# Screening of PTEN Gene Mutations in Gastric Cancer

## Gastrik Kanserde PTEN Geni Mutasyonlarının İncelenmesi

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Geliş Tarihi/*Received:* 26.09.2013
Kabul Tarihi/*Accepted:* 24.01.2014

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**ABSTRACT Objective:** It is not exactly defined how many known gastric cancer causative factors cause cancer in stomach lining cells. PTEN gene is an important tumor suppressor gene that is frequently activated in many cancer cells. In this study, we aimed to investigate the molecular changes of PTEN gene in patients with gastric adenocarcinoma. **Material and Methods:** Forty seven paraffin-embedded tumor tissues from patients diagnosed with gastric adenocarcinoma and adjacent normal tissues were screened for frequency of PTEN mutations using polymerase chain reaction-single strand conformation polymorphism and high resolution melting curve analyses, and DNA sequencing. **Results:** There were no mutations in exons 3,4,5,6 in the PTEN gene. However, in 8 patients, a single-nucleotide polymorphism (rs1903858) was revealed by sequencing in the junction of intron 1-exon 2, and its clinical significance is not known. **Conclusion:** There are no data about the function and frequency of this single-nucleotide polymorphism in gastric cancer patients. Therefore, single-nucleotide polymorphism seen in gastric cancer should be evaluated in a larger population of patients to determine its frequency and function in this group.

**Key Words:** Stomach neoplasms; PTEN phosphohydrolase; polymorphism, single nucleotide; sequence analysis, DNA

**ÖZET Amaç:** Gastrik kansere sebep olan pek çok faktör vardır. Ancak bunların mide mukoza hücrelerinde kansere nasıl yol açtığı tam olarak bilinmemektedir. PTEN geni pek çok kanser hücresinde sıklıkla inaktif halde bulunan bir tümör baskılayıcı gendir. Bu çalışmada gastrik adenokarsinomalı hastalarda PTEN genindeki moleküler değişiklikleri araştırmayı amaçladık. **Gereç ve Yöntemler:** Gastrik adenokarsinoma tanısı almış 47 hastanın parafine gömülü tümör dokuları ve komşu normal dokularında, polimeraz zincir reaksiyonu-tek sarmal konformasyon polimorfizmi, yüksek rezolüsyonlu erime eğrisi ve DNA dizileme yöntemleri kullanılarak, PTEN geni mutasyonlarının frekansı incelendi. **Bulgular:** PTEN geni ekzon 3, 4, 5 ve 6'da hiçbir mutasyon saptanmadı. Ancak, dizileme yöntemi sonucunda 8 hastanın intron1 ekzon 2 kesişim bölgesinde klinik önemi bilinmeyen tek nükleotid polimorfizmine (rs1903858) rastlandı. **Sonuç:** Gastrik kanserli hastalarda bu tek nükleotid polimorfizminin fonksiyonu ve frekansı hakkında hiçbir veri yoktur. Bu nedenle, gastrik kanserde görülen bu tek nükleotid polimorfizminin sıklığını ve fonksiyonunu belirlemek için, daha geniş bir hasta popülasyonunda değerlendirilmelidir.

**Anahtar Kelimeler:** Mide tümörleri; PTEN fosfohidrolaz; polimorfizm, tek nükleotid; dizi analizi, DNA

**Turkiye Klinikleri J Med Sci 2014;34(1):104-10**

Gastric cancer is one of the most frequent malignancies, affecting about one million people per year worldwide. In the recent years, the incidence and mortality rates for gastric cancer decreased in many countries due to improved nutritional habits.[1,2] Studies have shown that the

doi: 10.5336/medsci.2013-37635

development of gastric cancer is a complicated process, involving interactions between polygenes and environmental factors, such as a high intake of salted foods and also possibly smoked, cured and pickled foods, as well as alcohol intake, tobacco use and *Helicobacter pylori* (HP) infection.[3,4] Despite extensive research, the genetic mechanisms which regulate the initiation and progression of gastric cancer are not clearly defined. Recent advances have implicated a defined set of oncogenic pathways in the underlying biology of gastric cancer.[5] Among these crucial signaling networks, the AKT pathway and its negative regulator phosphatase and tensin homolog (PTEN) gene have emerged as particularly important factors in the pathogenesis of gastric adenocarcinoma.[6] PTEN is a tumor-suppressor gene located on human chromosome 10q23.3 that encodes a 403 amino-acid, multifunctional, dual-specificity phosphatase, whose major substrate is the lipid second messenger molecule phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 activates numerous downstream targets and regulates phospholipid phosphatase activity.[7,8] PTEN-induced phospholipid phosphatase activity promotes cell proliferation and motility, and has the potential to influence the cellular process associated with gastric cancer.[9,10] Genetic alterations in the PTEN gene, often due to a complete loss of its locus on chromosome 10q, are observed in a large percentage of sporadic tumors, including endometrial carcinoma, glioblastoma, melanoma, and prostate and breast cancer.[11-15] Given the central role of PTEN in the pathogenesis of diverse cancers, the identification of alternative mechanisms of PTEN regulation would be of significant interest. In the present study, we aimed to investigate the frequency of PTEN gene alterations in gastric cancer and the corresponding normal tissues using polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP), high resolution melting curve (HRM) analyses and DNA sequencing techniques.

