RESEARCH COMMUNICATION

# Genetic Changes in the PTEN Gene and their Association with Breast Cancer in Pakistan

**Ruqia Mehmood Baig, Ishrat Mahjabeen, Maimoona Sabir, Nosheen Masood, Saniya Hafeez, Faraz Arshad Malik, Mahmood Akhtar Kayani***

## Abstract

**The PTEN gene, a candidate tumor suppressor, is one of the more commonly inactivated and extensively studied genes in cancer. However, few data are available about the role of germ line mutations of this gene in sporadic breast cancer cases. The purpose of this study was to determine extent of involvement of this gene in breast cancer in Pakistan. To test the hypothesis that genetic variations of PTEN play a role in the etiology of breast cancer, a population based case-control study was conducted in 350 breast cancer patients along 400 healthy controls. After extracting DNA from blood, the whole coding sequence of PTEN along with intron/exon boundaries was genotyped by polymerase chain reaction-single stranded conformational polymorphism. Sequencing analysis revealed nineteen different types of mutations in different regions of PTEN (in exon 2, 4, 5, 6, 7 and splicing sites of intron 2 and 4 and also in the 3' UTR region), including 3 silent, 8 missense, 2 frame shift and 6 splice site variations. Among the observed variations in this study, three missense mutations have already been reported i.e. 319G>A (Asp106Asn), 389G>A (Arg129Gln) and 482G>A (Arg160Lys) in different populations. The present results suggest that a wide range of germline PTEN mutations may play a role in the pathogenesis of breast cancer.**

**Key words:** PTEN - breast cancer - germline mutations - PCR - SSCP

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## Introduction

PTEN gene (phosphatase and tensin homologue deleted from chromosome 10) (Li et al., 1997), also termed as MMAC1 (mutated in multiple advanced cancers 1) (Steck et al., 1997), or TEP1 (TGF b regulated and epithelial cell enriched phosphatase 1) (Li and Sun 1997) is a candidate tumor suppressor located on chromosoma 10q23.3 (Steck et al., 1997). PTEN encodes a dual-specificity phosphatase that dephosphorylates focal adhesion kinase (FAK), which results in inhibition of cell migration, spreading, and focal adhesion formation. PTEN regulates cell cycle progression and cell survival (Tamura et al., 1998; Tamura et al., 1999). PTEN plays an important role in the modulation of phosphatidylinositol 3-kinase pathway (PI3K) that is involved in cell proliferation and survival (Besson et al., 1999). PI3K pathway aberrations play a distinct role in the pathogenesis of different breast cancer subtypes (Stemke-Hale et al., 2008). Genetic alterations at PTEN locus has also been described in a variety of neoplasms, including primary central nervous system, breast, prostate, colon and bladder tumors, Glioblastoma and non-Hodgkin's lymphoma (Li et al., 1997; Cairns et al., 1998; Nakaharavet al., 1998; Bismar et al., 2001; Jhawer et al., 2008; Zheng et al., 2008). Studies of embryonic stem cells have shown that cells featuring mutations of the PTEN gene exhibited an increased growth rate and displayed an advanced entry into S-phase (Sun et al., 1999). In breast cancer, cell line analyses have shown that PTEN appears to suppress breast cancer growth by down-regulation of PI3K, with resultant G1 arrest and cell death (Li et al., 1997; Weng et al., 1999). PTEN acts as a transcriptional repressor which inhibits cell-mediated survival signaling pathway and negatively regulates human breast carcinoma cell growth (Ghosh et al., 1999). Germ line mutations of PTEN gene have also been found associated with rare, autosomal-dominant, familial cancer syndrome known as Cowden disease having risk of developing breast cancer (Lynch et al., 1997; Carroll et al., 1999; Nelen et al., 1999).

