2006) and some mutations have been found unique to Pakistan. The prevalence of polymorphisms and haplotypes of *TP53* has been studied in Pakistani ethnic groups (Khaliq *et al.*, 2000) and the pro allele has been found to be dominant. The present study was designed to investigate allele frequency and genetic or geographical value of *TP53* gene codon 72 polymorphism in normal subjects, sporadic breast cancer patients and in those with genetic lineage of Pakistan.

MATERIALS AND METHODS

Subjects

The study project was evaluated by bioethics committees of School of Biological Sciences, University of the Punjab, Lahore, Mayo Hospital Lahore and Shaukat Khanum Memorial Cancer Hospital & Research Centre (SKMCH&RC), Lahore, Pakistan. One hundred and fifty female patients with sporadic breast cancer patients were included in this study, of these one hundred female patients were recruited at SKMCH&RC, Lahore and fifty patients were recruited at Mayo Hospital. The median age of the patients was 40 years (range 18-65). Blood, normal and tumor tissue samples were collected from all the participants of this study, so total number of examined specimens from sporadic patients were 450 (150x3). Besides the sporadic breast cancer patients, three families, two from Lahore and one from Multan were also included in the study. Fifty normal females belonging to different areas of Pakistan, ranging from 18-65 years of age were also included in this study for comparison purpose.

Pedigrees of families included in the present study

Blood samples were collected from the families having incidence of breast cancer in the family. Numbering of icons is according to the blood samples taken from the patients and processed.

Family no. 1

Blood samples were taken from seven members of a Lahore family no 1, who were extensively prone to breast cancer (Fig. 1).

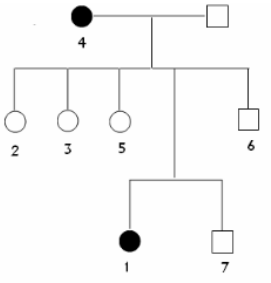

Fig. 1. Pedigree of familial breast cancer patient of family 1: Square, male; Circle, Female. The solid icons represent the breast cancer patients. No. 1 represents 30 years old female with breast cancer, who married her 40 years old first cousin (#7).

Family no. 2

The blood samples of another Lahore family 2, suffering from breast cancer (Fig. 2), were collected for analysis.

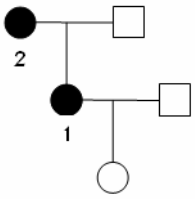

Fig. 2. Pedigree of familial breast cancer patient of family 2. Relationship of numbering is 1, daughter (40 years) and 2, mother (70 years).

Family no. 3

During data collection, we came across a family belonging to Multan having Li-Fraumeni

syndrome (LFS) like characteristics (Fig. 3). The spectrum of tumors, early age of cancer onset and pathology reports were strongly suggestive of the Li-Fraumeni syndrome. The following criteria were also used to further confirm the syndrome (Li and Fraumeni, 1969). (i) A proband diagnosed with sarcoma when younger than 45 years, (ii) A first-degree relative with any cancer diagnosed when younger than 45 years, and (iii) Another first or second-degree relative of the same genetic lineage with any cancer diagnosed when younger than 45 years or sarcoma diagnosed at any age.

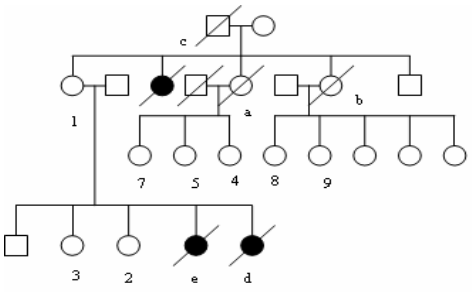

Fig. 3. Li. Fraumeni Syndrome like characters in Family 3. **1** is mother of five children, four daughters and one son, of which two daughters died at the age of 4 years (e) with brain tumor and the other at the age of 18 years (d) with soft tissue sarcoma metastasized as breast cancer. Blood was taken from two daughters (**2, 3)** and a son (**6**). Blood samples were taken from three daughters **(4, 5, 7)** of (a) who died at the age of 25 years with brain hemorrhage. Out of five daughters of (b) who died at the age of 40 years with myocardial infection) two (**8, 9)** were available for sampling**.** Normal male = □ , normal female = ◯ , died with (a) brain hemorrhage, (b) myocardial infarction = ⊘, died with cancer (c): brain tumor, (d): soft tissue sarcoma metastasized to breast, (e): brain tumor= ●.