## MATERIAL AND METHODS

Forty seven gastric adenocarcinoma and adjacent paracancerous normal tissue samples were obtained from the Department of Pathology. Of 47 patients included, 29 were males and 18 were females, with an average age of 64.45±1.9 years. The study protocol was approved by the Ethics Committee of the Meram School of Medicine, Education and Research Hospital. All patients signed an Informed Consent Form before enrollment. All samples were formalin-fixed, paraffin-embedded, and classified according to the Lauren's and World Health Organization's histological classification criteria.

### DNA EXTRACTION

Formalin-fixed paraffin samples were cut into 10-μm sections. Each sample was resuspended in 1 ml of xylene, and left at 50°C for 1 hour. The suspension was then centrifuged at 13 000 rpm for 15 minutes. The pellet was suspended in 0.1 mL of xylene (Sigma, cas no. 1330-20-7) and processed as above, for the second time. The resulted sediment was mixed with 100% ethanol (Sigma, E7023) and left at 37°C for 30 minutes. The suspension was then centrifuged at 13 000 rpm for 10 minutes. This process was repeated keeping in 80%, 60% and 40% ethanol for 15 minutes for each sample. The pellet was suspended in 0.1 mL $ddH_2O$ and left for at 37°C 15 minutes. The suspension was then centrifuged at 13 000 rpm for 10 minutes and the above parts were discarded. The pellet part was minced in smaller pieces by using surgical scissors. A commercial DNA isolation kit (Roche High Pure PCR Template Preparation Kit, cat no.11 796 828 001) was used according to manufacturer's protocol. To the pellet, 40 μL Proteinase-K (10mg/mL,Roche, cat no.11 796 828 001) and 200 μl tissue lysis buffer were added, and it was incubated at 37°C for overnight. In the next day, spin colon procedure was applied for DNA extraction.

### PCR-SSCP ANALYSIS

Tumor and normal DNAs were subjected to PCR-SSCP analysis to search for PTEN gene mutations. Five exons of the PTEN gene were amplified using primers (Table 1) with some modifications.[16,17] PCR amplification was performed in 15 μL reaction volume including 1xMg++ free PCR buffer, 0.125 mM

**TABLE 1:** Oligonucleotide primers for PTEN analysis.

| No | Exon | Primer | Product size (bp) |
|---|---|---|---|
| 1 | 2 | 5'-TGA CCA CCT TTT ATT ACT CC-3' | 367 |
| | | 3'-TAC GGT AAG CCA AAA AAT GA-5' | |
| 2 | 3 | 5'-ATA TTC TCT GAA AAG CTC TGG-3' | 434 |
| | | 3'-TTA ATC GGT TTA GGA ATA CAA-5' | |
| 3 | 4 | 5'-TTC AGG CAA TGT TTG TTA-3' | 225 |
| | | 3'-CTC GAT AAT CTG GAT GAC TCA-5' | |
| 4 | 5 | 5'-GCA ACA TTT CTA AAG TTA CCT A-3' | 386 |
| | | 3'-TCT GTT TTC CAA TAA ATT CTC-5' | |
| 5 | 6 | 5'-TTG GCT TCT CTT TTT TTT CTG-3' | 201 |
| | | 3'-ACA TGG AAG GAT GAG AAT TTC-5' | |

dNTPs, 1.5 mM MgCl++, 0.375 units of Taq polymerase (Fermentas, K0171), 2-15 pmol for each primer and 50 ng of genomic DNA. Amplification was carried out in a Gene-Amp PCR System 2700 thermocycler (PE Applied Biosystems) by using following profile: 4 min. at 95°C, 15 sec. at 94°C, 30 sec. at 48°C or 60°C for 35 cycles, then 15 sec. at 72°C, which was followed by a final 3 min. extension at 72°C.