Prognostic significance of PTEN protein in breast cancer initiation and progression, however, is not well established. PTEN is involved in Cowden syndrome. A

*Cancer Genetics Lab, COMSATS Institute of Information and Technology, Islamabad, Pakistan *For correspondence: mkayani@comsats.edu.pk*

familial cancer disease while additional work is needed to confirm its role in sporadic breast cancer cases (Martin and Weber, 2000). Immunohistochemical analysis of sporadic primary breast carcinomas has shown no or decreased expression in 33% of tumors. Loss of PTEN may therefore play an important role in the development of sporadic breast cancer (Perren et al., 1999). Expression of PTEN in a variety of breast cancer cell lines caused growth suppression via apoptosis (Li et al., 1997). These mentioned studies suggest that PTEN is an important gene mutated in many cancers but very little data is available regarding prognostic significance of PTEN germ line mutations in breast cancer. Current study investigated mutations of PTEN gene and the prognostic significance of these mutations in breast cancer from 350 Pakistani female patients with the disease. The results of this study may aid in early diagnosis and help in understanding the correlation of regulation of its expression with breast cancer.

## Materials and Methods

*The Identification of Patients and Sample Collection*

The present case-control study consisted of 350 pathologically confirmed breast cancer cases along with age and gender matched 400 healthy and disease free normal individuals as controls. Blood samples were recruited from National Oncology and Radiotherapy Institute (NORI) and Pakistan Institute of Medical Sciences (PIMS) Pakistan. These samples were collected with a prior approval from Ethical Committees of both CIIT and hospitals. All study subjects participated on a volunteer basis with informed consent. Subject's blood was collected in EDTA-containing tubes and stored at –20 °C until further use.

*DNA Isolation and Quantification*

DNA was isolated from leukocytes, using organic method as previously described (Nosheen et al., 2010; Masood et al., 2011). Electrophoresis was performed on isolated DNA in 1% ethidium-bromide stained agarose gel and photographed (BioDocAnalyze Biometra). Five ng dilutions of DNA were made for amplification and stored at 4°C until further usage.

*Primer designing and PCR Amplifications*

Primers for whole coding region of PTEN gene were designed using primer 3 input software versions 0.4.0. Intron/ exon junctions were also included in this study for identification of splice site variants. Exons 1–9 were amplified in separate PCR assays. PCR amplifications were performed in 20$\mu$l PCR mixture containing 2$\mu$l PCR buffer, 2$\mu$l of each primer (10mM), 0.24$\mu$l deoxynucleotide triphosphate (25mM) 0.2$\mu$l Taq polymerase (5u/$\mu$l) and 2$\mu$l (10 ng/$\mu$l) extracted DNA. The reaction mixture was placed in 9700 thermal cycler of ABI systems with amplification conditions consisting of initial denaturing step of 5 minutes at 94˚C, followed by 35 cycles of 45 sec. at 94˚C, annealing temperature for 45 sec. and 1 min at 72˚C, with a final extension step of 10 minutes at 72˚C. All patient and control DNA samples was amplified for Pthe TEN gene with exon specific primers.

Amplification products were resolved on a 2% ethidium bromide–stained agarose gel along with 100bp DNA ladder.

