Association of promoter methylation and 32-bp deletion of the *PTEN* gene with susceptibility to metabolic syndrome

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Abstract. Metabolic syndrome (MeS), a cluster of several metabolic disorders, is increasingly being recognized as a risk factor for type II diabetes (T2D) and cardiovascular disease. Genetic and epigenetic alteration of the phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) has been associated with components of MeS. The aim of the present study was to investigate the possible association of a 32-bp deletion polymorphism and promoter methylation of the *PTEN* gene with MeS. DNA was extracted from the peripheral blood of 151 subjects with and 149 subjects without MeS. The 32-bp deletion variant of *PTEN* was detected by polymerase chain reaction (PCR) and *PTEN* promoter methylation was defined by a nested methylation-specific PCR (MSP) method. No significant differences were found in the allelic and genotypic frequencies of the 32-bp deletion variant of *PTEN* between the groups [odds ratio (OR), 0.77; 95% confidence interval (CI), 0.41-1.45; P=0.431]. However, patients with MeS were identified to have lower levels of *PTEN* promoter hypermethylation than subjects without MeS. Promoter methylation may be a protective factor against susceptibility to MeS (OR, 0.52; 95% CI, 0.29-0.92; P=0.029). Our findings suggest that *PTEN* promoter methylation may be a mechanism for *PTEN* downregulation or silencing in MeS, which remains to be fully clarified.

Introduction

Metabolic syndrome (MeS) is described as a combination of clinical disorders that increases the risk of obesity (central adiposity), insulin resistance, glucose intolerance, dyslipidemia, non-alcoholic fatty liver disease and cardiovascular diseases, including atherosclerosis, stroke and hypertension (1,2). During the past decades, the prevalence of MeS has markedly increased worldwide, and is becoming a significant health problem (3). The etiology of this syndrome is complex and is considered to be the result of interaction between genetic and environmental factors.

Phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) was the first phosphatase to be characterized as a tumor suppressor (4). *PTEN* is mapped on chromosome 10q23.3 and encodes a dual lipid/protein phosphatase that acts as a negative regulator of the phosphoinositol-3-kinase (PI3K)/AKT pathway (5,6). A number of studies have indicated that *PTEN* deficiency causes an accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3), a lipid product of PI3K, which in turn allows constitutive activation of the PI3K/AKT signaling pathway (7,8). In muscle and adipocytes, PI3K/Akt pathway activation is critical for insulin-induced metabolic actions, including GLUT4 translocation, glucose uptake and glycogen synthesis (9), whereas in the liver, the PI3K/Akt pathway mediates suppression of glycogenolysis and glucose release (10). Furthermore, it has been demonstrated that PI3K/*PTEN* signaling pathways are significant in a variety of abnormalities associated with MeS, including diabetes (11), obesity (12,13), hypertension and cardiovascular diseases (13,14). Also, it has been shown that the depletion of *PTEN* in the liver and adipocytes by the systemic administration of *PTEN* antisense oligonucleotide improved blood glucose concentrations and insulin sensitivity in diabetic mice and protected them from developing diabetes (15). However, through its protein tyrosine phosphatase activity, *PTEN* is able to inhibit integrin/FAK and Ras/MAPK/Erk1/2 signaling pathways under certain circumstances, hence negatively regulating cell cycling, proliferation, focal adhesion and cell migration (16). *PTEN* deficiency has been identified in a variety of human cancers, including glioblastoma, melanoma and cancers of the prostate, breast, lung and bladder as a result of germline or somatic mutations (17). *PTEN* harbors a 32-bp deletion variant located on intron 2, but its role in disease remains unclear. Promoter hypermethylation of *PTEN* is considered to be an alternative to mutations and deletions as a mechanism of gene inactivation (18). Therefore, in the current study, we aimed to evaluate the association of *PTEN* 32-bp

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deletion polymorphism and *PTEN* promoter methylation with MeS in a sample of an Iranian population.

Materials and methods

Patients. This case control study involved 151 patients with and 149 without MeS. MeS was defined using the National Cholesterol Education Program Adult Treatment Panel III (NCEP ATP III) criteria (19) as previously described (20,21). Ethical approvals for recruitment were obtained from the local ethics committee of the Zahedan University of Medical Sciences and informed consent was obtained from all individuals. The data included weight, height, waist circumference, systolic and diastolic blood pressure; blood levels of glucose, triglycerides, total cholesterol, HDL cholesterol and LDL cholesterol were collected as previously described (20-22). Blood samples were collected in EDTA-containing tubes and genomic DNA was extracted using the salting-out method as previously described (23).