PCR products were diluted 1:10 in denaturing loading buffer (95% formamide, 0.5 M EDTA pH 8.0, 0.02% xylene cyanol, and 0.02% bromophenol blue), heated at 95°C for 5 min, placed on ice, and 10 µl aliquots of this denaturated mixture was subjected to electrophoresis. Single strand conformation polymorphism (SSCP) analysis was done in polyacrilamide gel containing 8% polyacrylamide and 5% glycerol. Any shift in the pattern of single-strand migration in the gel electrophoresis was considered PCR-SSCP positive.

### HRM ANALYSIS

In order to confirm SSCP analysis, a HRM analysis was accomplished in 8 tumor tissues and adjacent normal tissues. PCR amplification and HRM were performed on the LC 480 II (Roche Applied Science). The same primer sequences were used but different PCR conditions were done as in 20 µL reaction volume including HRM master mix (Roche, cat no.04909631001), 50 mM $MgCl^{++}$, 10 pMol each primer, 50 ng of genomic DNA and 3 µl double distilled water. Each experiment was performed in triplicate. The cycling conditions were as follows: SYBR Green 1/HRM dye detection format; 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 10 seconds, 60°C for 10 seconds, and 72°C for 20 seconds; followed by an HRM step of 95°C for 1 minute, 40°C for 1 minute, 65°C for 1 seconds, and continuous acquisition to 90°C at 25 acquisitions per 1°C. Plate included water blanks were used as a negative control. The melting curves were normalized by calculation of two normalization regions before and after the major fluorescence decrease representing the melting of the PCR product. This algorithm allows the direct comparison of the samples that have different starting fluorescence levels. Output plots are in the form of normalized temperature-shifted melting curves that show the decrease in fluorescence against increasing temperature.

### DNA SEQUENCE ANALYSIS

Genome DNAs which repeatedly showed a bands-hift by SSCP analysis were amplified using the same primers as those used for PCR-SSCP analysis for DNA sequencing analysis.

These PCR products were purified and sequenced by using the ABI 3730 48 capillary (Big Dye Cycler sequencing Version 3.1). Sequencing data were collected and analyzed by using the FinchTV 1.4.0 (Geospiza, INC).

### STATISTICAL ANALYSIS

Descriptive statistics were obtained for patient characterization and TNM classification. DNA sequencing analysis results were summarized. HRM data were analyzed using the Gene Scanning Software (Roche Applied Science).

## RESULTS

Forty seven samples of gastric adenocarcinoma and adjacent paracancerous normal tissue samples were evaluated in this study. PTEN gene mutations were seen in 8 gastric adenocarcinoma samples and the paired adjacent normal tissues by PCR-SSCP. The PCR-SSCP assays demonstrated that 8/47 (17%) patients displayed aberrant migration band

shifts for exon 2 of PTEN in both the tumor and normal tissues, indicating the presence of genetic alterations (Figure 1). No abnormal SSCP shifts were observed in exons 3, 4, 5 or 6 of PTEN.

In order to confirm these results, HRM analysis was performed (Figure 2) and further characterized by bidirectional sequencing technique of DNA from the tumor and adjacent normal tissues (Figure 3). Analysis of the sequencing data revealed the presence of a C>T mutation at the intron1-exon2 junction site in both the tumor and normal tissues in 8/47 (17%) patients (Figures 3A, 3B). This mutation was previously described as the rs#1903858 SNP (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=1903858).

The clinical and prognostic data and the TNM stages of the patients were grouped according to presence or absence of the polymorphism (Table 2, Figure 4). The tumors were pathologically classified by the depth of tumor invasion: 3 cases were T1, 3 cases were T2 and 33 cases were T3 in polymorphism negative group. In addition, one case was T2 and 7 cases were T3 in the polymorphism group. According to the pathological analysis of lymph node metastasis, 3 cases were classified as N0, 23 cases as N1, and 13 cases as N2 in polymorphism negative group. Two cases were classified as N2, and 6 cases as N3 in the polymorphism group. Eight cases had liver metastasis in polymorphism group and 39 cases had no metastasis in polymorphism negative group. With respect to tumor location, 8 cases had tumors located in the gastric corpus, 5 cases in the greater curvature, 3 cases in the pylorus, 2 cases in the pyloric antrum and 1 case in the lesser curvature in the polymorphism group. On the other hand, 10 cases had tumors located in gastric corpus, 7 cases in greater curvature, 3 cases in pylorus, 4 cases in pyloric antrum and 4 cases in lesser curvature in polymorphism negative group (Table 2).

