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ORIGINAL ARTICLE

Profiling of Germline Mutations in Major Hotspot Codons of *TP53* Using PCR-RFLP

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Abstract

Tumor suppressor protein, TP53 also known as the "guardian of the genome" plays a key role in preventing malignant transformation. Almost 50% of human tumors carry mutations in this gene; in the remaining tumors, the *TP53* network is functionally inoperative. The majority of *TP53* mutations are missense mutations and more than 90% of the missense mutations affect specific codons in the DNA-binding domain, called "hotspot codons." The present study was aimed at analyzing the germline mutation status of four hotspot codons in *TP53* namely, codon 175, codon 245, codon 248 (within the DNA binding domain) and codon 72 (outside the DNA binding domain) in cancer cases encountered in a tertiary care hospital in South India by PCR-RFLP. The case-control study included 85–10 subjects respectively. The results of the study indicated that majority of the cancer cases did not harbor germline mutations in the four hot spot codons of *TP53.* The study further highlights the usefulness of PCR-RFLP as a simple and cost effective tool for checking gene mutations.

Keywords Tumor suppressor protein TP53 · Mutation · Hotspot codon · Homoallelic mutation · Heteroallelic mutation · Cancer

Introduction

The tumor suppressor protein TP53 is a nuclear phosphoprotein, which mainly functions as a transcription factor [1]. Various mutations including point mutations and single nucleotide polymorphisms (SNP) lead to variations in the human genome and this finally predisposes individuals to cancer susceptibility [2]. The *TP53* orchestrates a myriad of signaling pathways aimed at prevention of a damaged cell from undergoing malignant transformation [1]. In normal conditions, TP53 is expressed at a low level in the cells. However, the expression of *TP53* gets up regulated under stress conditions such as hypoxia, DNA damage, oncogenic activation and low ribonucleotide level [3]. In response to the cellular stress signals, *TP53* is activated and mediates important cellular responses such as cell cycle arrest, apoptosis, inhibition of angiogenesis and DNA repair [4]. Thus *TP53* is aptly termed as "the guardian of the genome" [5]. Tumor suppression is the most important function attributed to *TP53* and this is reflected in the fact that more than 50% of all cancer cases carry mutations in *p53* gene. Among these, 90% of the mutations have been observed in the DNA binding domain of *TP53* [6]. Majority of these mutations are missense mutations that result in change of amino acid sequence. Mutations in DNA binding domain of the gene interferes with its transcription activity leading to uncontrolled cell growth and proliferation.

Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is a DNA-based technique, which can be used to study gene mutations. The RFLPs are the restriction fragments generated by subjecting PCR- amplified DNA for digestion with specific restriction enzymes. This is based on the principle that the mutation in DNA sequence can either generate or abolish a restriction site thus leading to the generation of different fragments, which can be observed when electrophoresed in an agarose gel. Depending on the number of bands generated, the zygosity of the mutation can be ascertained. Conventionally, gene mutations are detected by direct sequencing of the gene. RFLP is an alternative and cost-effective method for detecting gene mutations.

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Methodology

Sample Collection

A total of eighty-five blood samples were collected from patients from a Tertiary care Hospital, Mangalore. Blood samples of different cancer types were collected with the maximum number being lung carcinoma samples. Other cancer types included breast, cervix, pancreas, ovary, and oropharynx. Blood samples collected from ten healthy individuals were considered as the control samples.

Isolation of Genomic DNA

The DNeasy® blood & tissue kit (Qiagen, Germany) was used for DNA extraction according to manufacturers' instructions. The purity of genomic DNA was determined using a Spectrophotometer (Eppendorff, Germany).

PCR Amplification of *TP53* Gene Fragments

Amplification of *TP53* DNA fragments was done by PCR. Primers were designed to amplify four different fragments of *TP53* gene, with each fragment containing at least one of the four hotspot codons, i.e. codon 72, 175, 245, 248. Primer sequences were obtained from published reports [7–10]. PCR was carried out in a programmable thermocycler (Applied Biosystem, India). The primer sequences and PCR conditions are provided in Table 1.