DNA isolation and genotyping

DNA was isolated from blood by using Grimberg *et al.* (1989) method. In the case of sporadic breast cancer patients, DNA was extracted from frozen tissues [tumor (T) and normal tissue (N)] according to Deb and Palit (2003). DNA was analyzed for the genetic variation in codon 72 in exon 4 of the *TP53* gene using RFLP (Dellacale-Martin *et al.*, 1990). Genomic DNA (100 ng) was amplified in 50 µl of PCR reactions (Eppendorf Mastercycler Gradient), containing 10 pmol of each primer (F: 5′-TTGCCGTCCCAAGCAATGGATGA-3′, R: 5′-TCTGGGAAGGGACAGAAGATGAC-3′), 5 µl of 10× buffer (Fermentas, containing 100 mM Tris-HCl, 500 mM KCl and 0.8% Nonidet P40), $MgCl_2$ 1.5mM, 2.5 mM deoxynucleotide triphosphate, and 2.5 units of Taq DNA polymerase (Fermentas). A 199-bp fragment was amplified using a PCR program starting with denaturation for 3 min at 95°C, followed by 30 cycles each of 20 sec at 94°C, 20 sec at 56°C, and 30 sec at 72°C. Restriction analysis was performed mixing 8 µl of PCR product, 9 µl of H_2O, 2 µl of 1× NE Buffer 2, 1 µl of (10 units/µl) *Bst*UI (New England BioLabs), and incubated for 3h at 60°C. Agarose gel electrophoresis (2%) was used for resolution of unrestricted fragment and 4% agarose gel electrophoresis was used for resolving the *Bst*UI restricted fragments.

RESULTS

A 199bp unrestricted fragment after restriction with *Bst*UI gave the following pattern for three different polymorphisms by electrophoresis in 4% agarose gel (Fig. 4): (i) presence of one band (199 bp) for homozygus proline (pro72/pro72), (ii) presence of two bands (113 bp, 86 bp) for homozygus arginine (arg72/arg72), and (iii) presence of three bands (113 bp , 86 bp, 199 bp) for heterozygous arginine-proline (Fig. 5).

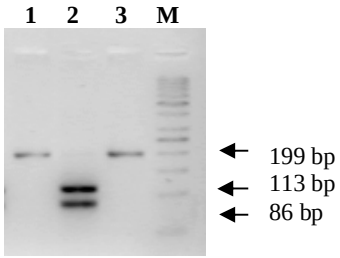

Fig. 4. RFLP gel (4%) showing *TP53* codon 72 polymorphism in blood samples of normal subjects**.** Pattern of two alleles of 113 bp + 86 bp (arg) and 199 bp (pro), lane 1, uncut fragment (199bp); lane 2, homozygus arginine (arg/arg); lane 3, homozygus proline (pro/pro); M, 50 bp marker.

Normal subjects

In 50 normal females, frequency of homozygous arginine was 10%, for homozygous proline it was 40%, and for heterozygotic arg/pro it was 50%. Figure 4 shows the banding pattern of two normal subjects as a reference.

Sporadic breast cancer patients

One hundred and fifty breast cancer patients were observed for arg/pro genotype. Frequency of homozygotic arginine at codon 72 was 12%, for homozygotic proline it was 35%, and for heterozygotic arg/pro it was 533%. Codon 72 polymorphisms in blood (B), tumor tissue (T) and normal tissue (N) was checked. Figure 5 shows two samples of sporadic breast cancer patients (SKH-85 and SKH-86). Table I shows the comparison in frequencies of *TP53* genotypes in normal subjects and breast cancer patients. There was no significant genotypic difference between patients and controls (normal subjects) and frequency of pro allele is prominent both in patients and normal subjects.

