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Analysis of Genetic Alterations in *TP53* Gene in Breast Cancer

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Abstract: Tumor protein p53 (TP53) mediates DNA repair and cell proliferation in growing cells. The *TP53* gene is a tumor suppressor that regulates the expression of target genes in response to multiple cellular stress factors. Key target genes are involved in crucial cellular events such as DNA repair, cell cycle regulation, apoptosis, metabolism, and senescence. *TP53* genetic variants and the activity of the wild-type p53 protein (WT-p53) have been linked to a wide range of tumorigenesis. Various genetic and epigenetic alterations, including germline and somatic mutations, loss of heterozygosity, and DNA methylation, can alter TP53 activity, potentially resulting in cancer initiation and progression. This study was designed to screen three reported mutations in the DNA-binding domain of the p53 protein in breast cancer, to evaluate the relative susceptibility and risk associated with breast cancer in the local population. Genomic DNA was isolated from 30 breast tumor tissues along with controls. Tetra and Tri ARMS PCR were performed to detect mutations in the *TP53* coding region. For SNPs c.637C>T and c.733C>T, all analyzed cases were homozygous for the wild-type allele 'C,' while for SNP c.745A>G, all cases were homozygous for the wild-type allele 'A.' These results indicate no relevance of these three SNPs to cancer progression in our study cohort. Additionally, the findings from whole exon sequencing will help to predict more precise outcomes and assess the importance of TP53 gene mutations in breast cancer patients.

Keywords: Breast cancer; p53; Gene expression; Mutation; SNPs

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1. Introduction

Breast cancer (BC) is the most prevalent type of cancer among women and has the second highest death rate after lung cancer [1]. It is multidimensional, poorly understood, and difficult to treat if diagnosed in advanced stages. Old age and female sex are the most common risk factors [2]. The prevalence of breast cancer is remarkably lower in men as compared to women as it accounts for about 1 % of all BC cases in men [3]. BC is one of the leading causes of death among women worldwide. In 2022, 250 deaths are projected to occur per day [4]. BC occurs all around the world in women at any age after puberty. Recent studies have shown an increased prevalence of breast cancer in developed countries as compared to developing countries [5]. Australia

and New Zealand have the highest cases of BC, followed by America and Europe. However, the incidence of breast cancer is comparatively low in South Asia when compared with the developed world. BC is one of the leading causes of death in Pakistani women. Approximately, 90,000 patients are diagnosed with BC with 16,170 deaths each year in Pakistan [6]. Approximately 10% of breast cancer is due to genetic mutation, particularly in the *BRCA1* and *BRCA2* genes. In addition, there are a variety of factors that increase the risk of development and progression of BC such as gender, age, hormonal changes, alcohol consumption, lifestyle, and mutation in genes [7].

Commonly lobules or ducts of breasts are considered the prone areas for developing a tumor. Frequently, the tumor further penetrates via the chest skin, known as the pectoralis muscle, which links the chest wall with the shoulder. Breast cancer can begin in several different locations, including the ducts, lobules, and intermediate tissues. It has been hypothesized that stem cells in the breast are responsible for cancer development due to striking molecular resemblances between normal growth and tumor progression [8]. The two genes that are crucial for breast cancer studies are *BRCA1* and *BRCA2*. They act as tumor suppressors in normal conditions [9]. However, any mutation in these genes results in cancer. Approximately 10% of breast cancer is due to genetic mutations, particularly in the *BRCA1* and *BRCA2* genes. For patients above age 80, the risk of developing BC was recorded to be 72% for the *BRCA1* mutation and 69% for the *BRCA2* mutation carrier [10]. The disease is caused by numerous large mutations in *BRCA-1*, *BRCA-2*, *TP53*, *PTEN*, *STK-11*, and *CDH-1* and a much larger number of medium variations in *CHK-2*, *ATM*, *RAD-51C*, *BRIP-1*, and *PALB-2* is the most well-known approach to breast cancer risk. Cancer is caused by the overexpression of oncogenes and the under-expression of tumor suppressor genes [11]. DNMT, chromatin remodeling, histone modification, and non-coding RNAs are just a few examples of the many epigenetic factors that play a role in tumorigenesis [12].