Restriction Fragment Length Polymorphism (RFLP)

The restriction enzymes used in the study included *BstU1*, *Hha*1, *Aci1* and *Msp1* for codon 72, 175, 245 and 248 respectively. PCR products were subjected to restriction digestion and incubated for about 2 h and 30 min followed by heat inactivation at high temperature for 10 min in a dry bath. The presence or absence of mutation in the respective codon was analyzed after performing gel electrophoresis. The mutations were categorized as no mutation or wild type (*W*/W), mutation in single allele or heteroallelic mutation (W/M), mutation in both the allele or homoallelic mutation (M/M). The details of restriction enzymes used for the experiment is provided in Table 2.

Results

Amplification of P53 Fragments

The primers were designed to amplify hotspot codons at amino acid positions 72, 175, 245, and 248. In total, 4 fragments of *TP53* gene were amplified with each fragment encompassing atleast one hotspot codon. The expected band size for the fragment containing codon 72 was 186 bp whereas it was 108 bp for codon 175, 177 bp for codon 245 and 677 bp for codon 248 respectively.

Analysis of RFLP for *TP53* Hotspot Codons

Restriction digestion was performed for the PCR products obtained from both patient and control samples using specific restriction enzymes for each hotspot codon. Eighty-five patient samples and ten control samples were subjected to restriction digestion for all the four hotspot codons.

Our analysis for codon 72 revealed that 21 out of 85, associated with heterozygous mutation and 25 with homozygous mutation. The RFLP analysis of codon 175 indicated that the 20 out of 85 samples harbored homozygous mutations and only 9 had heterozygous mutation with a majority showing a wild type status. Surprisingly, for codon 245, 65 samples out of 85 analyzed, showed a wild-type status; only 20 samples showed heterozygous mutation status. None of the samples showed homozygous mutation at this site. Interestingly for codon 248, all the samples analyzed showed wild-type status indicating an absence of mutation at this site. As expected, none of 10 control samples showed mutations in any of the codons. The representative images of PCR-RFLP for all the four codons analyzed are shown in Fig. 1.

Table 1 Primers used for the amplification of the specific codon of p53 gene

Targeted codon	Primer sequence	Amplicon size	Annealing Temperature	Reference
72	F-ATGATTTGATGCTGTCCCCG R-AGGTTTTCTGGGAAGGGACA	186 bp	64 °C	Matei et al. [7]
175	F-GCACCCGCGTCCTCGCCATG R-GCTCCACCATCGCTATCTGAGCATCG	108 bp	60 °C	Chen et al. [8]
245	F-AGGCGCACTGGCCTCATCTT R-TGTGCAGGGTGGCCAGTGGC	177 bp	58 °C	Chittmittrapap et al. [9]
248	F-TGGTGCTGGGCACCTGTAGTCCCAGCTACTCG R-ACTACTCAGGATAGGAAAAGAGAAGCAAGAGGC	677 bp	64 °C	Liming et al. [10]

Table 2 Restriction enzymes used in restriction digestion of the hotspot codons

Name of the restriction	Targeted codon	Recognition site	Concentration	Incubation temperature
BstU1	72	CGCG	5U	60°C for 2:30hr
Hha1	175	GCGC	10U	37°C for 2:30hr
Aci1	245	CCGC	5U	37°C for 2:30hr
Msp1	248	CCGG	10U	37°C for 2:30hr