**Table1. Mutations in the PTEN gene in Breast Cancer Patients**

| No of Cases | Frequency of Variation | Location | Nucleotide/ Position in Transcript | Amino Acid /Codon | Alteration | Change | Effect |
|---|---|---|---|---|---|---|---|
| 27 | 0.056 | Exon 2 | 92 | 31 | A/- | deletion | Frame shift |
| 12 | 0.025 | Exon 2 | 153, 163 | 50,54 | T>C, A>C | Asp to Asp, Arg to Arg | Silent |
| 12 | 0.025 | 3'splice site, Exon 2 | 31598 | intron | T>C | Splice site | Splice site variation |
| 36 | 0.075 | 5'splice site, Exon 4 | 68527 | intron | -/T | insertion | Splice site variation |
| 20 | 0.042 | Exon 5 | 274 | 91 | G>A | Asp to Asn | Missense |
| 32 | 0.067 | Exon 5 | 319 | 106 | G>A | Asp to Asn | Missense (rs57374291) |
| 10 | 0.021 | Exon 5 | 343 | 114 | G>A | Asp to Asn | Missense |
| 23 | 0.048 | Exon 5 | 396 | 132 | T>G | Gly to Gly | Silent |
| 33 | 0.069 | Exon 5 | 389 | 129 | G>A | Arg to Gln | Missense (rs121909229) |
| 30 | 0.063 | Exon 5 | 457 | 153 | G>A | Asp to Asn | Missense |
| 18 | 0.037 | Exon 5 | 482 | 160 | G>A | Arg to Lys | Missense (37) |
| 42 | 0.088 | Exon 6 | 572 | 190 | T>G | Val to Gly | Missense |
| 28 | 0.059 | Exon 6 | 621 | 206 | T>G | Ser to Arg | Missense |
| 63 | 0.132 | Exon 7 | 676 | 225 | -/A | insertion | Frame shift |
| 66 | 0.139 | 3'UTR | 2634 | Non coding | T>A | substitution | 3'UTR variation |
| 22 | 0.046 | 3'UTR | 266,226,642,665 | Non coding | -/G, T>C, A>T | Insertion, substitution | 3'UTR 2 variation |

**Table2. Multiple Mutations in Different Samples**

| No. of Samples | Multiple Mutations |
|---|---|
| 20 | 92InsA, 153 T>C, 163A>C, 31598T>C |
| 23 | 389G>A, 482 G>A |
| 25 | 274 G>A, 319 G>A, 343 G>A, 396 T>G, 457 G>A |
| 39 | 274 G>A, 343 G>A, 457 G>A |
| 43 | 68526-68527InsT, 389 G>A |
| 62 | 572T>G, T>G621 |

*SSCP Analysis*

PCR product was analyzed by single stranded conformational polymorphism (SSCP) (Amalio et al., 1998) and results were analyzed with gel documentation system (BioDocAnalyze Biometra) after ethidium bromide staining and photographed for further analysis.

*Sequencing*

Amplification products showing abnormal SSCP patterns were selected for sequencing. Samples were prepared as per instructions and shipped to MCLab (USA). The sequenced results were made forward complementary before analysis using BioEdit v 7.0.5 software and analyzed.

## Results

We have screened 350 patients, diagnosed with breast cancer for mutations in the coding region and at intron/exon boundaries of the PTEN gene. Sixteen different types of novel mutations were identified in this study which includes three known mutations.

PTEN mutations were located in exon 2, 4, 5, 6, 7 and the splicing sites of intron 2 and 4 and also in 3' UTR region of PTEN (Table 1). No mutations were detected in exon 1, 3, 8 and 9 while multiple mutations were also detected in some samples. Also no mutation was detected in any of control samples. In this data, out of nineteen variations 3 silent, 8 missense, 2 frame shifts, 2 splice site and 4, 3' UTR mutations were observed in 35, 213, 90, 48 and 88 samples respectively, whereas 212 samples among these have multiple mutations (Table 1, 2). Substitution at 3' UTR region of PTEN 2634 T>A had highest mutation rates of all detected mutations. It was detected in 66 cases and has highest frequency among all variations that is 0.139. Exon 5 has highest rate of mutations as compared to other exons (Table 1).

*Synonymous Substitution (Silent):*

12 samples were found to have substitution mutations at exon 2 with no change in resultant amino acid i.e., 153 T>C (Asp50Asp) and 163 A>C (Arg54Arg) with frequency 0.025. Another substitution was found at exon 5 in 23 samples i.e., 396 T>G (Gly131Gly) having frequency 0.048 (Figure 1A-C).

*Non synonymous Substitution:*

Exon 5 have shown different types of missense substitutions i.e. 274G>A (Asp91Asn) in 20 samples, 319G>A (Asp106Asn) in 32 samples, 343G>A (Asp114Asn) in 10 samples, 389 G>A (Arg129Gln) in 33 samples, 457G>A (Asp153Asn) in 33 samples, 482G>A (Arg160Lys) in 18 samples with frequencies 0.042, 0.067, 0.021, 0.069, 0.063, 0.037 respectively. In exon 6, 2 types of missense substitutions were observed i.e. 572T>G (Val190Gly) in 42 samples and 621T>G (Ser206Arg) in 28 samples with respective frequencies 0.088 and 0.059 (Figure 2 A-H).

