Online journal ISSN - 1676-5680
GMR Genetics and Molecular Research
EVOLUTION AND TECHNOLOGY

TP53 Pro47Ser and Arg72Pro polymorphisms and colorectal cancer predisposition in an ethnic Kashmiri population

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Genet. Mol. Res. 9 (2): 651-660 (2010)
Received December 16, 2009
Accepted January 14, 2010
Published April 13, 2010
DOI 10.4238/vol9-2gmr751

ABSTRACT. Two *TP53* gene polymorphisms at codon 47 (*TP53* Pro47Ser) and at codon 72 (*TP53* Arg72Pro) have been associated with susceptibility to various cancers. We carried out a case-control study and examined the genotype distribution of *TP53* Pro47Ser and Arg72Pro single nucleotide polymorphisms (SNPs), using a PCR-RFLP approach, to determine if these two SNPs are risk factors for colorectal cancer (CRC) development and to look for a possible correlation of these two SNPs with clinicopathological variables of CRC. We investigated the genotype distribution of these SNPs in 86 CRC cases in comparison with 160 healthy subjects in an ethnic Kashmiri population. *TP53* Arg72Pro SNP genotype frequencies differed significantly (P = 0.000001) between the groups; the frequency of the Pro/Pro mutant was almost 20% in the general population. We also found significant association of the Pro/Pro mutant with tumor location, nodal status/higher tumor grade and bleeding

per rectum/constipation. We conclude that Arg72Pro SNP is associated with susceptibility to developing CRC in this ethnic Kashmiri population.

Key words: Colorectal cancer; *TP53*; Polymorphism; RFLP; Restriction digestion; Kashmir

INTRODUCTION

Colorectal cancer (CRC), being the most common cancer, is the major cause of mortality and morbidity worldwide, where there are nearly one million new cases of CRC diagnosed each year along with half a million deaths (Boyle and Leon, 2002). The incidence of this malignancy shows considerable variation among racially or ethnically defined populations in multiracial/ethnic countries. Kashmir has been reported lately as being a high-incidence area of gastrointestinal (GIT) cancers (Mir et al., 2005; Murtaza et al., 2006). In Kashmir Valley, CRC represents the third most common GIT cancer after esophageal and gastric (Sameer et al., 2009) as per Sher-I-Kashmir Institute of Medical Sciences (SKIMS) record registry, with almost 104 cases reported in 2009, as also reported previously by Shah and Jan (1990).

TP53, a representative tumor suppressor, located on chromosome 17p13, is one of the most commonly mutated genes in all types of human cancer (Iacopetta, 2003; Whibley et al., 2009). It is 19 kb in size and consists of 11 exons resulting in a transcript of 2629 bp and a protein of 393 amino acids (Bojesen and Nordestgaard, 2008).

TP53 is responsible for the transcription of a site-specific DNA-binding protein and acts as a transcription factor of cell growth-regulator genes (Soussi and May, 1996). In CRC, both germ line and somatic mutations of this gene have been reported by numerous studies up to a frequency of 45%. In half of all human cancers, the tumor suppressor p53 is damaged by somatic mutation in tumor cells (Iacopetta, 2003; Sameer et al., 2009).