Table I.- Frequencies (%) of *TP53* genotypes in control and breast cancer patients.

Genotypes	Patients (%)	Controls (%)
arg/arg	18 (12%)	5 (10%)
pro/pro	52 (35%)	20 (40%)
arg/pro	80 (53%)	25 (50%)
Total	150	50

Table II.- Frequencies of *TP53* genotype among F1 and F2 family members.

Relative	F1	F2
Patient	arg/pro	pro/pro
Mother	arg/pro	pro/pro
Husband	pro/pro	
Brother	arg/arg	
Sister	pro/pro	
Sister	arg/pro	
Sister	arg/pro	

The incidence of somatic *TP53* polymorphism was checked in blood, tumor and normal tissue. Figure 6 shows the normal (46N) and tumor (46T) tissue with heterozygous (arg/pro) codon 72 polymorphism of *TP53* gene. The blood sample of the same patient however, showed arg/arg, which may be the case of wrong labeling by concerned hospital worker.

No significant association between *TP53* codon 72 genotypes and clinicopathological features was detected (Table IV).

Familial breast cancer

Family 1

PCR for codon 72 polymorphism detection of familial breast cancer patients (Fig.7A) gave unrestricted fragments (Fig. 7B). Figure 7C shows an allel pattern for the heterozygus samples (arg/pro) which is different from homozygous arginine (arg/arg) and proline (pro/pro). Family1 shows all the three genotypes of codon 72 polymorphism. The breast cancer patient, her two sisters and mother were heterozygous arginine-proline (arg/pro), whereas brother was homozygous arginine (arg/arg), one sister and husband were homozygous proline (pro/pro) (Fig.7C).

Family 2

The samples of family 2 were amplified and the product was loaded on the gel (lanes 8-9) (Fig.7B). Figure 7C shows the RFLP results. The family shows the genotype, homozygous proline (pro/pro) in both samples (lanes 8 and 9). Table II represents comparison of frequencies of *TP53* genotype among F2 and F3 family members. In family 1 arg/pro genotype was found dominant, while both patients of family 2 had pro/pro genotype.

Family no. 3 (*Li–Fraumeni Syndrome family (LFS)*

Figure 8A shows the pedigree of LFS family, Figure 8B shows the PCR amplification before restriction with *Bst*UI and Figure 8C shows RFLP pattern of two types of polymorphisms *i.e.* arg/pro and pro/pro. The family members, f1 (mother of proband), f4 (f1's niece), f5 (f1's niece), f6 (f1's son) and f7, f8 and f9 (f1's nieces) showed heterozygus genotype, arginine and proline (arg/pro), while f2 (f1's daughter), f3 (f1's daughter) shows homozygous genotype, proline (pro/pro). Table III shows the clinical and genetic status of LFS family. It is obvious from the Table that the clustering of heterozygous alleles arg/pro may conform the phenomenon of genetic anticipation.

Table III.- Clinical and genetic status of LFS family.

No.	Sex	Family members (in relation to F1)	Age(y) at blood sampling	Effect of any type of tumor	Codon 72 polymorphism
f1	Female	mother	56	Non effected	arg/pro
f2	Female	F1's daughter	21	Non effected	arg/pro
f3	Female	F1's daughter	18	Non effected	arg/pro
f4	Female	F1's niece	10	Non effected	pro/pro
f5	Female	F1's niece	6	Non effected	arg/pro
f6	Male	F1's son	27	Non effected	arg/pro
f7	Female	F1's niece	3	Non effected	arg/pro
f8	Female	F1's niece	16	Non effected	pro/pro
f9	Female	F1's niece	20	Non effected	arg/pro