The “Guardian of the Genome” protein, known as a p53 tumor suppressor is involved in cell differentiation, aging, and development [13]. The p53 protein acts as a transcription factor, influencing the outcome of numerous biological processes, depending on the type of cellular stress signal input. The p53 protein controls cell cycle, apoptosis, DNA repair, and senescence by being triggered by hypoxia, oncogene activation, DNA damage, and food deprivation [14]. The deregulation of the *TP53* gene promotes cell survival, invasion, and proliferation, which promotes cancer development and progression [15]. *TP53* is the breast cancer gene that is most often altered, accounting for 30% of all breast cancer cases [16].

Genomic instability is a crucial hallmark of human cancers and is largely driven by the gain-of-function activity of mutant p53. Additionally, mutant p53 can bind to chromatin-regulated genes and promote methylation and acetylation of histones, which contribute to genetic instability and the development of cancer [17]. The transcription of multiple different genes linked to cell proliferation is triggered by mutant p53 proteins those regulated by the wild-type protein. The genes *e-MYC*, *MAP2K3*, *CXCL1*, and *CCNE2* are among them. Furthermore, SREBP via stimulation of the mevalonate pathway, PARP, NF-Y, and DAB2IP are just a few of the proteins or transcription factors that interact with mutant p53 to encourage breast cancer growth. The main objectives of the present study are to analyze the frequency of *TP53* SNP rs587778720 c.637C>T and rs28934575 c.733C>T using ARMS-PCR and to assess the clinical, pathological relevance, and prognostic value of these SNPs in breast cancer.

2. Material and methods

2.1. Study design

The **TP53** gene mutation profile in breast cancer was examined in the current investigation. Throughout this research work, all the lab apparatuses that were used are given in the following **Table 1**.

Table 1. Lab apparatus

Sr.No.	Equipment Name	Company Name	Model
1	Thermostat	Eppendorf	5352
2	Weighing Balance	Sartorius	CP 153
3	Thermocycler	Applied Biosystem	Gene-amplification PCRSystem-9700
4	Centrifuge	Eppendorf	5417 R
5	Autoclave Machine	Hiclave	HV-25
6	Step 1 Plus PCRSystem	Applied Biosystem	EN60822-1
7	Thermo cycler	Applied Biosystem	Veriti 96 well
8	Gel Tank	Progen Scientific	HU-13 midi horizontal 9902
9	Ice Machine	Scotsman	AF 30
10	Bio Doc analyzer	Biometra	BDA BOX2
11	Power supply	Consort	EV-243
12	Centrifuge machine	Eppendorf	5452
13	Power Supply	CBS Scientific	300Series

2.2. Methods

(1) The solution was made by mixing 0.254 g of $MgCl_2$, 27.36 g of sucrose, and 0.302 g of Tris in 150 mL of distilled water, then raising the volume to 247 mL while maintaining the pH at 7.5–7.6 before autoclaving. After autoclaving, 2.5 mL of Triton was added to the solution, which was stored at -4°C until the foam settled. The solution was then ready to use.

(2) To prepare another solution, 0.182 g of Tris, 3.51 g of NaCl, and 0.11 g of EDTA were added to 150 mL of distilled water, the pH was maintained at 7.5 to 7.6, and then the solution was autoclaved. After autoclaving and cooling, the solution was ready to use.

(3) Solution C was prepared at a ratio of 24:1 by mixing 24 mL of chloroform and 1 mL of iso-amyl alcohol.

(4) Solution D (PCI) was prepared in a ratio of 25:24:1 by mixing 25 mL of phenol, 24 mL of chloroform, and 1 mL of iso-amyl alcohol.

(5) For the preparation of 33 M sodium acetate, 12.3 g of sodium acetate was mixed into 40 mL of distilled water and stirred.

(6) To prepare another solution, 48.4 g of Tris base, 11.4 g of glacial acetic acid, and 3.7 g of EDTA were mixed in approximately 900 mL of distilled water. The pH was maintained at 8, and the final volume was adjusted to 1 liter before autoclaving.

(7) For the next solution, 107.81 g of Tris base, 55.03 g of boric acid, and 7.4 g of EDTA were mixed in about 900 mL of distilled water. The pH was maintained at 8, and the volume was raised to 1 liter before autoclaving.