*Frame shift Mutations:*

Frame shift mutations were observed at frequencies 0.056 and 0.132. These were found to be at exon 2 due to a deletion i.e. 92delA (Figure 3C) in 27 samples and exon 7 i.e. 675-676insA in 63 cases (Figure 3D).

*Splice site variations:*

Splice site variants were found in different regions

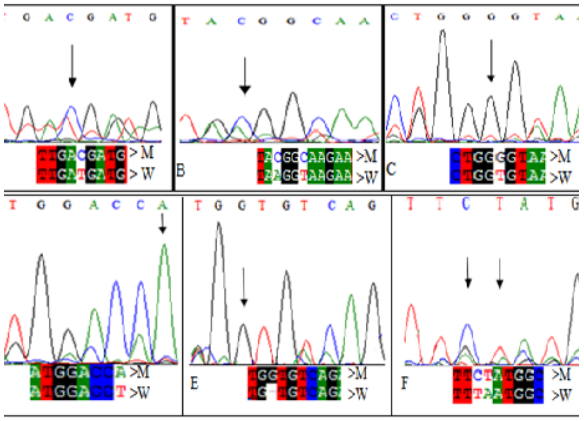

**Figure 1. PTEN Sequences from Genomic DNA Showing Synonymous Substitutions A- 153 T>C (Asp50Asp), B- 163A>C (Arg54Arg), C- 396 T>G (Gly131Gly) and 3' UTR Variations D- 2634T>A, E- 2661-2662insG, F-2664T>C and 2665 A>T.** Arrows shows observed change, M is for mutated sequence while W is wild sequence.

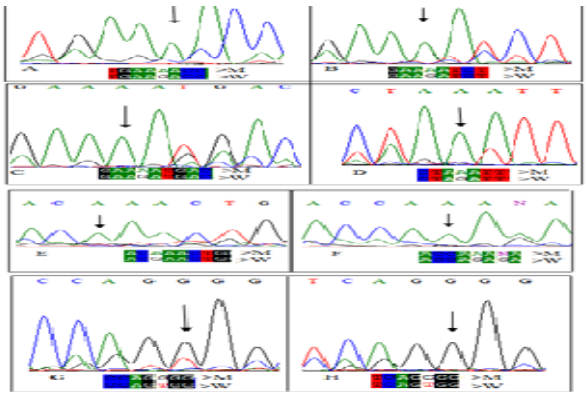

**Figure 2. Sequences Showing Non Synonymous Substitution Mutations in PTEN Gene in Breast Cancer Patients.** A-274G>A (Asp91Asn), B-319G>A (Asp106Asn), C-343G>A (Asp114Asn), D-457G>A (Asp153Asn), E-389 G>A (Arg129Gln), F-482G>A (Arg160Lys), G-572T>G (Val190Gly) and H- 621T>G (Ser206Arg). Arrows shows observed change, M is for mutated sequence while W is wild sequence.

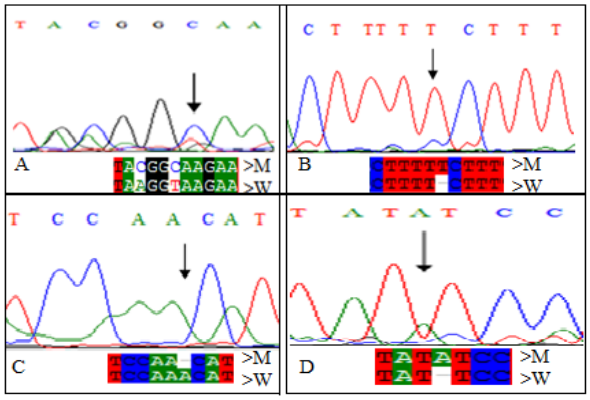


**Figure 3. Sequencing Analysis Showing Splice Site Variations (A- 31598 T>C and B- 68526-68527insT) and Frame Shift Mutations (C- 92delA and D-675-676insA).** Arrows shows observed change, M is for mutated sequence while W is wild sequence.