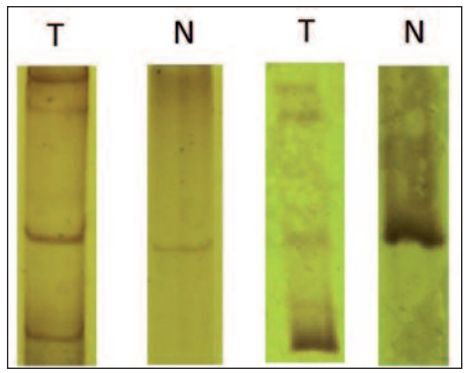


**FIGURE 1:** Polymerase chain reaction- single strand conformation polymorphism analysis of exon 2 of the PTEN gene.
T: Gastric cancer tissue samples; N: Paired normal tissue samples.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

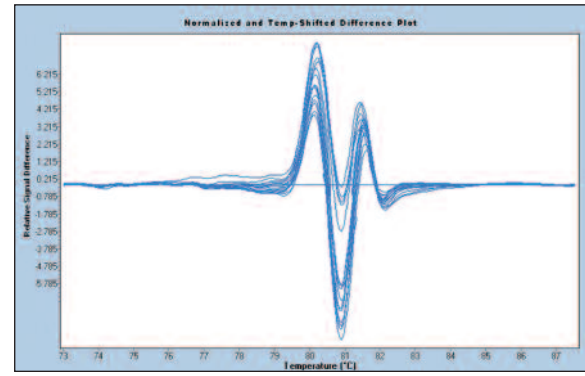


**FIGURE 2A:** Normalized and temperature-shifted difference plot for the mutation screening of polymorphism negative group.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

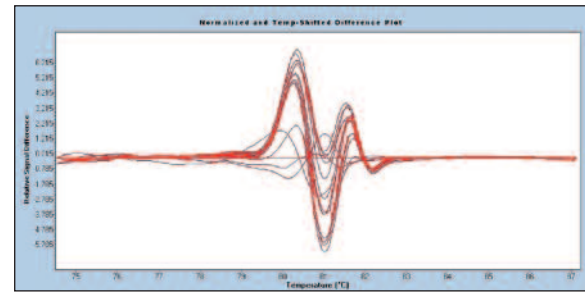

**FIGURE 2B:** Normalized and temperature-shifted difference plot for the mutation screening of polymorphism positive group.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

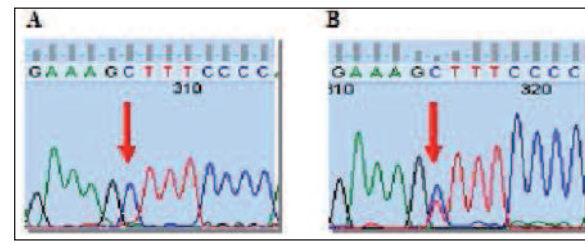


**FIGURE 3:** Sequencing results **A.** Wild-type allele (CC) of the PTEN gene in control DNA sample **B.** Heterozygote allele C>T of the PTEN gene in the patient sample.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

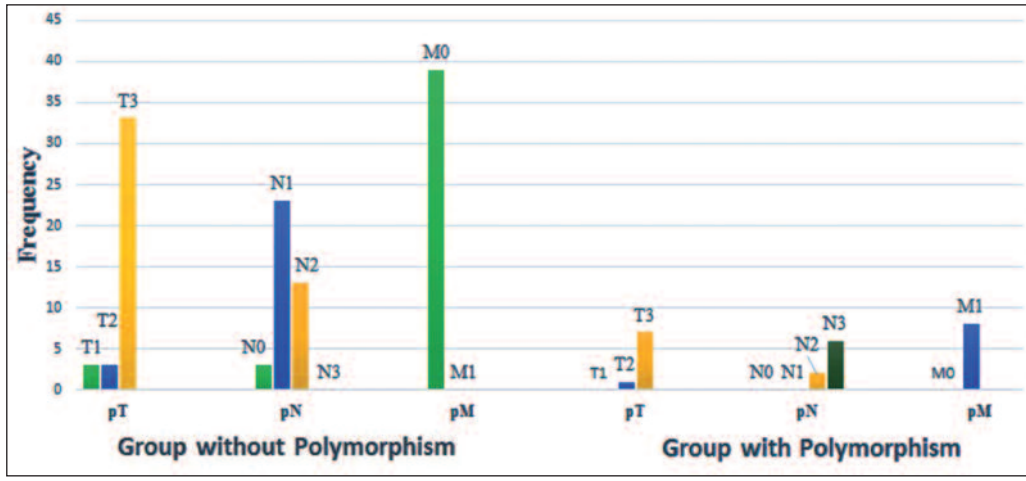


**FIGURE 4:** Distribution of tumor tissues according to the TNM classification in polymorphism positive and negative groups.
(See color figure at http://www.turkiyeklinikleri.com/journal/tip-bilimleri-dergisi/1300-0292/)