(8) Finally, 15.7 g of HCl and 2.9 g of EDTA were dissolved in 700 mL of distilled water, the pH was maintained, and the volume of the prepared solution was adjusted to 1 liter.

2.3. Sampling of patients

The cohort contains 40 to 50 pairs of women's breast cancer samples, ranging in age from 28 to 90 years old

with control and their consent. The collected tissues were treated with an RNA-later solution to prevent the RNA from degradation and stored at -20°C for further utilization.

2.4. Mutation analysis of *TP53* gene

Mutation analysis of the *TP53* gene was determined by collecting breast samples from the patient along with adjacent control tissue. DNA was extracted according to an optimized DNA extraction protocol. A tetra-amplification refractory mutation system was used to do mutation profiling.

2.5. DNA extraction

The phenol-chloroform method was used to extract genomic DNA, following the procedures outlined below:

(1) A small portion of 0.2 g of tissue was extracted from the stored sample and transferred to a sterile Petri plate, where it was chopped with a sharp spatula.

(2) The chopped tissue was transferred to a glass homogenizer along with 0.5 mL of Solution A and homogenized properly.

(3) The homogenized mixture was transferred to a new Eppendorf tube and left for 1 hour at room temperature.

(4) After 1 hour of incubation, the tube was centrifuged for 7 minutes at 13,000 rpm. The supernatant was discarded, and the pellet was resuspended in 0.5 mL of Solution A. The pellet was broken up with a pipette tip, and the tube was centrifuged for 4 minutes. This step was repeated.

(5) The supernatant was discarded again, and 0.5 mL of Solution B with 15 µL of 20% SDS and 60 µL of diluted proteinase K was added. The tube was incubated overnight at 37°C for protein digestion.

Day 2:

(6) The dissolved pellet was treated with 0.5 mL PCI in each Eppendorf tube after overnight incubation. The suspension was mixed well by inversion and then centrifuged for 10 minutes at 13,000 rpm.

(7) The upper aqueous layer was collected and transferred to a fresh Eppendorf tube. 0.5 mL of Solution C was added, and the tube was centrifuged for 10 minutes at 13,000 rpm.

(8) The upper layer was transferred to another Eppendorf tube, and 0.5 mL of chilled isopropanol and 55 µL sodium acetate were added. The tube was placed at -20°C for 20 minutes of incubation.

(9) After incubation, the tube was centrifuged for 10 minutes at 13,000 rpm, and the supernatant was carefully discarded.

(10) The pellet was washed with 0.5 mL of 70% ethanol and then centrifuged for 5 minutes at 750 rpm. The supernatant was carefully discarded, and the pellet was allowed to air dry at room temperature.

(11) Finally, the dried pellet was eluted in 40 µL of water for injection or TE buffer.

2.6. Quantitative analysis of DNA

A Nano-drop spectrophotometer was used to quantify the isolated DNA. After loading 1 µL of DNA onto a Nano-drop, the absorbance ratio of 260/280 was calculated. All extracted DNA samples underwent a qualitative examination using the gel electrophoresis technique. The gel was created by combining 70 mL of 1× TAE buffer with 2 µL of ethidium bromide and 1% agarose. The gel was poured onto a gel caster equipped with a 24-tooth comb once it had cooled and been given time to solidify. 2 µL of 6× DNA loading dyes and 4 µL of DNA were mixed and then loaded into the wells of the solidifying gel. For 30 minutes, the gel was operated at 110 V, 350 mA, and 50 W. The gel was then visualized on the gel dock system after 30 minutes.

2.7. Tetra and tri primer designing

Pairs of tetra primers for two SNPs and tri ARMS primers for one SNP of the TP53 gene were designed. The specific SNPs were selected from the NCBI-dbSNP database. These SNPs were rs587778720, rs587782082 and rs28934575. Two SNPs were assessed to make a set of tetra primers and one for tri ARMS primers. Tetra primers contained pairs of forwarding outer, forward inner, reverse outer, and reverse inner primers, while tri ARMS primers contained forward common, reverse common, and reverse primers specific for mutation. The Primer-1 Tool was used to create primers for the specific alleles. Primers were tested for specificity using a primer blast as given in **Table 2**.