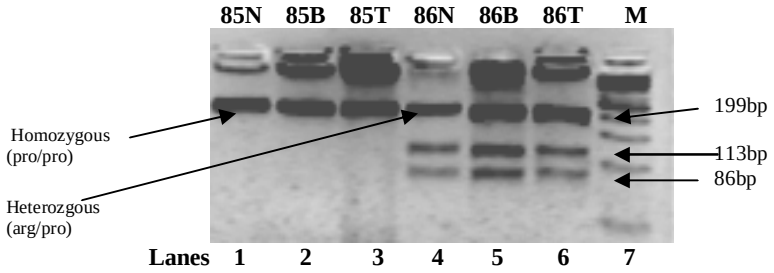

Fig. 5. RFLP gel (4%) showing *TP53* codon 72 polymorphism in blood (B), tumor (T) and normal (N) samples of sporadic breast cancer patients**.** SKH-85 and SKH-86. Lane 1, normal tissue 85; lane 2, blood 85; lane 3, tumor tissue 85; lane 4, normal tissue 86; lane 5, blood 86; lane 6, tumor tissue 86 and M, 50 bp marker.

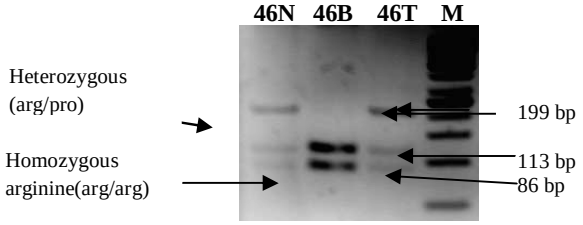

Fig. 6. RFLP gel (4%) showing *TP53* codon 72 polymorphism in blood (B), tumor (T) and normal (N) samples of a sporadic breast cancer patient (SKH46) having different results. Lane 1, normal tissue 46; lane 2, blood 46; lane 3, tumor tissue 46 and lane 4 M, 50 bp marker.

DISCUSSION

Gene polymorphism in normal population

Codon 72 polymorphism is considered important because the presence of homozygous arginine (arg/arg) increases the chances of development of cancer (IARC, 2011). In this study polymorphism at codon 72 in exon 4 was studied both in the normal and breast cancer patients. pro/pro showed the enhanced frequency in normal population compared to homozygous arginine (arg/arg). The frequency of homozygous arginine was 10%, for homozygous proline it was 40%, and for heterozygotic arg/pro it was 50%. The differences in allele frequencies of three polymorphisms of TP53 gene including codon 72 polymorphism among different ethnic groups of Pakistan has been studied previously (Khaliq *et al.*, 2000). For comparing the results of present work with previous study, allele frequencies were determined using genotypic frequencies for codon 72 polymorphism. It was observed that the allele