The *PTEN* 32-bp deletion was detected using a pair of forward and reverse primers (5'-CCAGCCCTCACTAAAA ACAAA-3' and 5'-CAAGTGTCCAAGCAGCAAA-3', respectively). Polymerase chain reaction (PCR) was performed using a commercially available PCR premix (AccuPower PCR PreMix; Bioneer Corp., Daejeon, Korea) according to the manufacturer's instructions. Briefly, 1 μl template DNA (~100 ng/ml), 1 μl each primer (10 pmol/ml), and 17 μl DNase-free water were added to AccuPower PCR PreMix. Amplification was performed with an initial denaturation step at 95˚C for 5 min, followed by 30 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 23 sec with a final extension at 72˚C for 10 min. Each reaction was verified on a 2% agarose gel. The PCR products for insertion and deletion alleles comprised 241 and 209 bp, respectively (Fig. 1). Random samples were regenotyped to verify the accuracy of the genotyping. We identified no genotyping mistakes.

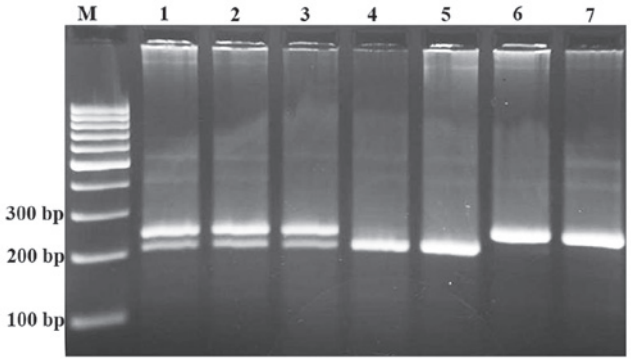

Figure 1. Representative polymerase chain reaction (PCR) products resolved by agarose gel electrophoresis to detect the presence or absence of the 32-bp deletion of the phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) gene. M, DNA marker. Lanes 1-3, ins/del; lanes 4 and 5: del/del; lanes 6 and 7: ins/ins.

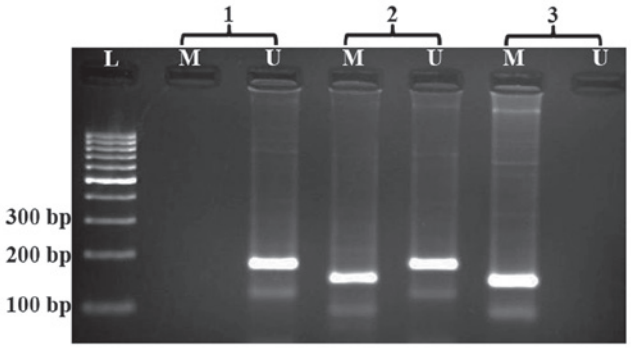

Figure 2. Electrophoresis pattern of promoter methylation of phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) using methylation-specific PCR (MSP). L, DNA marker; M, methylated; U, unmethylated. Methylated primers amplify a 155-bp product while unmethylated primers amplify 173-bp products. Sample 1, UU; sample 2, MU; sample 3, MM.

PTEN promoter methylation. The samples of DNA were treated with sodium bisulfite, which converts unmethylated C to U while methylated C residues resist the treatment. The method of Tiwari *et al* (24) was used for the bisulfite treatment of DNA with certain modifications. Briefly, NaOH solution was added to 10 μl DNA (~1 μg) to a final concentration of 0.3 M. The mix was incubated at 50˚C for 15 min to achieve effective denaturation of the DNA strands. This mixture was then mixed with 50 μl 2% low melting point (LMP) agarose and incubated at 50˚C for 15 min. A 15-μl drop of this mixture was pipetted into 300 μl cold mineral oil (Sigma, St. Louis, MO, USA). The agarose/DNA drop solidified in the oil and agarose beads formed during incubation at -4˚C for 30 min. Aliquots comprising 700 μl 5 M bisulfite reagent (5 M sodium bisulfite and 125 mM hydroquinone; both Merck KGaA, Darmstadt, Germany; pH 5.0) were added to each reaction tube containing a single bead. The tube was gently inverted to move the bead into the aqueous phase and was incubated at 55˚C in a water bath for 16 h under exclusion of light. Treatments were stopped by equilibration against 1 ml 1X TE (2x15 min) followed by desulfonation in 500 ml 0.2 M NaOH (2x10 min). Finally, the beads were washed with 1 ml 1X TE followed by equilibration against 1 ml ddH_2O (1x15 min). The beads were used directly for PCRs. We have also kept beads at -20˚C for several weeks and never detected any loss of quality.