Table 2. Primers for *TP53* gene SNPs

Primers	Primer sequence	Product size (bp)	Annealing temp. (°C)
SNP1 rs28934575 (c.733C>T)			
FI (T Allele)	ATGGGCCTCCGGTTCAGGT	143	58
RI (C Allele)	GTAACAGTTCCTGCATGGGAGG	196	
FO	GGATGTGATGAGAGGTGGAT	299	
RO	CCTGCTTGCCACAGGTCT		
SNP2 rs587778720 (c.637C>T)			
FI (CAllele)	AGGGCACCACCACACTATTTC	196	59
RI (TAllele)	GGATGACAGAAACACTTTGCA	243	
FO	GGCCCTTAGCCTCTGTAAGCT	392	
RO	ATAGCGATGGTGAGCAGCTG		
SNP2 rs587782082(c.745A>G)			
FC	ATCTTGGGCCTGTGTTATC		63
RC (Allele A)	GTGTGATGATGGTGAGGATGGAC	122	
RM (Allele G)	GTGTGATGATGGGAGGATGGGAA	259	

Abbreviation: FI, forward inner; FO, forward outer; RO, reverse outer; RI, reverse inner; FC, forward common; RC, reverse common; RM, reverse mutation

2.8. Polymerase chain reaction

Tetra-primer amplification refractory mutation system PCR (ARMS) was performed to amplify specific regions of selected genes. For the PCR reaction, a total volume of 10µl of the reaction mixture was prepared, along with 1 µL of DNA sample, 3 µL PCR water, 2 µL of master mix, and 1.0 µL of each primer. The conditions set for the PCR reaction included initial denaturation (95°C for 5 minutes), 30 cycles of denaturation (94°C for 40 seconds), annealing (optimized temperature for 45 seconds), extension (72°C for 45 seconds), final extension (72°C for 10 minutes), and holding at 4 °C.

2.9. Horizontal gel electrophoresis

The amplified product was visualized using 2% agarose gel electrophoresis. 2 g of agarose powder was used to make the gel, which was then thoroughly dissolved in 100 mL of 1× Tris-boric acid buffer (TBE) and heated in the oven. The pre-staining gel contained 2.0 µL of ethidium bromide. The gel was poured into a ready-made caster and kept for solidification at room temperature. The solidified gel was transferred into the running buffer (1× TBE). 4.5 µL of PCR product was loaded along with 1.5 µL of 100-bp DNA ladder. The gel was run for 45

minutes at 120 V and 300 mA. The PCR products were visualized with the help of the UV trans-illuminator Bio Doc Analyze (Biometra, UK).

3. Results

In the present study, mutation profiling of the *TP53* gene was performed to evaluate the susceptibility as well as the risk associated with breast cancer occurrence in the local population of Pakistan. The study cohort comprised 30 primary female breast tumor tissues and their adjacent normal control tumor tissue samples. In our study cohort female breast cancer patients were aged from 34 to 94 years. The mean age of the cohort was 48. The frequency of the participants was divided into two age groups. The Frequency of patients aged below 50 years was 46.67 % while patients with age above 50 years were 53.33 % involved in breast cancer progression. Stage II, III & IV frequency in our cohort study was highly significant (90%), while patients with Stage I were 10%. According to metastasis status, patients were categorized into two groups, M0 and M1. 83.33% of patients had non-metastatic breast cancer M0. 16.67% were metastatic with status M1. A significant portion of our study cohort had non-metastatic breast carcinoma. The majority of patients in this study had a positive Involvement of lymph nodes. 76.67% of patients were determined positive node involvement while 23.33% of patients had negative nodal involvement.

3.1. DNA extraction

Genomic DNA was extracted from both tumor and control tissues through the phenol-chloroform method. The extracted DNA was quantified using 1% agarose gel and visualized on a UV Trans Illuminator. The banding patterns of extracted DNA are shown in the given **Figure 1** where T is for the Tumor and C is used for the control sample.