Genetic polymorphisms are an important cause of the predisposition to several human cancers. In the *TP53* gene, several polymorphisms have been identified, both in non-coding and coding regions (Murphy, 2006; Bojesen and Nordestgaard, 2008; Costa et al., 2008; Whibley et al., 2009). Most of these polymorphisms are single-nucleotide polymorphisms (SNPs) affecting a single base. Within the coding regions of *TP53*, only two important polymorphisms are present, which alter the amino acid sequence of their products (Pietsch et al., 2006); these are located at codon 47 and codon 72 in exon 4. Codon 72 (Arg72Pro) is a frequent functional SNP that leads to an arginine-proline amino acid change, which has been reported by many authors (Thomas et al., 1999; Dumont et al., 2003). Dumont et al. reported that the Arg72 allele, if in homozygous, has an apoptosis-inducing ability 15-fold higher compared to Pro72 allele. According to Leu et al. (2004), this high apoptosis-inducing ability of the Arg72 allele is in part due to its mitochondrial location, which makes it possible for *TP53* to have a direct interaction with pro-apoptotic BAK protein. Studies on this SNP function were the basis for testing its impact on the risk and progression of tumors, where the less apoptotic allele Pro72 was associated with increased risk for development of tumors (Marin et al., 2000; Ignaszak-Szczepaniak et al., 2006; Toyama et al., 2007). Codon 47 (Pro47Ser), is the second most common polymorphism in *TP53* and leads to a substitution of proline with serine, first identified by Felley-Bosco et al. (1993). The Ser47 polymorphic variant is very rare, with an allele frequency less than 5% in populations of African origin (Pietsch et al., 2006; Murphy, 2006; Whibley et al., 2009). In a pioneer study by Li et al., in 2005, it was found that

the serine 47 polymorphic variant, which replaces the proline residue, necessary for recognition by proline-directed kinases, is a markedly poorer substrate for phosphorylation. Codon 47 encodes proline (CCG) in wild-type p53, but in a small subset of individuals it can encode serine (TCG).

In the present research, we conducted a case-control study and examined the genotype distribution of *TP53* Pro47Ser and Arg72Pro SNPs, using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach, to evaluate its possible relevance in susceptibility to CRC and to study the correlation of these two SNPs with the clinicopathological variables of CRC cases.

MATERIAL AND METHODS

Study population

This study included 86 consecutive primary CRC patients. All CRC patients were recruited from the Department of Surgery, Sher-I-Kashmir Institute of Medical Sciences, from March 2008 to August 2009. Tumor types and stages were determined by two experienced pathologists. Blood samples of 160 age- and gender-matched cases with no signs of any malignancy were collected for controls. The mean age of both patient and control groups was 52 years old, and 56 patients and 104 controls were >50 years old or older. See Table 1 for details.

Table 1. Frequency distribution analysis of selected demographic and risk factors in colorectal cancer cases and controls.

Variable	Cases (N = 86)	Controls (N = 160)	P
Age group			1
≤50 years	30 (34.9%)	56 (35.0%)	
>50 years	56 (65.1%)	104 (65.0%)	
Gender			0.764177
Female	37 (43.0%)	72 (45.0%)	
Male	49 (67.0%)	88 (55.0%)	
Dwelling			0.565659
Rural	59 (68.6%)	104 (65.0%)	
Urban	27 (31.4%)	56 (35.0%)	
Smoking status			0.102256
Never	31 (36.0%)	75 (46.8%)	
Ever	55 (64.0%)	85 (53.2%)	
Pesticide exposure			0.200325
Never	33 (38.4%)	75 (46.8%)	
Ever	53 (61.6%)	85 (53.2%)	

Data on all CRC patients were obtained from personal interviews with patients and/or guardians, medical records and pathology reports. The data collected included gender, age, dwelling, tumor location, Dukes stage, lymph node status, pesticide exposure, and bleeding per rectum. All patients and/or guardians were informed about the study, and their consent to participate in this study was obtained on a predesigned questionnaire (available on request). The collection and use of tumor and blood samples for this study were previously approved by the appropriate Institutional Ethics Committee.

DNA extraction and polymerase chain reaction-restriction fragment length polymorphism

DNA extraction was performed using any one of the previously described techniques. Previously reported primers (Pinto et al., 2008; Katkoori et al., 2009) were used for the am-

plification of the target regions of the *TP53* Pro47Ser and Arg72Pro polymorphisms. Table 2 shows the primers and PCR product sizes.

Table 2. Primers for *TP53* codon 47 and codon 72 polymorphism.