We developed a nested methylation-specific PCR (MSP) method for the detection of promoter methylation of *PTEN* with increased MSP sensitivity. First-stage PCR primers in the nested MSP recognize a bisulfite-treated template but do not discriminate between methylated and unmethylated alleles. In the second stage, two pairs of primers are used; one pair is specific for an unmethylated template and the other is specific for a methylated template. The forward and reverse primers for the first stage were 5'-TTTAGGGAGGGGGTTTGA-3' and 5'-CCATCCTCTTAATATCTCCT-3', respectively, producing a 529-bp amplicon that was used as a template for the second PCR stage once diluted 1:5. The second stage was performed as previously described (25). Primer sequences used to amplify an unmethylated product were 5'-TGGGTTTTGGAGGTT GTTGGT-3' (sense) and 5'-ACTTAACTCTAAACCACAA CCA-3' (antisense), which amplify a 173-bp product, and primer sequences for the methylated reaction were 5'-GGTTTCGGAGGTCGTCGGC-3' (sense) and 5'-CAACCG AATAATAACTACTACGACG-3' (antisense), generating a 155-bp product.

Table I. Association of the 32-bp insertion/deletion polymorphism of the *PTEN* gene in individuals with and without MeS.

Polymorphism	MeS (yes)	MeS (no)	OR (95% CI)	P-value
PTEN (32-bp ins/del)				
Ins/ins	44 (29.1)	37 (24.8)	Ref.	-
Ins/del	70 (46.4)	72 (48.3)	0.81 (0.47-1.41)	0.471
Del/del	37 (24.5)	40 (26.8)	0.77 (0.41-1.45)	0.431
Ins/del+del/del	107 (79)	112 (75.1)	0.80 (0.48-1.34)	0.436
Alleles				
Ins	158 (52)	142 (48)	Ref.	-
Del	144 (48)	152 (52)	0.84 (0.61-1.16)	0.288

PTEN, phosphatase and tensin homolog deleted on chromosome ten; MeS (yes), patients with metabolic syndrome; MeS (no), patients without metabolic syndrome; OR, odds ratio; CI, confidence interval.

Table II. Frequency distribution of the *PTEN* promoter methylation in individuals with and without MeS.

PTEN promoter methylation	MeS (yes)	MeS (no)	OR (95% CI)	P-value
UU	129 (85.4)	112 (75.2)	Ref.	-
MU	16 (10.6)	30 (20.1)	0.46 (0.24-0.89)	0.024
MM	6 (4.0)	7 (4.7)	0.74 (0.24-2.28)	0.744
MM+MU	22 (14.6)	37 (24.8)	0.52 (0.29-0.92)	0.029

PTEN, phosphatase and tensin homolog deleted on chromosome ten; MeS (yes), patients with metabolic syndrome; MeS (no), patients without metabolic syndrome; OR, odds ratio; CI, confidence interval; U, unmethylated; M, methylated.

The cycling conditions were as follows: 95˚C for 10 min, and 35 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 10 min with a final extension for 10 min. The PCR products were verified on 2% agarose gel containing ethidium bromide and observed under UV light (Fig. 2).

Statistical analysis. The differences between the variables were assessed by the χ^2 test or t-tests according to the data. The association between genotypes and MeS was assessed by computing the odds ratio (OR) and 95% confidence interval (95% CI) from logistic regression analyses. $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 18 software.

Results

A total of 151 cases with MeS (50 male, 101 female; age, 41.98±14.65 years) and 149 controls (45 male, 104 female; age, 43.53±15.96 years) were enrolled in the study. There were no significant differences between the groups with regard to gender and age ($P>0.05$). As shown in Table I, no statistically significant difference was identified between the groups concerning 32-bp insertion/deletion polymorphism of the *PTEN* gene ($\chi^2=0.74$, $P=0.69$). Our finding demonstrated that the 32-bp deletion polymorphism of *PTEN* is not a risk factor for MeS (Table I).