of PTEN. 3'splice site variation of exon 2 was due to substitution, 31598 T>C, in 12 cases with a frequency 0.025 (Figure 3A). Insertion was observed in 36 samples at 5'splice site of Exon 4 i.e. 68526-68527insT with frequency 0.075 (Figure 3B). Different types of variation were found in 3'UTR region of PTEN that were 2634T>A in 66 samples (Figure 1D) and 2661-2662insG, 2664T>C and 2665 A>T in 22 samples with respective frequencies 0.139 and 0.046 (Figure1E and 1F).

## Discussion

In PTEN/MMAC-1 is a candidate tumor suppressor that appears to have a multifunctional role in cellular proliferation, migration, and invasion (Tamura et al., 1998; 1999). Alterations at the PTEN locus have been described in numerous malignancies and reports have shown a regulatory role of PTEN in growth of breast carcinoma cells (Ghosh et al., 1999; Perren et al., 1999; Weng et al., 1999). Previous studies have demonstrated that inactivation of PTEN closely relate to the poor prognosis of breast cancers (Zhu et al., 2007). A loss of PTEN expression in 32–48% of breast cancers has been observed (Perren et al., 1999; Depowski et al., 2001; Bose et al., 2002; Chung et al., 2004). Although Cowden disease, a breast cancer susceptible syndrome, has higher frequency of PTEN germ-line mutation (Marsh et al., 1998; Bussaglia et al., 2002) as compared to sporadic breast cancers (Rhei et al., 1997; Guenard et al., 2007).

The relationship between PTEN mutation and carcinogenesis of breast cancer remains unclear. Present study was undertaken in order to investigate the role of germ line mutations in PTEN gene on sporadic breast carcinogenesis. All 1–9 exons and splicing sites (intron/exon boundries) of PTEN were analyzed in 350 cases of breast cancer along with 200 normal individuals as control from Pakistani population. The results describe the association of genetic changes in this gene in Pakistani population.

In this study PTEN mutations were observed in exons 2, 4, 5, 6, 7 and the splicing sites of intron 2 and 4 and also in 3' UTR region of PTEN (Table 1). No mutations were detected in exons 1, 3, 8 and 9, with most of the mutations found in exon 5. These findings are in concordance with previous findings that germ line mutations of PTEN occur in exons 2–8, are highest in exon 5, and seldom occur in exon 1 and 9 (Bonneau et al., 2000; Dicuonzo et al., 2001).

Synonymous substitutions having silent effect were found at exon 2 and 5 (Figure 1A, 1B and 1C). In exon 2, 2 substitutions, 153 T>C and 163 A>C were observed which result in the formation of same amino acids at positions 50 and 54 that are asparagine and arginine respectively. Both of these substitutions were found in Phosphatase tensin domain of PTEN (fig 4). Another substitution was found at exon 5, 396 T>G at amino acid 132 and results in same amino acid that is glycine. This glycine is part of important core phosphatase motif (fig 4) found in tyrosine phosphatases and dual specificity phosphatases (Tonks et al., 1996).

Most of the missense mutations are localized at the NH2-terminal portion especially in exon 5 (fig 4). Among these, three mutations have already been reported that are 319G>A, Asp106Asn (rs57374291). Its effect is reported to be deleterious and possibly damaging, resulting in the complete loss of PTEN functions (Goliaei et al., 2009). 389 G>A (Arg129Gln) (rs121909229) is another already reported mutation in Japanese (Kanaya et al., 2005) and in Chinese population (Yang et al., 2010) that is located in the phosphatase core motif where high mutational frequencies have been detected in various tumors. Base 389 is also among the dinucleotide CpG. The mutation resulted in the change of arginine to glutamic acid. This is consistent with the observation that frequent C to T (or G to A) transition occurs at a much higher rate in methylated CpG dinucleotide than in unmethylated bases (Yang et al., 2010).