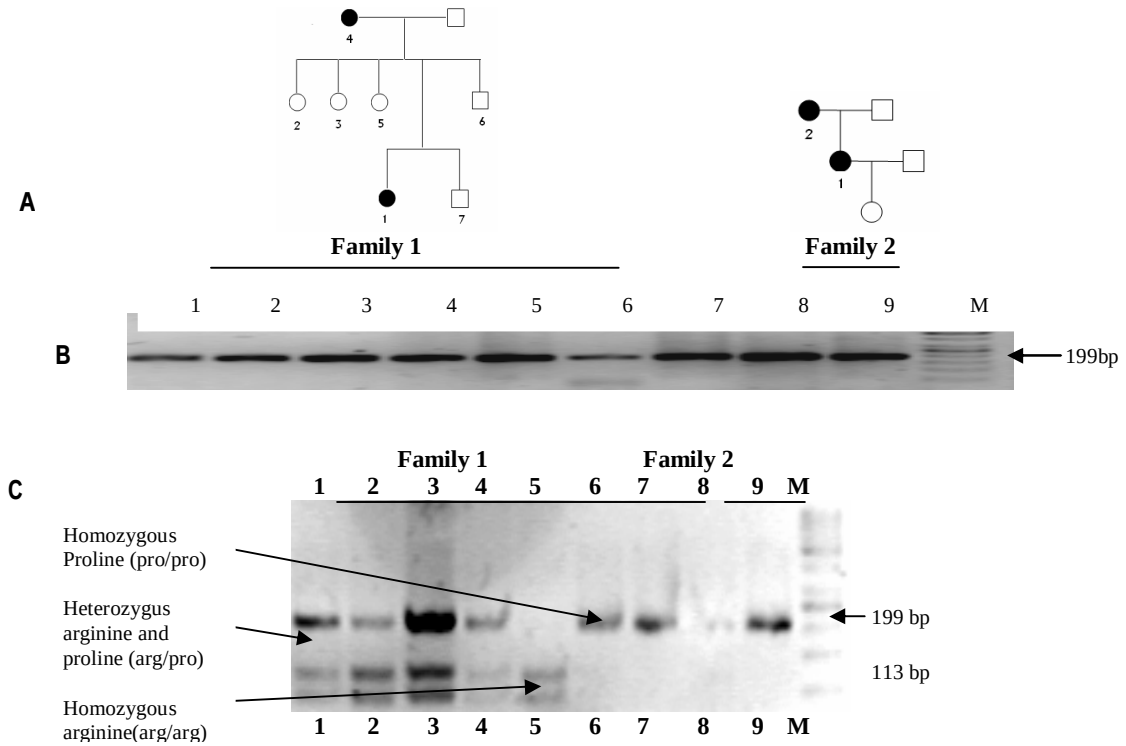

Fig. 7. RFLP gel showing TP53 codon 72 polymorphism in family 1 and 2. A, shows pedigree of families; B, unrestricted 199bp PCR product and C shows fragment restricted with *Bst*UI of codon 72, exon 4 of *TP53* gene of families. Family 1: 1, Patient; 2, sister; 3, sister; 4, mother; 5, brother; 6, sister; 7, patient's husband. Family 2: 8, Mother; 9, daughter. M is 50 bp marker (lane 10).

frequency of pro allele in normal persons was 0.65, whereas in previous work it was 0.50 for Punjabi population. So it may be concluded that apparently higher frequency of pro allele reported in present work may be due to small sample size of normal persons (50) and gender bias as only female subjects were included in the present study. pro allele frequency in normal population is reported to be 0.55 (Ghasemiu *et al.*, 2010) from Iran.

Frequent reports of pro allele in old civilization (Africans who have 95% frequency of proline and the frequency of arginine allele increases in percentage due to migration of populations in far areas) may be the reason of pro allele frequency in Pakistan mostly Punjab which is representative of the Indus valley civilization, a Bronze Age civilization (3300–1300 BCE; mature period 2600–1900 BCE) consisting of Pakistan centered along the river Indus and the Punjab region (Ratnagar, 2006).

Different reports have shown that the frequency of pro allele varies from 0.17–0.63 in populations with different ethnic backgrounds (Mojtahedi *et al.*, 2010). In the Northern hemisphere, the pro allele shows a North-South gradient, from 0.17 in Swedish Saamis to 0.63 in African Blacks (Beckman *et al.*, 1994). In Western Europe (France, Sweden, and Norway), North America (USA), Central and South America (Mexico, Costa-Rica, Peru) and Japan, the most common allele is arg72, with frequencies ranging from 0.60 to 0.83. However, frequencies of pro72 superior to 0.40 have been observed in African-Americans (Jain *et al.*, 2005). A study suggests that these latitude-dependent variations may be due to selection related to winter temperature and not to UV radiation. It has been observed that low average temperature, but not UV radiation, was associated with high frequency of arg72 in Eastern Asia (Shi *et al.*, 2009).