The promoter methylation status of the *PTEN* gene in the case and control groups is shown in Table II. The findings indicate that promoter methylation is a risk factor for MeS (OR=2.51, 95% CI =1.38-4.58, $P=0.002$) in our population.

Discussion

In the present study, we analyzed the impact of genetic and epigenetic alterations of *PTEN* on the risk of developing MeS in a sample of an Iranian population. The *PTEN* 32-bp deletion demonstrated no effects on the predisposition to MeS since the frequency distribution of the del allele was similar in the MeS cases and the controls. However, *PTEN* promoter methylation was more prevalent in the individuals without MeS than the subjects with the syndrome. To the best of our knowledge, we report for the first time the correlation between promoter methylation of *PTEN* and decreased risk for MeS. Our findings suggest that hypermethylation of CpG sites in the promoter region of the *PTEN* gene is a possible mechanism accounting for downregulated or silenced *PTEN* expression.

MeS comprises a combination of risk factors for cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM). These factors include hyperglycemia, raised blood pressure, dyslipidemia primarily characterized by increased levels of triglycerides and low HDL-cholesterol and obesity (particularly with abdominal localization) (20). MeS is associated with a 2-fold increased risk for cardiovascular disease, 5-fold increased risk for T2DM and 1.5-fold increase

in all-cause mortality (26). The prevalence of MeS varies worldwide and depends, in part, on lifestyle, gender, age and ethnicity (20,27).

The expression and activity of the tumor suppressor *PTEN* are regulated by numerous and complex mechanisms, specifically in pathological conditions. These mechanisms include genetic alterations or epigenetic silencing of the gene by promoter methylation, which affect *PTEN* stability, localization, activity and interactions with other cellular partners (28). *PTEN* is mutated or deleted in 30% of all human cancers, and is thus, after p53, one of the most common tumor suppressors, the expression or activity of which is altered during carcinogenesis (29). A few studies have evaluated the 39-bp deletion variant located on intron 7, close to the inton 7-exon 8 splice site deletion of the *PTEN* gene. Ding *et al* (30) identified no association between the frequencies of this del variant in patients with HCC. By contrast, Zhou *et al* (31) suggested that this intronic deletion variant is not a mutation, but more likely a normal polymorphic variation, which has no functional effect on the expression of *PTEN*. In the present study we evaluated the 32-bp deletion of *PTEN* in intron 2 and identified no association with MeS.

In addition to germline mutations or deletion, the epigenetic silencing of *PTEN* through its promoter hypermethylation has been suggested as a potential mechanism contributing to *PTEN* downregulation. *PTEN* promoter methylation has been reported to be positively associated with increased risk for several types of cancer, including breast cancer (32,33), cervical carcinoma (34) and thyroid tumors (35). However, no study has yet investigated this polymorphism in non-cancerous disease.

The liver-specific deletion of *PTEN* has been reported to correlate with insulin hypersensitivity, decreased serum leptin and body fat content and progression of fatty liver via insulin-induced fatty acid synthesis (36). Moreover, a number of studies indicate that *PTEN* is a major dysregulated cellular factor contributing to the development of a wide spectrum of disorders related to MeS, including T2DM, steatosis, steatohepatitis, fibrosis, cirrhosis, cardiac hypertrophy, heart failure, preconditioning and hypertension (1,28,29,37).

In MeS, in addition to an excess of circulating free fatty acids, the release of inflammatory cytokines or various adipokines, including leptin, resistin and adiponectin, have been reported to either up- or downregulate *PTEN* expression or activity in non-hepatic cells, thus leading to pathological conditions related to MeS (28).

PTEN is a tumor suppressor that regulates multiple cellular functions, including cell growth and survival, differentiation and proliferation, apoptosis, focal adhesion, invasion, migration and angiogenesis (4,29). As a dual-specificity enzyme, *PTEN* is capable of dephosphorylating proteins and lipids. Through its phosphatase activity, *PTEN* downregulates the PI3K/Akt signaling pathway, while the protein phosphatase function of *PTEN* suppresses the integrin/FAK and Ras/MAPK/Erk1/2 pathways which are involved in the progression of cell cycle, migration and invasion (5,8).

In conclusion, our findings demonstrate for the first time that the 32-bp deletion polymorphism of *PTEN* is non-functional in predisposing individuals to MeS, but *PTEN* promoter hypermethylation protects against susceptibility to MeS. Larger studies with individuals of different ethnicity are required to validate our findings.

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