

Mutational analysis of p53 gene in sporadic breast carcinoma

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Abstract: Breast cancer is the most common malignancy among females worldwide and more than 1,000,000 new cases are diagnosed every year. p53 accounts for 12-46% of sporadic carcinoma of breast. In this study all analyzed tumors were sporadic. The PCR-SSCP technique is economical, convenient, fast, safe and popular in clinical research for the mutational analysis. In the present study, mutational analysis of p53 gene in exon 5, 7, 8 and 9 has been done by Single Strand Conformational Polymorphism (SSCP). DNA was isolated from 30 tumor samples and after PCR followed by SSCP, ten samples showed mutations in exon 8 (mutation rate 33.3%), eight mutations were observed in exon 7 (mutation rate 26.6%) and mutation in one sample was found in exon 5 (mutation rate 3.3%) in these 30 tumors. While exon 9 shows no mutation. For exon 8, four samples showing additional band, mobility shift and band deletion as compared to control in SSCP were selected for sequencing. Out of these four samples, two showed polymorphism when compared with the sequencing pattern of the control, one showed deletion of C after codon 281 and other showed insertion of T between codon 293 and 294. Overall, 19 different potential mutations had been detected by SSCP in present study showing 63.3% mutation rate and two mutations (6.66%) were confirmed by sequencing and proved this as a significant data. Suggested mutation of exon 5 and exon 7 by SSCP would be confirmed through DNA sequencing.

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INTRODUCTION

p53 (TP53) is a tumor suppressor gene, also known as the 'guardian of the genome'¹. The biological consequences of p53 activity include cell-cycle regulation, induction of apoptosis, development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and cellular senescence². Presently, p53 is known to play a key role in practically all types of human cancers, and the mutation or loss of the p53 gene can be identified in more than 50% of all human cancer cases worldwide³. In 1993, p53 protein has been voted molecule of the year by the Science magazine. Although the wt p53 gene is a tumor suppressor gene, some mutants of p53 (mt p53) can be considered to be oncogenes. Patients with the Li-Fraumeni syndrome, who have an inherited germline mutation in one of the two p53 alleles, are at very high risk of developing cancer throughout their lifetimes^{4,5}.

The subsequent loss of the wt p53 allele leads to tumors of the brain, breast, connective tissue, hematological system and adrenal gland. p53 inactivation also leads to cancer development in tumors without p53 mutation. For example, mice deficient in wt p53 are susceptible to spontaneous tumorigenesis⁶. Mutations in the p53 gene can result in abolition of protein function and this loss of function may be linked to tumor progression and genetic instability. Clearly, inactivation of p53 is a key event in carcinogenesis. The presence of mt p53 protein, rather than the complete lack of wt p53 activity may confer a selective advantage to evolution of tumor cells⁷. Mutations of p53 gene in hypoplastic marrow represent an early indicator of

significant DNA genetic alteration with cancer propensity⁸. Mutants of p53 proteins observed in many different types of tumors⁹. The result of mutational inactivation of p53 by single amino acid substitutions is that many tumor cells retain the ability to express the mt p53 protein. These proteins are often more stable than wt p53, and are present at very high levels in tumor cell¹⁰.

Wild-type p53 protein has a very short half-life as compared to mutated p53 which is stable and can accumulate at high concentration in the nuclei of tumor cells. Detecting the precise alteration occurring at the genetic level is very laborious and costly. The most widely used molecular approach is single strand conformation polymorphism (SSCP) analysis of DNA fragment amplified by polymerase chain reaction (PCR), with subsequent sequence analysis. The sensitivity of SSCP is influenced by the length of the amplified fragment under study¹¹. Somatic mutations in mononucleotide repeats of genes involved in cell cycle and DNA damage response has been successfully done by SSCP^{12,18}.

The aim of the present study is to screen p53 that is the most commonly mutated gene in human cancers and its mutation has been extensively documented in many solid cancers like breast cancer. The objective is to gain definitive and reliable assessment of the mutational status of the p53 gene in sporadic breast cancer.

