PCR-SSCP-DNA SEQUENCING METHOD IN DETECTING PTEN GENE MUTATION AND ITS SIGNIFICANCE IN HUMAN BREAST CANCER IN TURKISH POPULATIONS

Hasibe Cingilli Vural
Selcuk University, Department of Biology, Molecular Biology, Konya, Turkey
Correspondence to: Hasibe Vural
E-mail: hcingilli@selcuk.edu.tr

ABSTRACT
The aim of this study was to analyze the possible effect of PTEN gene mutations on the occurrence and development of human breast cancer. PCR-SSCP-DNA sequencing indicated that two kinds of mutation sites were found in 4 out of 80 breast cancer samples. One kind of mutation was found in exons. AA-TCC mutation was located 40 bp upstream of the 3’ lateral exon 2 (115946 AA-TCC). Such mutations led to terminator formation at codon 267 of the PTEN gene. The other mutation was found in an intron, including a C-T point mutation 91 bp upstream of 2’ lateral exon 2 (1903858 C-T). The PTEN protein expression in tissues with breast cancer indicated that the total positive rate of PTEN protein expression was 68 % in breast cancer tissue, which was significantly lower than that (100 %) in paracancerous tissues (P < 0.005). PTEN gene mutation may play an important role in the occurrence and development of breast cancer.

Keywords: breast cancer, PTEN gene, PCR-SSCP, mutation

Introduction
PTEN is a tumor suppressor gene located on human chromosome 10q23. This gene encodes a dual substrate-specific phosphatase and is frequently deleted or mutated in a wide range of human tumors and tumor cell lines. Allelic losses and somatic mutations at the PTEN locus are present in aggressive brain tumors, endometrial and breast cancers and melanomas, reiterating its role as a tumor suppressor gene. Allelic losses at 10q23.3 in breast cancers have been correlated with an increased incidence of lymph node metastases, and poor histologic grade. A candidate tumor suppressor gene PTEN (also known as MMAC1 or TEP1) has recently been isolated from chromosome 10q23.3 (9, 18). However, there are only few reports on PTEN gene mutation and protein expression in breast cancer (6, 19). The aim of this study was to analyze the possible role of the PTEN gene in the occurrence and development of breast cancer.

Materials and Methods
Subjects
Paraffin blocks of breast pathologies were derived from the archives of the Department of Pathology in the Faculty of Medicine at the University of Cumhuriyet, Turkey. Namely, paraffin-embedded breast cancer tissue specimens of 80 breast cancer patients were used in this study. The age range was 35–72 years, all patients were females. Ten patients had family history of cancer and 40 patients were excluded from the study due to the lack of a detailed family history. Their breasts were examined by one or more of the following means: the tests used for screening, diagnosis, and monitoring, including mammograms, ultrasound, MRI, CAT scans, PET scans. Biopsy was performed for the patients who were suspected to have breast cancer, and all specimens were from archived paraffin blocks that had been used in routine diagnosis of cancer, and none of them were collected specifically for this study. Control samples were obtained from natural or healthy tissue of the same cases. We carried our tumor, nodule and metastasis grading as postoperative anamnesis and pathological reports of all the patients. All assays were tested as localization of tumor (T); T1, T2, T3 and T4; formation of nodule (N); N0, N1, N2, N3 and the case of metastasis (M); M0 and M1. The clinicopathological characteristics of the tumors or pTMN stage I was identified in 22 patients, stage II in 8 patients, stage III in 32 patients, and stage IV in 18 patients. Furthermore, in this study we used exon 1 and exon 2 spesific primers for the PTEN gene for polymerase chain reaction (Table 1).

Genome DNA isolation from breast cancer and paracancerous tissues
Formalin-fixed paraffin samples were cut into 10 μm sections. The sections were pulverized under liquid nitrogen condition using Mikro-dismembrator (B. Braun Biotech, Melsungen, Germany). For each sample, 0.1 g of pulverized tissue powder was resuspended in 1 ml of 0.25 % xylene and left for 15 min at 55 °C. The suspension was then centrifuged at 14 000 g for 5 min. The pellet was suspended in 0.1 ml of xylene and processed as above for a second time. The resulted sediment was mixed with 100 % ethanol and processed with xylene lysis buffer (Tris, sodium dodecyl sulfate, ethylenediaminetetraacetic acid, or EDTA). A lysis buffer containing 300 μg/mL of proteinase K was added to the
PCR primers of PTEN gene exon 1 and 2 including locales and annealing temperature $T_m$ values