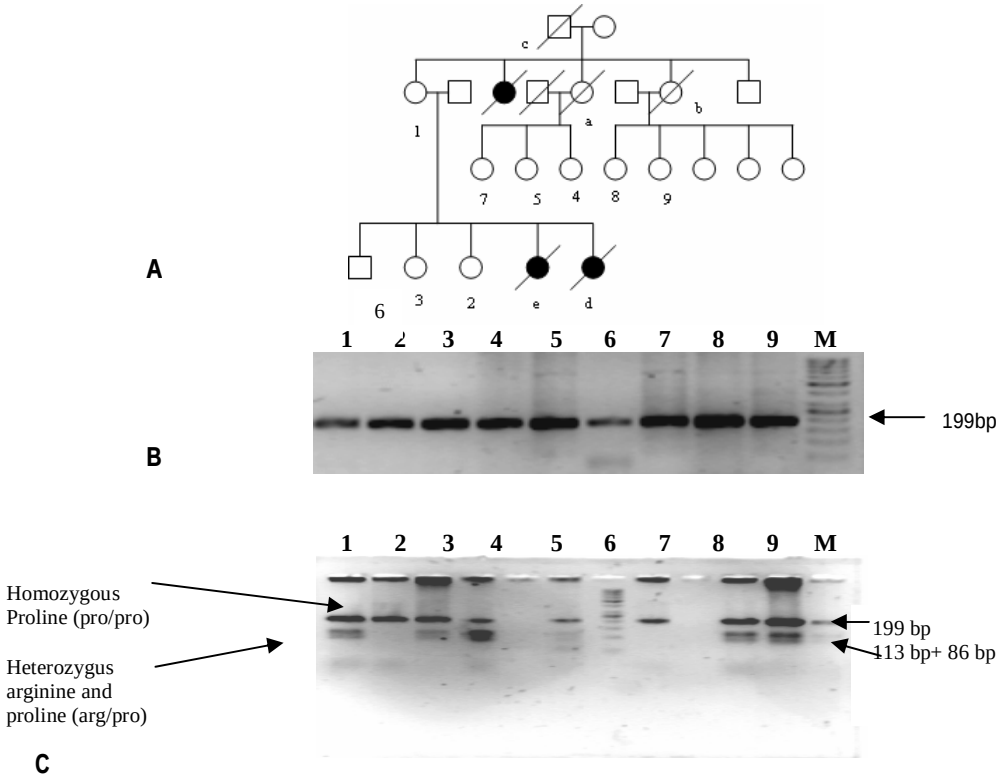

Fig. 8. RFLP gel showing *TP53* codon 72 polymorphism in family 3 (LFS). A, shows pedigree of LFS family , B, shows unrestricted 199bp PCR product and C shows fragment restricted with *Bst*UI of codon 72 in exon 4 of *TP53* gene. 1, mother; 2, daughter; 3, daughter; 4, niece; 5, niece; 6, son; 7, niece; 8, niece and 9, niece.

Gene polymorphism in sporadic breast cancer patients

Overall, the status of codon 72 polymorphism in one hundred and fifty breast cancer patients of present study showed that the genomic frequency was 12% arg/arg, 34.6% for pro/pro and 53.3% for arg/pro. Incidence of somatic *TP53* polymorphism on the arg72 allele is considered as the enhancer of breast carcinomas (Dicomo *et al.*, 1999). The breast epithelial cells malignancy growth (Martin *et al.*, 2003). In Pakistani population, however pro allele was detected prominently in breast cancer patients.

Most of the clinic-pathological characteristics of sporadic breast cancer patients were different compared to reports from developed countries (IARC, 2011) like early age breast cancer, no active smoking, less percentage of familial breast cancer and tumor characteristics. Since there was found no significant difference between frequencies of genotypes of patients and controls so it may be predicted that polymorphisms in codon 72 of *TP53* gene was not associated with breast cancer in Pakistani patients. Our results are in agreement with similar studies on bladder cancer (Toruner *et al.*, 2001) and breast cancer (Beckman *et al.*, 1994) reported from other laboratories. These results also coincide with those of Khadang *et al.* (2007) on breast cancer in Iran but are contrary to some other reports on prevalence of this polymorphism in cervical cancer (Siddique *et al.*, 2005), lung (Wang *et al.*, 1998; Pierce *et al.*, 2000), colon (Sayhan *et al.*, 2001), bladder (Kuroda *et al.*, 2003; Soulitzis *et al.*, 2002), skin (Dokianakis *et al.*, 2002) and breast (Langerod *et al.*, 2002).