Target codon	Sequence	Amplicon (bp)	T_m (°C)
P47	F 5'-CTG GTA AGG ACA AGG GTT GG-3' R 5'-TCA TCT GGA CCT GGG TCT TC-3'	*201/185*	62
P72	F 5'-TCC CCC TTG CCG TCC CAA-3' R 5'-CGT GCA AGT CAC AGA CTT-3'	*279*	60

PCR was carried out in a final volume of 25 µL containing 50 ng genomic DNA template, 1X PCR buffer (Biotools) with 2 mM $MgCl_2$, 0.4 µM of each primer (Genescript), 50 µM dNTPs (Biotools), and 0.5 U DNA polymerase (Biotools). For PCR amplification, the standard program was used as follows: one initial denaturation step at 94°C for 7 min, followed by 35 denaturation cycles of 30 s at 94°C, 30 s of annealing at 54°C, and 30 s of extension at 72°C, followed by a final elongation cycle at 72°C for 5 min.

For RFLP, the PCR products of *TP53* Pro47Ser and Arg72Pro SNPs were digested with *Msp*I (1 U at 37°C for 16 h) and *Bst*UI (1 U at 37°C for 16 h) (Fermentas), respectively. In the case of *TP53* Pro47Ser polymorphism Pro/Pro wild was identified by two bands (156/140 and 45 bp); Ser/Ser variant was identified by one band (201/185 bp), and heterozygous Pro/Arg variant displayed three bands (201/185, 156/140, and 45 bp) (size divergences due to a 16-bp in/del intronic polymorphism near the *TP53* SNP, as shown in Table 1). In the case of *TP53* Arg72Pro polymorphism, the Arg/Arg wild produced two bands (160 and 119 bp); the Pro/Pro variant was identified by a single band (279 bp), and heterozygous Pro/Arg variant displayed three bands (279, 160, and 119 bp) (Figures 1 and 2).

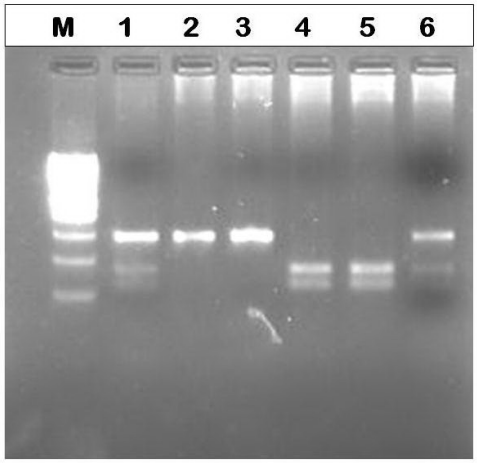

Figure 1. Restriction fragment length polymorphism analysis of *TP53* Arg72Pro single nucleotide polymorphism (SNP) using *Bst*UI enzyme. *Lane M*: 100-bp ladder. *Lane 1-6*: Restriction digestion products; wild (Arg/Arg) is cleaved by *Bst*UI yielding two fragments, of 119 and 160 bp, while mutant (Pro/Pro) yields a 279-bp fragment. *Lanes 1* and *6* show the heterozygous (Arg/Pro) form of SNP. *Lanes 2* and *3* show the mutant form. *Lanes 4* and *5* show the wild (Arg/Arg) form.