<table>
<thead>
<tr>
<th>No</th>
<th>Exon</th>
<th>Locus</th>
<th>Sequence of Primers</th>
<th>Primer length (bp)</th>
<th>Amplified fragments (bp)</th>
<th>Annealing temperature ($^\circ$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>Exon-2F</td>
<td>TGACCACCTTTTATTACTCCA</td>
<td>21</td>
<td>367</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>TACGGTAAGCCAAAAATGA</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>Exon-1F</td>
<td>TCTGCCATCTCCTCCTCCTC</td>
<td>20</td>
<td>177</td>
<td>60.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CCGCAGAAATGGGATAAAGT</td>
<td>20</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Restriction enzyme reaction with Taq I**

The restriction enzyme reaction (7) was composed of 3.2 μL ddH$_2$O, 1.5 μL buffer Taq I, 10.0 μL PCR product, 0.3 μL Taq I (10 units/μL) in a total volume of 15.0 μL. The mixture was centrifuged for 15 s and heated for 3.5 h at 65 °C. Ten microliters of the restriction enzyme reaction product were run for 45 min at 100 V in a 2 % agarose gel containing 0.5 g/L EB, with a 100 bp DNA ladder as a standard reference. The results were observed with an ultraviolet transmission analysis instrument and photographs were taken with an automatic gel documentation system.

**Heteroduplex analysis by SSCP**

Each of the primary breast cancers was first screened for mutations by using the PCR-SSCP approach. Primers used for each PTEN exon were as described by Lu et al. (12). The intron-exon structure of PTEN has been reported previously and primers were designed to amplify each of the two exons (primer details and conditions are available on request). PCR products (5 μl) were denatured and electrophoresed in an 8 % polyacrylamide gel containing 5 % glycerol, using 0.5 TBE as the running buffer. Electrophoresis was at 1 W per gel for 12–18 h depending on the size of the PCR products. DNA bands were visualised by silver staining. According to the PCR-SSCP results of genome DNA, the difference in the single strand strip number and electrophoresis transference location, also known as the mobility shift, was considered PCR-SSCP positive (7).

**DNA sequencing**

Genomic DNA from positive PCR-SSCP samples was amplified again in 60 μL reaction system. The product was identified by electrophoresis for bidirectional DNA sequencing.

**Histopathology study**

In the study (from 2002 to 2008) 80 new cases which were sent to Cumhuriyet University Medical Faculty, Department of Pathology, with the prediagnosis of malignant tumors and whose fresh tissue samples had been investigated were analyzed. Fresh tissue samples about 3 mm were taken from the tumor pieces for the purpose of molecular genetic analysis and were stored in a freezer (-20 °C). For histopathologic investigation, tissues were fixed in 10 % formalin and were embedded in paraffin, then 5 μm cross sections were prepared and they were stained with routine haematoxylin-eosin dye. A
sample histopathological image of breast cancer types (stained using hematoxylin-and-eosin technique) is shown in Fig 1.

Fig. 1. Sample histopathological image of breast cancer types stained using hematoxylin-and-eosin technique. Bar = 10 μM.

**Statistical analysis**
Fisher’s exact probability and chi-square test were used for statistical analysis. $P < 0.05$ was considered statistically significant.

**Results and Discussion**

**Mutation of exons 1-2 in the PTEN gene in breast cancer tissue**
Detection of the PTEN gene exons 1-2 of genome DNA in 80 paired breast cancer and paracancerous tissue samples indicated that the amplified PCR product had no gene homozygous alteration and no large alteration in the alleles. The results indicated that the number and size of the bands were in accordance with those theoretically expected. The 367 bp, 330 bp, 30 bp segments were relatively justified as the complete restriction enzyme reaction.

**SSCP detection**
Positive PCR-SSCP in terms of mutation of exon 1 and exon 2 in the PTEN gene was considered as abnormal single strand number and mobility location. Two kinds of mutations were detected in 4 of the 80 breast cancer tissue samples (mutation rate of 9.6 %). A shift in a band of exon 2 was found in 4 samples (mutation rate of 7.8 %) (Fig. 2).

Fig. 2. Representative image of mutations in exon 2 of the PTEN gene identified by SSCP. Lane 1: 100 bp DNA ladder; Lanes 2, 3, 4, and 5: positive SSCP.

**DNA sequencing**
Bidirectional DNA sequencing was applied on amplified genomic DNA from positive PCR-SSCP samples. The results suggested that only one mutation was found in exons. PCR-SSCP-DNA sequencing indicated that there were two kinds of mutation sites in 4 out of 80 breast cancer samples. One kind of mutation was found in exons. AA-TCC mutation was located 40 bp upstream of the 3’ lateral exon 2 (115946 AA-TCC). Such mutations lead to terminator formation codon 267 of the PTEN gene (7). The other mutation was found in an intron, including a C-T point mutation 91 bp upstream of 2’ lateral exon 2 (1903858 C-T) and a single base A mutation 7 bp upstream of 2’ lateral exon 2.