Discussion

TP53 was discovered by David Lane and Arnold J. Levine independently in 1979 [11, 12]. The study on TP53 antibodies in the sera of patients with various types of cancer revealed that the most common mutations in *TP53* gene includes point missense mutations, which are frequently found within the coding region of the gene [13]. Such mutations are mainly found to correlate with major histogenetic groups, which include cases of colon, stomach, breast, esophagus and lung. The commonly mutated regions in *TP53*, known as the "hotspot regions" include codon 72, codon 175, codon 245, codon 248, codon 249, and codon 282. The mutations in these hotspot regions have shown a strong correlation with the disease incidence [10]. It has been shown that mutations in *TP53* gene occur in diverse human tumor types and that these mutations are clustered in four hotspots that coincide with the conserved regions [14]. Considering that *TP53* is a tumor suppressor gene, germline mutations in *TP53* would predispose an individual to a greater risk of cancer. Our study was focused on profiling germline mutational status of four hot spot codons (codon 72, 175, 245 and 248) in *TP53* gene in a

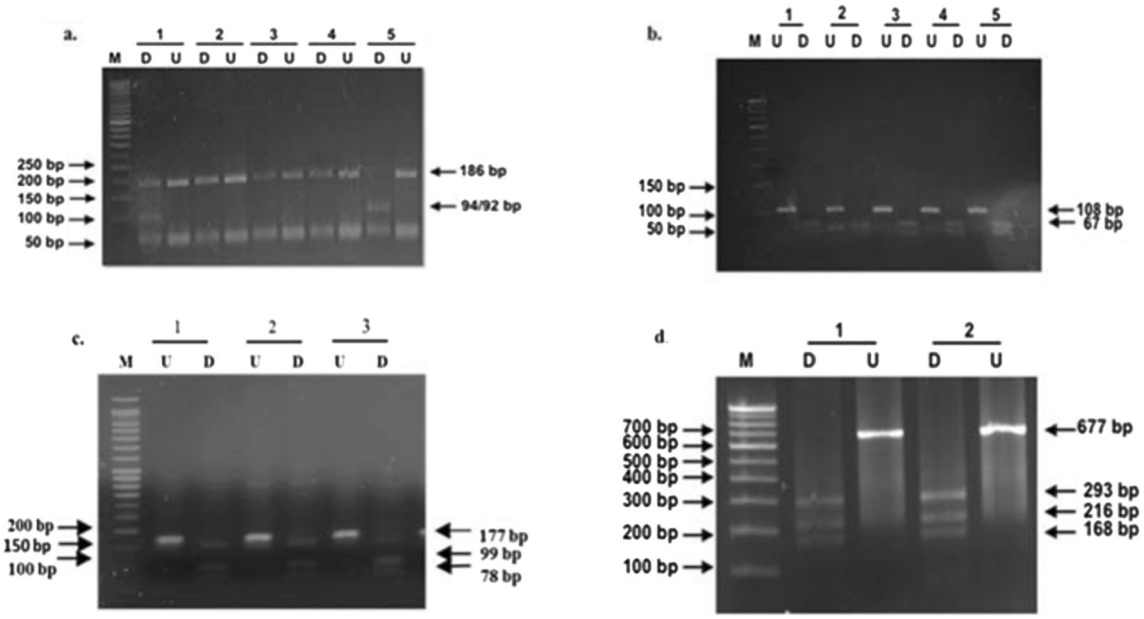

Fig. 1 A representative image of RFLP analysis of hotspot codon 72 (1a), codon 175 (1b), codon 245 (1c) and codon 248 (1d). The PCR amplified *TP53* gene fragment was analyzed by restriction digestion. The RFLP pattern obtained from each sample was compared with the standardized RFLP pattern of codon 72,175,245 and 248 to examine their mutation statuses. Sample codes are indicated. Abbreviations: M: 50 bp ladder; U, undigested; D, digested. 1–5: Number of samples