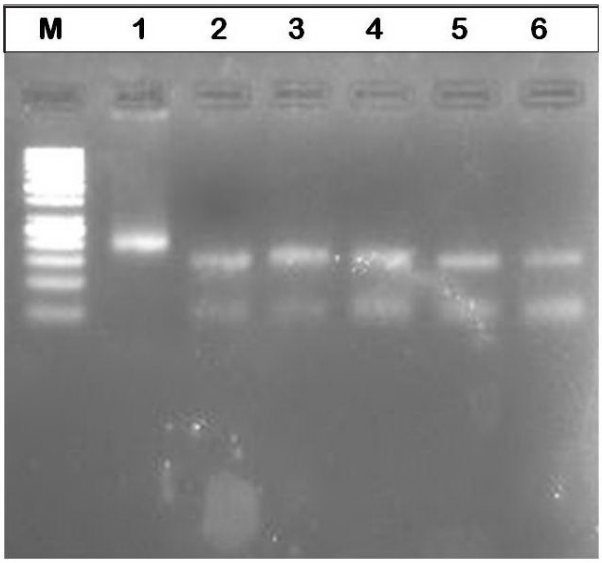

Figure 2. Restriction fragment length polymorphism analysis of *TP53* Pro47Ser single nucleotide polymorphism (SNP) using *Msp*I enzyme. *Lane M*: 50-bp ladder. *Lane 1-6*: Restriction digestion products; wild (Pro/Pro) is cleaved by *Msp*I yielding two fragments of 156/140 and 45 bp, while the mutant (Ser/Ser) yields a 201/185-bp fragment. *Lane 1* shows the mutant (Ser/Ser) form of SNP, and *lanes 2-6* show the wild (Pro/Pro) form.

DNA fragments were electrophoresed through a 2-3% agarose gel for resolution. The genotypes of >20% of the samples were double blindly reassessed to confirm the results by two independent researchers. A positive control for each polymorphism was used for 50% of samples.

Statistical analysis

Observed frequencies of genotypes in CRC were compared to controls using chi-square or Fisher exact tests when expected frequencies were small. The chi-square test was used to verify whether genotype distributions were in Hardy-Weinberg equilibrium. Statistical significance was set at $P < 0.05$. Statistical analyses were performed with the PASW version 18 software.

RESULTS

A total of 86 CRC patients and 160 control subjects were included in this study. The patients comprised 49 males and 37 females (M/F ratio = 1.32), and the control subjects consisted of 88 males and 72 females (M/F ratio = 1.2). Mean age in patient and control groups was 52 years. No significant gender- or age-related differences were observed between the groups ($P > 0.05$). Furthermore, of the 86 confirmed cases of CRC, 81 cases were sporadic, 4 were familial adenomatous polyposis and one case was hereditary non-polyposis colorectal cancer. All but one case were adenocarcinoma and only one was squamous cell carcinoma of basal cell type. There were 59 rural and 27 urban cases.

Carcinoma of the colon occurred in 36 cases, and 50 patients had rectal cancer. There were 55 smokers and 31 non-smokers. See Tables 3-5 for further details.

Table 3. Genotype frequencies of *TP53* gene polymorphisms in cases and controls.

		Cases (N = 86)	Controls (N = 160)	P
TP53 Pro47Ser	Pro/Pro	81 (94.2%)	156 (97.5%)	0.166
C > T	Pro/Ser	0	0	
	Ser/Ser	5 (5.8%)	4 (2.5%)	
TP53 Arg72Pro	Arg/Arg	10 (11.6%)	65 (40.6%)	0.000001
G > C	Arg/Pro	37 (43.0%)	63 (39.4%)	
	Pro/Pro	39 (45.4%)	32 (20.0%)	

Table 4. Association between *TP53* codon 72 phenotypes and clinicopathologic characteristics.

Variables	Cases				χ^2; P
	N = 86	Arg/Arg 9 (10.5%)	Arg/Pro 37 (43.0%)	Pro/Pro 40 (46.5%)	
Age group					
≤50 years	30 (34.9%)	2	13	15	0.757; 0.685
>50 years	56 (65.1%)	7	24	25	
Gender					
Female	37 (43.0%)	4	16	17	0.013; 0.994
Male	49 (67.0%)	5	21	23	
Dwelling					
Rural	59 (68.6%)	4	25	30	0.200; 3.217
Urban	27 (31.4%)	5	12	10	
Smoking status					
Ever	55 (64.0%)	7	25	23	1.678; 0.432
Never	31 (36.0%)	2	12	17	
Tumor location					
Colon	36 (41.9%)	3	27	6	26.84; 0.000001
Rectum	50 (58.1%)	6	10	34	
Nodal status					
Involved	48 (55.8%)	4	11	33	22.23; 0.000015
Not involved	38 (44.2%)	5	26	7	
Tumor grade					
A + B	38 (44.2%)	5	26	7	22.23; 0.000015
C + D	48 (55.8%)	4	11	33	
Pesticide exposure					
Ever	53 (61.6%)	3	22	28	4.306; 0.116
Never	33 (38.4%)	6	15	12	
Bleeding per rectum/constipation					
Yes	60 (69.8%)	4	19	37	18.486; 0.0001
No	26 (30.2%)	5	18	3	
Tumor type*					
Mucinous	33 (38.5%)	6	14	13	3.448; 0.178
Non-mucinous	52 (60.5%)	3	23	26	

*One was squamous cell carcinoma.