MATERIALS AND METHODS

Patients and tissue samples

Tumor samples of breast carcinoma patients (only sporadic cases i.e. no other tumors were referred to in the family history) were collected from

Mayo Hospital and Sheikh Zaid Hospital, Lahore, Pakistan. All cases were invasive ductal carcinomas. Non tumourous tissues of patients were used as control. After informed consent of hospital authorities sampling was done at the time of surgery in 4 months time period. Sampling involved many visits to Hospital during which tumor samples were collected from 30 patients in properly labeled sterile containers. Complete information about the patients was recorded on the performa. Fresh tissues were used during this study and were kept tissue on ice at all times.

DNA extraction

DNA was extracted by PUREGENE DNA ISOLATION Kit protocol “DNA Isolation From 5-10 mg Solid Tissue” (Gentra systems).

PCR amplification of p53 gene

On the basis of published sequences (Gene Bank-EMBL database, accession number X54156). Primers specific to exon 5, 7, 8 and 9 were designed using Primer3 software (Table 1).

PCR amplification of all exons were performed in a total volume 50µl containing 500ng of template DNA, one unit of Taq polymerase, 1.5mM dNTPs, 15pmole primers, 3mM MgCl₂, 5µl of 1x reaction buffer.

SSCP analysis and Staining

Non-radioactive SSCP was performed as follows: A 5 µl volume of PCR product was denatured in 5 µl of formamide loading buffer by boiling for 10 min. Denatured samples were loaded onto 8% acrylamide non-denaturation gel and electrophoresed at a constant 75 V for 6-7 hours. Gels were stained by both methods silver and ethidium bromide staining. Images of the ethidium-bromide-stained gels were captured using the BioRad Gel Documentation system.

DNA sequencing

Table 1: Details of primers and cycling parameters

p53 gene	Sequence (5'-3')	Cycling parameters*	PCR product
Exon 5	F: TTCCTCTCCTGCAGTAGTC R: CTGGGGACCCCTGGGCAA	20sec denaturation at 94°C, 30sec annealing at 55°C, 30sec extension at 72°C	252bp
Exon 7	F: TGGCTCTGACTGTACCACCA R: CAAGTGGCTCCTGACCTGGA	45sec denaturation at 94°C, 30sec annealing at 58°C, 30sec extension at 72°C	145bp
Exon 8	F: ATCCTGAGTAGTGGTAATCT R: TACCTCGCTTAGTGCTCCCT	45sec denaturation at 94°C, 30sec annealing at 58°C, 30sec extension at 72°C	152 bp
Exon 9	F: AGGAGACCAAGGGTGCAGTT R: ACCAGGAGCCATTGTCTTTG	45sec denaturation at 94°C, 30sec annealing at 56°C, 30sec extension at 72°C	280bp

*Initial denaturation of 10 min at 94°C and final extension of 10 min at 72°C were used in all amplifications. Number of cycles in all amplifications was 45.

Samples with abnormal bands were sequenced on Genetic analyzer (ABI) in Centre of Excellence in Molecular Biology (CEMB), Lahore, Pakistan.

RESULTS

Detection of p53 gene mutation by SSCP analysis

The p53 exons 5, 7, 8 and 9 were successfully amplified from all 30 cases which gave expected PCR fragments of 252bp, 145bp, 152bp, and 280bp, respectively. After SSCP analysis, altered p53 was identified by deletion or presence of one or more extra bands migrating above or below the normal PCR products. Ten mutations in exon 8 (mutation rate 33.3%) (Figure 1), eight mutations in exon 7 (mutation rate 26.6%) (Figure 2) and 1 mutation in exon 5 (mutation rate 3.3%) (Figure 3) has been observed through SSCP, while exon 9 showed no mutation.

DNA sequencing of exon 8

Three samples which show potential variation in SSCP in were sequenced along with normal (control). The TP53 tumor suppressor gene is located on chromosome 17p13.1 and the sequencing result of the normal blast with human genome hit on chromosome 17p13 (Figure 4).