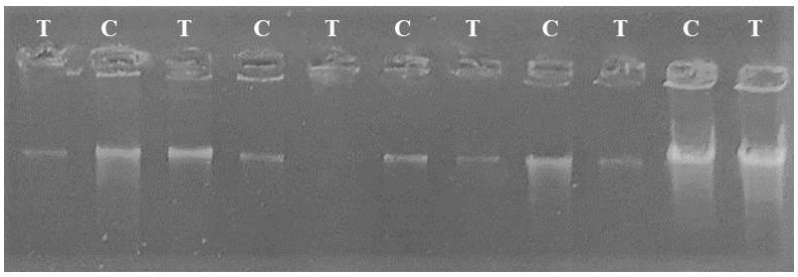

Figure 1. Electropherograms of extracted DNA

3.2. Primer optimization

3.2.1. Tetra ARMS PCR

Gradient PCR was carried out to optimize the sets of primers for the known SNPs (c.637C>T & c.733C>T) of the TP53 gene. The optimized temperature for SNP c.637C>T was 58°C while for the other SNP c.733C>T the optimum temperature was 59°C. PCR Amplified products were resolved on 2% agarose gel along with a 100-bp DNA ladder. The gel was then visualized on a gel dock system (Biometra, BDA BOX2). **Figure 2** shows the banding pattern of wild type band for both outer and inner alleles. At the 'A' panel the size of the outer bands is 299 bp while that of the inner bands is 196 bp. In panel 'B' the product length of the outer bands is 392 bp and the inner band is 199 bp for the wild allele.

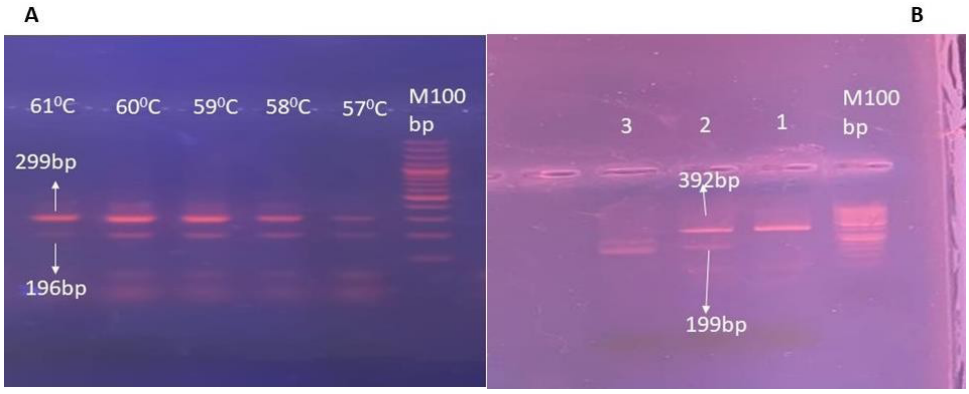

Figure 2. Primer optimization of two SNPs

3.2.2. Tri ARMS PCR

Gradient PCR was carried out to optimize the primers for known SNP c.745A>G of TP53. The optimized temperature at which primers give specified amplification was 63°C.The PCR amplified product was then resolved on 2% agarose gel along with a 50-bp DNA ladder and then visualized on a gel dock system. **Figure 3** shows the 122-bp band amplified after gradient PCR at 62℃ and 63℃.

Figure 3. Optimization of primer for SNP c.745A>G.

3.3. Amplification-refractory mutation system and polymerase chain reaction

We screened all patient and control samples of breast cancer for three mutations in the *TP53* gene. These missense mutations (c.637C>T, c.745A>G, and c.733C>T) are commonly located in the exonic part of the TP53 gene. However, no missense mutations of these positions were detected in our study cohort. In **Figure 4**, the upper bands represent the outer bands, indicating the entire amplicon length of a primer, while the lower bands represent the inner bands of wild types, showing the banding pattern of samples screened for SNPs c.637C and c.733C. Panel A shows the product size of c.733C>T and its banding patterns, with an outer band of 299 bp and an inner wild-type band of 199 bp. Panel B shows the banding pattern and product size of c.637C>T, with an outer band of 392 bp and an inner wild-type band of 196 bp. In **Figure 5**, there were only wild-type bands (122 bp) shown using tri ARMS PCR.