Table 5. Genotype frequencies of *TP53* codon 72 gene polymorphism in cases and controls and their associations with the risk of colorectal cancer.

TP53 codon 72 genotype	Cases (N = 86)	Controls (N = 160)	OR (95%CI)
Arg/Arg	10 (11.6%)	65 (40.6%)	1.00 (Ref.)
Arg/Pro	37 (43.0%)	63 (39.4%)	3.81 (1.75-8.32)
Pro/Pro	39 (45.4%)	32 (20.0%)	7.92 (3.51-17.87)
Arg/Pro + Pro/Pro	76 (88.4%)	95 (59.4%)	5.2 (2.5-10.8)

In this study, we found that the genotype frequencies in cases and controls were in Hardy-Weinberg equilibrium. Allele and genotype frequencies of *TP53* Pro47Ser in cases and controls did not differ significantly (P = 0.166) but did in *TP53* Arg72Pro (P = 0.000001). Fur-

thermore, for *TP53* Pro47Ser SNP, the frequencies of Pro/Pro and Pro/Ser genotypes among controls were 97.5 and 2.5%, while in cases these were 94.2 and 5.8%, respectively (P = 0.166) (Table 3). For *TP53* Arg72Pro SNP, frequencies of Arg/Arg, Arg/Pro, and Pro/Pro genotypes among controls were 40.6, 39.4 and 20.0%, while in cases these were 45.4, 43.0, and 11.6%, respectively (P = 0.000001).

The correlation of *TP53* Arg72Pro polymorphic status with the clinicopathological characteristics was carefully analyzed. It was found that the Pro/Pro mutant status was significantly related to tumor location, nodal status/higher tumor grade and occurrence of bleeding per rectum/constipation, but not to other variables (Table 4).

DISCUSSION

The incidences of polymorphism in genomic DNA, their susceptibility to genetic alterations, and the risk of tumor progression in patients with cancer can vary substantially between different racial groups (Perez et al., 2006; Bojesen and Nordestgaard, 2008; Katkoori et al., 2009). Although most polymorphisms are functionally neutral, some affect regulation of gene expression or the function of the coded protein (Costa et al., 2008). Several studies have been carried out on the association of *TP53* polymorphism with increased risk for various cancers (Sjalander et al., 1996; Thomas et al., 1999; Lee et al., 2000; Hiyama et al., 2002; Irarrazabal et al., 2003; Zhu et al., 2007). However, in CRC the data available on the *TP53* polymorphic status are somewhat ambiguous, where several studies found no association (Sjalander et al., 1995; Sayhan et al., 2001; Hamajima et al., 2002; Schneider-Stock et al., 2004). Other authors reported an inverse association (Perez et al., 2006), whereas another study indicated a positive association (Gemignani et al., 2004).

In this study, we assessed the two most common SNPs of *TP53*, Pro47Ser and Arg72Pro, in an ethnic Kashmiri population for the first time, since the role of *TP53* polymorphism in relation to CRC risk had not yet been reported from this part of the world.

In the case of *TP53* Pro47Ser SNP, we found that Ser/Ser frequency was 2.5% in controls, whereas 5.8% in cases, clearly showing a non-significant association of this SNP with CRC predisposition. This observation was in contrast to the previous study, showing a significant association between the mutant Ser47 phenotype and cancer risk (Felley-Bosco et al., 1993). This effect is because the mutant Ser47 phenotype has a decreased capacity to induce apoptosis, to transactivate two p53 target genes, p53AIP1 and PUMA, and to bind to MAPK1 protein as compared with the wild-type Pro47 phenotype (Li et al., 2005; Murphy, 2006; Katkoori et al., 2009).