For 482G>A (Arg160Lys), 18 samples were found to have this missense mutation with the substitution of arginine for lysine where the encoding amino acid is probably important for stabilizing the tertiary structure of the protein. This mutation has also been reported by Yang in Chinese population (Yang et al., 2010). Another missense mutation observed at base 457 is reported to show variation 457G>C, Asp153His (rs9651492). In this study variation found was 457G>A (Asp153Asn).

Novel missense mutations were also observed that are 274G>A which change amino acid aspartate in to asparagine at amino acid 91, 319G>A change amino acid aspartate in to asparagines, 106 amino acid and 114th amino acid is also changed due to 343G>A that also change amino acid aspartate in to asparagine. Exon 6 also contains 2 types of missense mutations that are 572T>G (Val190Gly) and 621T>G (Ser206Arg). All these missense mutations are very important in context of their consequence. As exons 5 and 6 contain WPD loop, P loop, and TI loop, which are making the active site pocket of the phosphatase domain in PTEN. Mutation

on the WPD, P, or TI loop of recombinant PTEN protein reduces its phosphatase activity compared with wild-type PTEN, and mutation on the CBR3 loop reduces affinity for membranes in vitro (Lee et al., 1999). Mutations that involve WPD, P, TI, or CBR3 loop disrupt the tumor suppressor function of PTEN more completely and thus contribute to the development of tumor with a more virulent phenotype (Ali et al., 1999).

Frame shift mutations were observed in exon 2 and exon 7 (fig 4). Deletion was observed at nucleotide 92 in exon 2 i.e. 92delA. Shifting of reading frame resulted in the amino acid changes at the polypeptide chain and early appearance of termination codon which in turn produced a truncated protein with 53 amino acids, leading to the loss of dual specific phosphatase catalytic domain located at amino-end phosphatase region. Thus, PTEN loses its normal protein function and tumor suppressive activity (Lee et al., 1999; Yaginuma et al., 2000).

Another frame shift mutation at exon 7 is 675-676insA, in C2 domain of PTEN gene. It has been proposed earlier that this region plays an important role in active protein expression as it is associated with phospholipid binding of both substrate and membranes. Mutations in the C2 domain also have decreased or no phosphatase activity (Sun et al., 1999; Eng et al., 2003). Furthermore, this deletion change the whole downstream coding sequence that might change the carboxyl terminal PDZ motif in PTEN. This motif is important in respect to PTEN ability to inhibit Akt activity (Wu et al., 2000).

Alterations at splicing site may contribute to a decreased expression of PTEN, due to abnormalities in RNA stability and splicing process (Francisco et al., 2008). In this study, splice site variations were observed that are 31598 T>C in 3' splice site of exon 2 and 68527-68527insT at 5'splice site of Exon 4. Variations at same position has earlier been reported, (CD003200) but in this study instead of deletion 68527delC, insertion of T is observed.

Genetic changes in 3' UTR of several genes have earlier been reported to be associated with higher susceptibility to particular tumor types (Vogelstein and Kinzler 2002). While screening 3' UTR region of PTEN different types of mutations were also found i.e. 2634T>A, 2661-2662insG, 2664T>C and 2665 A>T. Among these nucleotide 2634 is reported to have variation T>C (rs1044487) while in this study it was observed to be T>A. Another mutation i.e. 2664T>C has also been reported earlier (rs74535369) but with different nucleotide change i.e. 2664T>A.

In conclusion, a wide range of germ line mutations in PTEN gene observed in this study in sporadic breast cancer patients have shown a correlation of PTEN germ line variations with breast cancer. This shows that germ line mutations play important pathogenic role in sporadic carcinogenesis. The current findings can of prognostic and therapeutic implications for the management of patients with breast cancer. This demonstrates that PTEN could prove to be a good candidate of better diagnosis, treatment and prevention of breast cancer but obviously more detailed studies are warranted.

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