## DISCUSSION

Gastric cancer is one of the most frequent cancers resulting in cancer-related deaths. Despite reduced incidence and mortality rates, it still has an essential place in cancer-related deaths in some regions.[18,19] In our region, gastric cancer is the 6th most common cancer in both sexes.[20] The majority of gastric cancer cases are aged between 50 and 70 years. Males develop gastric cancer approximately twice more frequently compared to females.[21,22] Most of gastric cancers (90-95%) are composed of adenocarcinomas, and tumors are usually localized in pyloric part.[23,24] Combination of environmental and genetic factors play role in occurrence of gastric cancer and many other cancer types. In recent years, molecular genetic studies have shown that changes in the cell can be one of the effective factors in the mechanism of gastric carcinogens.[25-27] Molecular genetic alterations in gastric cancer cells, which are defined as activation of oncogenes and inactivation of tumor suppression genes, are known as main changes like PTEN gene.[28,29] PTEN gene is one of the landscaper gene playing roles in cell signal cascade which has some assignments in PI3K/AKT pathway. Structure alteration of this gene in tumor tissues and tumor cell lines, including deletion, insertion, point mutation, etc. indicates that the mutation rate of PTEN gene is higher than 50% in glioblastomas and endometrial cancers and less than 5% in soft tissue sarcomas.[30-32]

**TABLE 2:** Clinical and prognostic properties related to polymorphism positive and negative groups.

| | Polymorphism (+) group | Polymorphism (-) group |
|---|---|---|
| Sex (male/female) | 6/23 | 23/8 |
| Age average | 63,32±1,2 | 65,45±1,4 |
| pT | | |
| T1 | - | 3 |
| T2 | 1 | 3 |
| T3 | 7 | 33 |
| pN | | |
| N0 | - | 3 |
| N1 | - | 23 |
| N2 | 2 | 13 |
| N3 | 6 | - |
| pM | | |
| M0 | - | 39 |
| M1 | 8 | - |
| Gastric corpus | 8 | 10 |
| Greater curvature | 5 | 7 |
| Pylorus | 3 | 3 |
| Pyloric antrum | 2 | 4 |
| Lesser curvature | 1 | 4 |

In order to determine the role of mutations in gastric cancer, we screened the frequency of PTEN mutations by PCR-SSCP and HRM analyses, and

DNA sequencing. Our results showed that both PCR-SSCP and HRM analyses were capable of detecting variations. By using these techniques, we showed that 8 cases (8 tumors and 8 adjacent normal tissues) out of 47 samples had a C>T alteration in intron1-exon2 junction, indicating germline single-nucleotide polymorphisms (SNP). In the literature, there are no data about this SNP's function or frequency in gastric cancer patients. In addition, histopathological examination of our study showed that polymorphism positive group had advanced stage gastric cancers. Germline polymorphisms in these genes which are important in the pathogenesis of gastric cancer have been associated with an increased incidence of disease. In our study, we found that a SNP located in an intron-exon boundary of PTEN, and it may be a functional variant.

There are many conflicts about this SNP (rs1903858) in the literature. Haiman et al. investigated the relatio of rs1903858 with breast and prostate cancer risks in their multiethnic population study, and they could not find any relations.[33] Similarly, Treolar et al. reported that there was no association between SNP in PTEN and malignant transformation of endometriosis formation.[34] In the same way, Song et al. investigated the genetic association between rs1903858 and laryngocarcinoma in Chinese Han population, but they could not find any association of the SNP with laryngocarcinoma in this population.[35] In addition, Xie et al. studied germ-line genetic changes of PTEN on susceptibility to prostate cancer.[36] Nevertheless, they suggested that germ-line variants in PTEN did not have any important role in prostate cancer susceptibility. In contrast, Hosgood et al. reported that there was a correlation between frequency of rs1903858 and the occurrence of chronic obstructive pulmonary disease (COPD) risk.[37] They suggested that homozygote variant carriers of rs1903858 were associated with an approximate nine-fold decreased COPD risk.

These results indicate that rs1903858 may have different functions in different diseases. Therefore, rs1903858, what we found in gastric cancer, should be evaluated in a larger patient population in order to determine its frequency and function in this patient group. This may be a topic for researchers using more advanced genotyping technologies and a larger patient population.

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