In the case of *TP53* Arg72Pro SNP, we found that wild-type Arg/Arg frequency was 40.6% in controls whereas 11.6% in cases, and mutant Pro-Pro frequency was 20.0% in control whereas 45.4% in cases. Thus, Pro/Pro mutant phenotype shows a significant association with colorectal tumor development (P = 0.000001).

Codon 72 polymorphism of *TP53* is located within a proline-rich region of the p53 protein and is functionally important in growth suppression and apoptosis (Katkoori et al., 2009). Furthermore, it has been shown that the Arg72 form of p53 has a 15-fold greater capacity to induce apoptosis compared to Pro72, and that this is due to more efficient localization of the Arg72 form to the mitochondria (Thomas et al., 1999; Dumont et al., 2003; Murphy, 2006). Moreover, several earlier studies have reported a significant association between Pro/Pro mutant phenotype and the risk of developing colorectal cancers (Sjalander et al., 1995; Gemignani et al., 2004; Zhu

et al., 2007, 2008; Katkoori et al., 2009). Thus, our findings are in tune with these studies. Another remarkable fact that has arisen from this study is that our Kashmiri population has a significantly greater Pro/Pro allele frequency than Arg/Arg. This may be due to the fact that we reside at high altitudes under low-oxygen conditions and environmental extremes, especially high exposure to UV rays. This observation is similar to that in previously reported studies (Bojesen and Nordestgaard, 2008; Katkoori et al., 2009), where a high Pro/Pro mutant phenotype was found to be a race-specific prognostic molecular marker in CRC, especially in populations challenged by the environment; this is because Pro72 allele has been found to be a better protector against sunlight-induced diseases (Beckman et al., 1994; Zehbe et al., 1998; Katkoori et al., 2009). Furthermore, as the polymorphism is also affected by lifestyle, diet and/or environmental exposure, which vary according to race and ethnicity (Devesa and Chow, 1993; Gapstur et al., 1994), we suggest that as our population is exposed to a special set of environmental and dietary risks, which include the consumption of sun-dried and smoked fish and meat, dried and pickled vegetables, red chilli, hakh (a leafy vegetable of the Brassica family), hot noon chai (salted tea), and hukka (water pipe) smoke (Mir et al., 2005). As previously reported, the etiology and incidence of various GIT cancers in this population has been attributed to probable exposure to nitroso compounds, amines and nitrates, reported to be present in local foodstuffs, most of which have been shown to contain important irritants and carcinogens (Siddiqi et al., 1992; Murtaza et al., 2006). This might have caused the evolutionary preferential selection of Pro72 over Arg72 to deal with the challenge in a better way.

Furthermore, we also found in this study a significant association between the Pro/Pro mutant status and tumor location, nodal status/higher tumor grade and bleeding per rectum/constipation, but not with other clinicopathological variables (Table 4). These results were similar as previously reported by various authors (Sjalander et al., 1995; Schneider-Stock et al., 2004; Lung et al., 2004; Zhu et al., 2007, 2008; Katkoori et al., 2009).

Hence, in this study, which was carried out for the first time in Kashmir Valley, we observed a significant correlation between the Pro/Pro mutant status and tumor location, nodal status/higher tumor grade and bleeding per rectum/constipation, together with the preferential selection of the Pro/Pro mutant allele (frequency = 20%) in this ethnic Kashmiri population. However, this correlation needs to be authenticated in a large sample study in the future, so as to help in the better discernment of racial differences and in determining the aggressiveness of colorectal cancer, as suggested by Katkoori et al. (2009).

ACKNOWLEDGMENTS

Research supported by the Sher-I-Kashmir Institute of Medical Sciences, Kashmir. We would like to express our gratitude to Miss Mahoor S. Nanda of the Department of Biosciences, Jamia Millia Islamia, New Delhi, India, for her technical support. The authors also gratefully acknowledge the technical staff of the Department of General Surgery for helping in the procurement of tumor tissue samples from the Operation Theater. We also thank the anonymous pathologists of the Department of Pathology for the histopathological assessment of the tumor tissues.

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