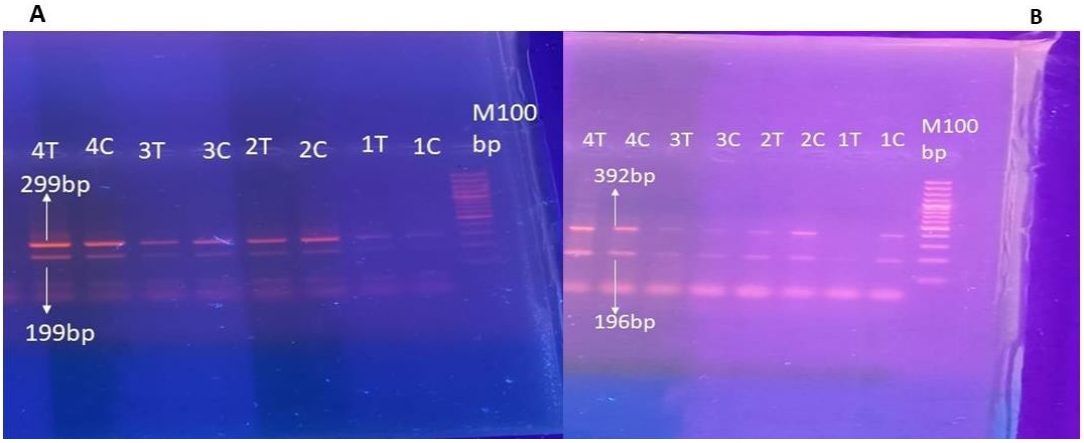

Figure 4. Banding patterns of samples for both SNPs c.733C>T (panel A) and c.637C>T (panel B)

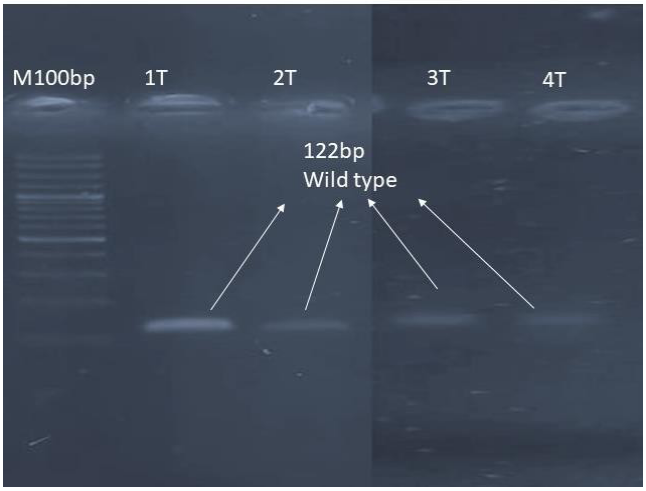

Figure 5. Banding pattern of SNP c.745A>G

3.4. Genotypic and allelic frequency of SNPs

In our study cohort, there were 30 tumor samples along with adjacent controls. All the tumor and control samples were screened through the known SNP c.637C>T, c.745A>G & c.733C>T. The given **Table 3**. shows the genotyping and frequency distribution of alleles. C represent the wild allele and T represent the mutant allele. According to genotyping, the frequency of all the samples was found to be homozygous wild. In the case of c.733C>T and c.637C>T allelic frequency was C=1 while T=0 and in the case of c.745A>G allelic frequency A=1 and G=0 was observed.

Table 3. Genotyping and allelic frequency of c.637C>T, c.733C>T, c.745A>G

Mutation	Patient no.	Control no.	Genotyping frequency			Allele frequency		OR	95% CI	*P*-value
			C/C	C/T	T/T	C	T			
c.637C>T	30	30	30	0	0	1	0	1.000	0.4889–2.0456	1.0000
c.733C>T	30	30	30	0	0	1	0	1.000	0.4889–2.0456	1.0000
			A/A	A/G	G/G	A	G			
c.745A>G	30	30	30	0	0	1	0	1.000	0.4889–2.0456	1.0000