The amino acid sequence of p53 (393 amino acids)

MEEPQSDPSVEPPLSQETFSDLWKLLPENNVLSPLPSQAMD
DLMLSPDDIEQWFTEDPGPDEAPRMPEAAPRVAPAPAAAPT
PAAAPAPAPSWPLSSSVPSQKTYQGSYGFRGLFHLHSGTAKSV
TCTYSPALNKMFCQLAKTCTPVQLWVDSTPPPGTRVRAMAI
YKQSQHMTEVVRRCPPHHERCSDSDGLAPPQHLIRVEGNLR
VEYLDDRNTFRHSVVVPYEPPEVGSDCSTTIHYNYMCNSSC
MGGMNRRLPILTIITLEDSSGNLLGRNSFEVRV**CACPGDRD**
RTEENLRKKKGEPHHELPPGSTKRALPNNTSSSPQPKKK
PLDGEYFTLQIRGRERFMFELNEALELKDQAQAGKEPGGS
RAHSSHLKSKKGQSTSRHKKLMFKTEGPDSD

(The highlighted amino acids are those encoded by exon 8 and also showed in chromatograms).

Codon 282 is hotspot mutated codon and, after codon 281 there was deletion of C as a result the frame was shifted, shown by arrow in figure 5 that might results in the formation of abnormal protein. Sequencing results shows that in codon 293 there is G → T transversion which leads to silent mutation of GGG (Gly) to GGT (Gly) shown as arrow 1 in figure 6. In codon 293-294 there is insertion of T which leads to frameshift of amino acids shown as arrow 2 in figure 6. In codon 294 there is G→T transversion shown as arrow 3 in figure 6. Sequencing of sample no. 10 showed no mutation detection.

Figure 1: SSCP gel of exon 8 PCR products after ethidium bromide staining (a) and after silver staining (b). In SSCP, sample no 10 and 3 show an additional band as compared to the control whereas, sample no 18 shows difference in mobility shift (a). Sample no 6 shows an additional band, whereas sample no sample 7, 8, 15, 19 and 12 show difference in mobility shift as compared to the Control (b). D=difference due to mobility shift D1=difference due to presence of additional band

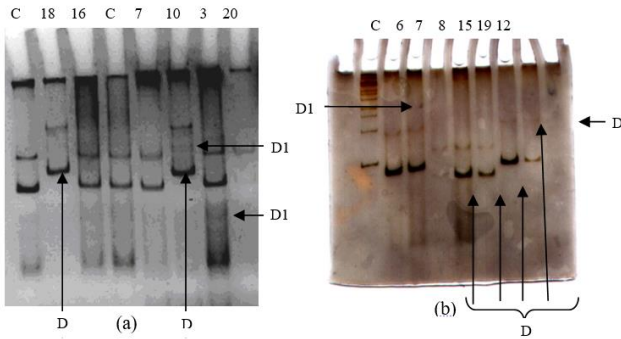
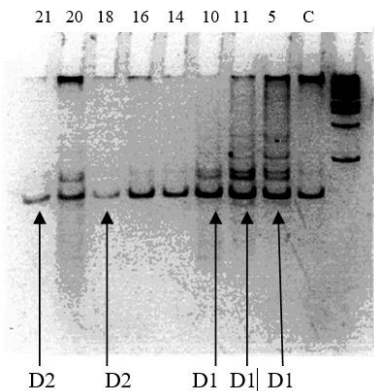


Figure 2: In SSCP of exon 7 sample no 5, 11, 10 show additional bands whereas sample no 18 and 21 show deletion of band as compared to the Control.



D1=difference due to presence of additional bands, D2=difference due to deletion of band.

Figure 3: SSCP gel of exon 5 PCR products after ethidium bromide staining. D1=difference due to presence of additional band

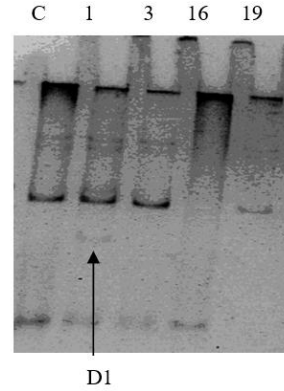


Figure 4: Ideogram showing sequence position of control sample on the chromosome 17.