The present study shows the dominance of proline genotype compared with arginine. Proline allele as a risk factor for breast cancer has also been shown by others (Sjalander *et al.*, 1996; Weston *et*

Table IV.- Association between *TP53* codon 72 status and clinicopathological characteristics,

	Total No. of patients 150	arg/arg. 18	pro/pro 52	arg/pro 80
Age (Years)				
≤ 40	120	15	40	65
≥ 60	30	3	12	5
Tumor size				
≤ 2 cm	15	2	5	8
2 – d ≤6 cm	135	16	47	72
Node status				
Negative	57	8	20	27
Positive	93	10	32	53
Histology				
IDC	129	12	47	70
ILC	14	5	5	5
Others	7	1	0	5
ER status				
ER+ / PR+	74	9	27	38
ER+ / PR -	48	5	16	27
ER- / PR +	23	3	7	13
E+ / PR-	5	1	2	2
Grades				
1	2	0	0	2
2	71	8	24	39
3	77	10	28	39
Age of menarche				
11-14 yrs	131	16	45	70
15-17 yrs	19	2	7	10
Uterality				
Rt. Breast	76	10	32	34
Lt. Breast	62	6	18	38
Both	12	2	2	8
Smoking				
Active smoking	6	1	1	4
Passive smoking	130	13	49	68
Non smoking	14	4	2	8

al., 1997). It has been suggested that codon 72 polymorphism, particularly the pro/pro genotype, is an independent prognostic factor in patients with breast cancer and provides evidence that patients harboring this genotype will have a reduced survival (Beckman *et al.*, 1994). According to a report breast cancer patients with the pro/pro genotype demonstrated less sensitivity to chemotherapy (Langerod *et al.*, 2002), while a strong association between the arg/arg genotype and breast cancer was reported in Turkish patients (Buyru *et al.*, 2003). In India, the arg/pro genotype in patients with lung cancer was associated with early progression of the disease, compared with arg/arg carriers. However, no relationship was found between *TP53* codon 72 polymorphism and risk of lung cancer after meta analysis (Matakidou *et al.*, 2003). Some investigators reported an increased frequency of the arginine allele in cancer patients compared to controls (Suspitsin *et al.*, 2003).

Stress hormones, specially cortisol exposure has been shown to be an underlying psycho-physiological pathway to breast cancer (Antonova *et al.*, 2011). As it is already stated that the pro allele favors a cell cycle arrest in response to stress (Van Heemst *et al.*, 2005) so the Pakistani women having a significantly higher level of stress than American due to economic and cultural differences (Afza *et al.*, 2011) may have affect on molecular level.

Both homozygotic polymorphisms arg/arg and pro/pro along with *TP53* gene mutation may cause worse prognosis. It may hence be claimed that both the arginine and proline genotypes affect the *TP53* gene mutation pattern but selection of either polymorphism (arg/arg or pro/pro) may be forced by differences in geographical variations which causes selective pressure on these alleles to fix it in the populations.

Gene polymorphism in familial breast cancer patients

The comparison of *TP53* polymorphisms has also been done in the present study. Family no. 1 has all the three categories of polymorphisms, whereas family 2 is homozygous for proline. The LFS family (family 3) showed both pro/pro and arg/pro polymorphisms.

It has been postulated that with the passage of time any of these polymorphisms may become more prominent at an earlier age, due to the phenomenon of genetic anticipation (McInnis, 1996)). Anticipation is a phenomenon, as a genetic disorder is passed on to the next generation, the symptoms of

disorder become more prominent at an earlier age. According to Bougeard *et al.* (2006), the distribution of the arg/arg, arg/pro, and pro/pro genotypes was 41%, 46%, and 13%, respectively in familial breast cancer patients and it was observed that the mean age of tumor onset in affected carriers of the arg allele was 21.8 years and in pro/pro patients 34.4 years. This age pattern coincides in the present research only in case of family 2 which is homozygous for proline.

CONCLUSIONS

Although the allele arg, codon 72, exon 4 of *TP53* gene is reported to contribute to breast cancer development in western countries yet in the present study, the allele pro remain more prominent in Pakistani normal subjects, sporadic breast cancer patients and genetic lineage. It may be due to environmental and geographical reasons.

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The authors have no conflict of interests to declare.

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