The PTEN protein expression in tissues with breast cancer indicated that the total positive rate of PTEN protein expression was 68 % in breast cancer tissue, which was significantly lower than that (100 %) in paracancerous tissues ($P < 0.005$). No statistically significant correlation was observed between PTEN expression and the clinicopathological parameters of the studied breast cancer patients, such as age, location and size of the carcinoma, distant metastases, and embolization ($P > 0.05$). Significant correlation ($P < 0.05$) was revealed with the infiltrating depth, lymph node metastasis, and pTMN staging.

Few studies are available on PTEN gene mutation and protein expression in breast cancer. However, cancer cells have not been isolated from normal cells which may lead to undetectable PTEN gene mutations because of plenty of normal genome DNAs. Furthermore, inherited changes in several other genes, including PTEN have been found to increase the risk of developing breast cancer and endometrium cancer. The results from this study also suggest that molecular testing is probably the only reliable method for making a correct diagnosis, especially in the case of rare clinically overlapping syndromes.

PTEN is a crucial negative regulator of breast tumorigenesis and loss of PTEN is associated with a poor outcome of breast cancer (5, 12). PTEN has been reported as one of the important factors controlling mammary epithelial cells during normal mammary gland development and mammary gland cycling (8). For example, analysis of the four histologic types of ovarian tumors (endometrioid, serous, mucinous, and clear cell) for the loss of heterozygosity on chromosome 10 and mutations in the PTEN/MMAC1 gene indicated that the gene is predominantly mutated, if not exclusively, in ovarian tumors of endometrioid origin (15, 20). Thus, an important observation is that PTEN/MMAC1 mutations are common in endometrial as well as ovarian tumors of endometrioid histology and that tumors of both types containing such mutations are well or moderately differentiated, suggesting the involvement of PTEN/MMAC1 tumor suppressor function in disease initiation (1). As outlined by Li et al. (10), PTEN/MMAC1 was originally isolated from a region homozygously deleted in several cancer cell lines, including gastric carcinoma and cancers of the breast and endometrium. Mutations in this gene have been reported
as endometrial carcinoma, breast tumors, and malignant melanoma. Germ-line mutation of PTEN/MMAC1 is also associated with two autosomal dominant disorders belonging to the family of hamartomous polyposis syndrome (2, 3, 14). Thus some cancers seem devoid of PTEN/MMAC1 alterations (e.g. serous carcinoma of endometrium and cervical cancer). Genetic changes of PTEN/MMAC1 occur in multiple types of cancer, suggesting that inactivation of PTEN/MMAC1 may play an important but probably somewhat general role in the pathogenesis of a variety of human malignancies (4, 21). It has been suggested that PTEN may be the most frequently mutated gene in prostate cancer and in cancer of the uterine lining (endometrial cancer). PTEN mutations also have been identified in human gastric cancer (7) and several other types of cancer, including certain aggressive brain tumors (glioblastomas and astrocytomas) and an aggressive form of skin cancer called melanoma (13). Mutations in the PTEN gene result in an altered enzyme that has lost its tumor suppressor function. The loss of this enzyme likely permits certain cells to divide uncontrollably, contributing to the growth of cancerous tumors. In some cases, the presence of PTEN mutations is associated with more advanced stages of tumor growth (11).

Conclusions
PCR-SSCP-DNA sequencing indicated that two kinds of mutation sites were found in 4 of 80 breast cancer specimens. One kind of mutation was found in exons. AA-TCC mutation was located 40 bp upstream of 3' lateral exon 2 (115946 AA-TCC) associated with terminator formation in codon 267 of the PTEN gene. The other mutation was found in an intron, including a C-T point mutation 91 bp upstream of 2' lateral exon 2 (1903858 C-T). The PTEN protein expression detected in tissues with breast cancer indicated that the total positive rate of PTEN protein expression was 68 % in breast cancer tissue, which was significantly lower than that (100 %) in paracancerous tissues (P < 0.005). PTEN gene mutation may play an important role in the occurrence and development of breast cancer.

Acknowledgements
The authors thank the members of Archeometry and Biotechnology, Konya, Turkey and the Department of Pathology in the Faculty of Medicine at the University of Cumhuriyet, Turkey, for their helpful comments and discussion. This study was partially supported by Selcuk University Archeometry-Biotechnology Laboratory and Scientific Research Foundation of Selcuk University (BAP).

REFERENCES