Figure 5: Chromatogram showing the sequence of tumor sample 8

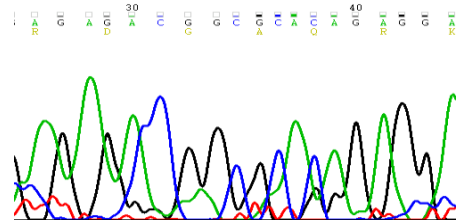
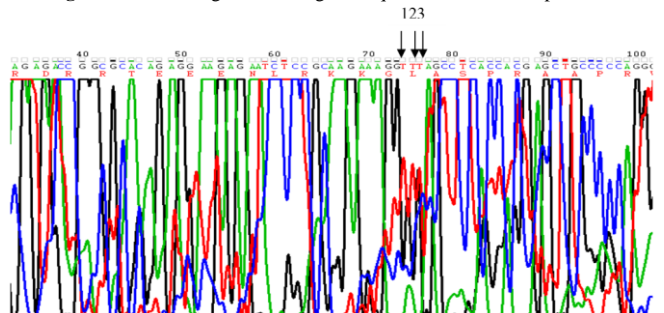


Figure 6: Chromatogram showing the sequence of tumor sample 18



DISCUSSION

Breast cancer is the most common malignancy among females worldwide and thousands of new cases are diagnosed every year. Approximately, 5% of the breast cancer occurs due to germline mutation in BRCA1 and BRCA2. The remaining 95% is due to genetic changes that takes place in a women's life. The genes which undergo sporadic mutation are mostly the tumour suppressor genes. Breast cancer is associated with different types of somatic genetic alterations such as mutations in oncogenes and tumor suppressor genes. To date, the most frequent sites of gene mutations are in the TP53 gene (MIM# 191170) with approximately 30% of the tumors having a mutation. Overview of reported mutations is found in various databases¹²⁻¹⁴. Mutation analysis methods have increased in variety during the past years but the classical methods, such as PCR-manual sequencing and PCR-SSCP, are still valuable and necessary¹⁵. The feasibility of using a rapid and simple polymerase chain reaction-SSCP screening procedure to detect p53 gene mutation in breast cancer for the provision of prognostic information has been demonstrated¹⁶. The PCR-SSCP technique is economical, convenient, fast, safe and popular in clinical research. For PCR products less than 200 bp or more than 400 bp in length, the detection rates by this technique will reach more than 90% and less than 80%, respectively¹⁷. The lengths of PCR products in this study were about 150-300 bp which were more detectable that's why this technique had been selected for mutation analysis.

One can usually identify a deletion or insertion from the SSCP results when all of the major bands shift together. A single nucleotide change could dramatically affect the strand's mobility through a gel by altering the intra-strand base pairing and its conformation. PCR-SSCP results for exon 7 showed the difference in banding pattern in six patients due to presence of additional bands as compared to control so there might be some transition or transversion (Figure 2). And two patients showed difference in the deletion of major band as compared to the control so there might be some deletion which should be confirmed through sequencing. In exon 5 only one sample shows presence of additional band as compared to control (Figure 3). According to PCR-SSCP results of exon 8, seven samples showed mobility shift in band pattern and three samples showed presence of additional band as compared to the banding pattern of control. Three samples were

selected for sequencing, which show potential differences (Figure 1).

Sequencing of sample 18 showed that in codon 293 there is G → T transversion which leads to silent mutation of GGG (Gly) to GGT (Gly) shown as arrow 1 in figure 6. The same mutation has been documented by Gao et al¹⁸ while working on mutational analysis of p53 gene in lung cancer tissues. Codon 293 and 294 are most commonly mutated codons in p53, deletion of G in GGGGAG (293-294 codon) has been studied by Gealy et al.¹⁹ in lung carcinoma patients which leads to frameshift of amino acids. In codon 293-294 there is insertion of T which leads to frameshift of amino acids shown as arrow 2 in figure 6. In codon 294 there is G → T transversion shown as arrow 3 in figure 6. Cooper et al.¹⁹ have documented C → T transition in codon 282 changing amino acid Arg → Trp and same mutation found by Soler et al.²¹ in ALL patients. Codon 282 is hotspot mutated codon and sequencing of sample no 8 showed that after codon 281 there is deletion of C as a result the frame is shifted which might results in the formation of truncated protein as shown in figure 5. In sporadic breast carcinoma deletion of C after codon 281 and insertion of T after codon 293 as showed by sample no. 8 and 18 have not yet been documented. Overall, 19 different mutations has been detected by SSCP in present study and suggested mutated samples would be confirmed through DNA sequencing.

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