4. Discussion

Breast cancer is one of the most frequent cancers in women globally and is the leading cause of death in women worldwide. Breast cancer is more common in developed nations. Every year, around 1.7 million new cases of breast cancer are diagnosed, accounting for nearly 500,000 (0.5 million) cancer-related deaths (Cao et al., 2021). Pakistan has the highest incidence of Breast Cancer among Asian countries and also it has been identified as the major cause of death among women in Pakistan (Khan et al., 2021). Breast cancer is more common in Pakistani women aged 45 and more, especially during the postmenopausal stage. The disease is distinguished by the disruption of many genomic pathways linked to malignancy. Breast cancer is defined by the uncontrolled proliferation and heterogeneity of aberrant tissue within the breast lobules and ducts (Li et al., 2003). The key to beating breast cancer is early detection. Hereditary breast cancer is caused by mutations in the BRAC1 and BRCA2 genes. Although the majority of breast cancer cases are not hereditary, male breast cancer accounts for around 1% of all BC cases. Because BC trials are uncommon, the bulk of them have solely included female patients (Leon-Ferre et al., 2018). The current COVID-19 pandemic has had a negative impact on breast cancer detection, research, and treatment. The primary function of TP53 is to prevent the formation of neoplastic growth by terminating and reducing unwanted cell proliferation. However, p53's negative regulatory functions are attenuated in cancers (Hassin & Oren, 2023). TP53 is the most often altered gene in cancer. Many researchers are interested in the TP53 protein since it is involved in many fundamental processes such as cancer, aging, senescence, and DNA repair (Barbosa et al., 2019). The loss of p53 function is a major contributor to cancer formation. *TP53* gene structural abnormalities have been discovered in approximately 30% of all breast cancer patients and 80% of triple-negative breast cancer cases (Yamaguchi & Murata, 2020).

The study's goal was to identify *TP53* gene mutation patterns and investigate the susceptibility and risk factors associated with breast cancer occurrence in the local community. We screened out 30 breast cancer samples and their adjacent control samples for mutations. Tetra and Tri-ARMS PCR were used to evaluate all of the breast cancer samples. To detect mutations, three known SNPs (c.637C>T, c.733C>T, and c.745A>G) were tested. According to the results, no mutation was detected in targeted SNP in our study cohort. The allelic frequency of all breast cancer patients and their ANCT had homozygous wild alleles. So our results show that there is no significant relevance of cancer progression with the mutation of targeted SNPs in our study cohort. The frequency of TP53 gene mutations differs between populations due to various risk factors. In the research conducted by Aziz et al. three harmful mutations in codon 238, codon 248, and codon278 were found in Pakistani breast cancer patients, which are the most common somatic mutations in breast cancer patients worldwide, while the frequency of TP53 gene mutations in exons 5-8 was low compared to those reported in the west (Aziz et al., 2013). As a result, it is possible to predict that environmental conditions and dietary habits of a specific community influence the frequency of TP53 gene variants (Dorling et al., 2021). However, our findings are limited by our study's small sample size and brief follow-up period (Li et al., 2019). TP53 gene is linked to cancer development in case of certain cases like mutations, loss of heterozygosity, and alteration in the complex structure. The mutation profile of TP53 in the Pakistani population should be studied. Furthermore, the complete TP53 mutation profile and its connection with other malignancies and therapeutic significance needs to be examined.

5. Conclusion

P53 Mutations in the DNA binding domain have been linked to cancer development and progression. The major theme of the present study was to investigate and predict the mutation profiling of the *TP53* gene concerning the local population. The study screened 30 breast cancer samples and their adjacent control samples for mutations. No missense mutation was detected in our targeted SNPs, all of the breast cancer patients and

their normal control had homozygous wild alleles. Hence, our study concluded that there was no significant association between breast cancer progression and mutation of targeted SNPs. The mutation profile of *TP53* in the Pakistani population must be studied and its therapeutic significance must be investigated. In addition, the findings of whole exon sequencing will help to predict more precise outcomes to assess the importance of *TP53* gene mutations in BC patients and their connection with other cancers.

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Author contribution

Conceptualization: Baqa ur Rehman, Muhammad Abubakar
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Writing – review & editing: Baqa ur Rehman, Muhammad Abubakar, Muhammad Naeem Kiani, Rooma Ayyoub

Disclosure statement

The authors declare no conflict of interest.

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