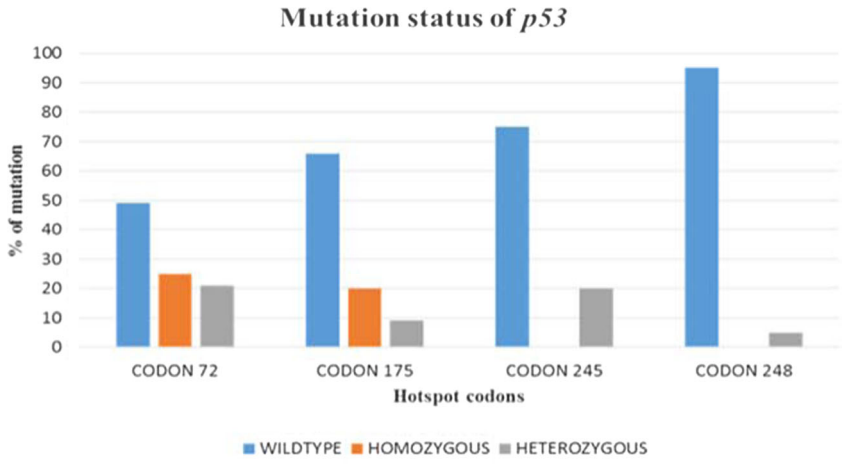

Fig. 2 The overall summarization of data obtained in the study using a bar graph

cohort of cancer cases obtained from a tertiary care hospital in Coastal Karnataka by PCR-RFLP. The applicability of PCR-RFLP in detection of mutations in the *TP53* gene has been shown in earlier studies [8]. The data obtained from our study supports the use of PCR-RFLP for routine checking of *TP53* mutation status in cancer cases.

One of the previous studies suggested that polymorphism at codon 72 increases the risk of ovarian cancer [15]. Another recent study showed that the mutation in codon 72 is indeed quite frequent (43% of the cases) in ovarian cancer in Romanian population [7]. In our data set, codon 72 was found to be the most frequently mutated codon with 48% of the cases showing mono or bi-allelic mutations at this site. However, unlike earlier studies, we have observed these mutations to be associated more with lung cancer cases. Nevertheless, the two ovarian cancer samples that we analyzed showed homozygous mutation at codon 72, indicating loss of heterozygosity at this site, which was in accordance with previous studies. Codon 72 was found to be mutated in few other types of cancer such as carcinoma of cervix, liver, oesophageal, oral cavity and pancreas. In our data set, codon 175 was found to be second most frequently mutated codon. A total of 30% of the cases showed mutation in codon 175 which included 21% with homozygous mutation and 9% with heterozygous mutations. Cancer types that showed polymorphism in codon 175 included thymoma, malignancy of post cricoids region, malignancy of lymph node, carcinoma of cervix, breast and ovary, liver and lung. In case of codon 245, only 21% of the cases were found with mono-allelic mutation and none with bi-allelic mutations. Majority of the cases did not show any mutation, which was a bit surprising given the fact that it is one of the hot spot codons in *TP53*. Data from a *TP53* mutation database (http://p53.free.fr) on human cancer patients (N = 25,902), indicated that 3.3% of the cases have mutation in codon 245 [16]. Quite surprisingly, none of the samples showed any mutation at codon 248.

To summarize, in this study a significant number of cancer cases showed no germline mutations in any of four hot spot codons analyzed, suggesting that germline mutations in *TP53* might not have contributed to the development of cancer in the samples that were analyzed. Although it might be true, considering that almost half of human tumors have wild-type *TP53* gene but an ineffective *TP53* signaling pathway [17], sequencing the entire *TP53* gene would enable identification of mutations in other codons.

Conclusion

In this study, attempts were made to detect mutations in *TP53* gene in cancer cases using a simple and cost-effective technique called PCR-RFLP. Although our dataset was small, the results obtained clearly demonstrated the usefulness of this technique in detecting gene mutations. Further studies with large sample size are necessary to evaluate the potential of this technique in routine molecular diagnosis of cancer.

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Compliance with Ethical Standards

Ethical Considerations The study was approved by the Institutional Ethics Committee. Blood samples were collected only after obtaining the individual consent from the patients.

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