STUDY OF SINGLE NUCLEOTIDE POLYMORPHISM (SNP) AND GENE EXPRESSION OF PTEN IN IRANIAN GASTRIC CANCER PATIENTS

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Abstract:
Gastric cancer is recognized as the fourth most common cancer in the world, and is the second leading cause of cancer death. PTEN is a tumor inhibitor gene. PTEN mutation is observed in most of the cancerous malignancies. In this study, the expression and common PTEN gene polymorphism in blood samples of patients with gastric cancer were studied using REAL TIME PCR and RFLP PCR techniques in Iranian population. 45 blood samples (36 patients and 9 controls) were studied in this study. After extracting the RNA and confirming its quality, cDNA synthesis was performed and the expression of the gene was evaluated by qPCR. In order to investigate the single-nucleotide change in rs10490920, the DNA was extracted from the specimens and then PCR was performed. After obtaining ct, the samples RQ was analyzed and analysis of samples RQ showed that the PTEN Gene expression was reduced in patients compared with controls. In patients with rs10490920 polymorphisms in the PTEN gene, the risk of the disease increased. The results of the study indicate a reduction in gene expression in cancer patients. Thus, it may be possible to use Pten gene expretion assesment as a possible biomarker in early diagnosis or in the treatment process.
Keywords: *Gastric cancer, PTEN, gene expression, polymorphism, Real time PCR*

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QR code

INTRODUCTION:

In Iran, stomach cancer Has the highest rate of cancers prevalence in men. In Iran, the malignancy is also known as the second cause of cancer deaths in men and women. Although the incidence of this cancer is lower in other areas of the world, the reasons for this difference are unclear. It is possible that the high rate of lethality in this cancer in Iran is related to its diagnosis in advanced stages of the disease, and this disease is more invasive in the Iranian population [1]. In recent decades, it has declined for making changes in lifestyle in developed It is possible that the high rate of lethality of this cancer in Iran is related to diagnosis in advanced stages and /or more aggressive behavior of tumor cells(1).recently the incidence of stomach cancer in developed countries is reduced due to changes in lifestyle such as the forbiddance of smoking and eradicating *Helicobacter pylori* [2]. Researches has shown that two types of genomic changes play a role in cancer development. 1. Changes causing the tumor suppressing genes to fail. 2. Changes that increase the gene or Changes that cause tumor suppression genes failure.(2) changes that lead to increasing the genetic products of oncogenic genes. *PTEN* is a tumor inhibitor gene. Most studies are evaluated *PTEN* in their negative regulation function in the PI3K/AKT messaging pathway, which are involved in cell viability, proliferation, and inhibition of apoptosis [3]. A balanced limit for *PTEN* expression is vital for the cell. Reports indicate that the expression and the PTEN enzymatic activity can be regulated by microRNAs during transcription or afterwards [4]. *PTEN* mutation is seen in most of the cancerous malignancies. This mutation has been seen alternately in the last stage of glioblastoma, melanoma, prostate cancer, the first stage of thyroid cancer, and endometrial Endometrial and cervical cancer. This mutation has not been identified in other types of tumors [5]. The inactivation of *PTEN* can lead to the diversification of tumor cells and tissue. Reports indicate that inactivation of *PTEN* is associated with the spread of disease and the progression of gastric cancer [6]. *PTEN* expression in metastatic lymph nodes and advanced gastric cancer was significantly less than the non-metastatic lymph nodes and primary stomach cancer. The *PTEN* expression level could be used as a pathologic diagnostic guide for gastric cancer [7]. Therefore, the purpose of this study was to detect *rs10490920* polymorphism in *PTEN* gene and to investigate the association of this polymorphism with gastric cancer, to detect *PTEN* in serum of gastric cancer patients, to investigate the relationship between the amount of *PTEN* expression in normal people and patients and the evaluation of using the *PTEN* expression level as a marker for diagnosis in order to screen in the early stages, which can be used In diagnosis and treatment if its efficacy and optimization is proved therapeutic and therapeutic diagnosis if it be efficacy.

MATERIAL AND METHODS:

Sample collection: For this study, 45 blood samples consist of 36 patients and 9 normal people were used. 5 ml of peripheral blood were taken from all people. Blood samples were collected in falcons containing EDTA at a final concentration of 1 mg/ml. Samples were kept at -20 c° until full extraction. Sampling was conducted under supervision of the Ethics Committee of Shahid Beheshti University of Medical Sciences under the WHO (World Health Organization) criterion. The statistical analysis of the information extracted from the questionnaire was done by GraphPad software (GraphPad prism5).

RNA extraction: For this purpose, an RNX PLUS solution was used according to the optimized protocol [8]. In order to investigate the quantity of RNA extracted from spectrophotometric apparatus and optical absorption in 280/260 nm was used.

Synthesis of cDNA: At first, 10 microliters of RNA template, together with 1-microliter dntp 10Mm and 1 microliter of Random Hexamer, followed by 1 microliter of oligo dt was mixed. The tube was placed at 65 c° for 5 minutes. Then, 2 μl of MMulv 10 X buffer and 5.0 μM of MMulv enzyme were added to the mixture, finally added the water and the final volume reached 20 μl. Finally, the final tube was incubated at 42 c° for 1 hour.

Design of specific primers for the GAPDH and PTEN genes: The *GAPDH* gene sequence used as internal control, as well as the *PTEN* gene gained from the NCBI gene database and specific primers was designed by primer express program. In order to confirm the specificity and precision of primers designed, their sequence was blasted into NCBI. The sequence of the listed primers include the target gene primers with the sequence of the ACAAGATATACAATCTTTGTGC *PTEN* Real time F /*PTEN* Real time R TCACAAAAGGGTTTGATAAG and the internal control sequences with sequences ATGGAGAAGGCTGGGGCT *GAPDH* (F) and *GAPDH* (R) ATCTTGAGGCTGTTGTCATACTTCTC were studied in this study.

Table 1: patient profile

Sex		Over 50 years old	Under 50 years old	Healthy	Patient	Total
Female	**Male**	57.77 %	42.22 %	20 %	80 %	100 %
33.33%	66.66%					

Optimizing the essential factors of the real time - PCR for the GAP DH and PTEN gene: For this purpose, separate reactions for the desired gene and internal control gene were made in the final volume of 20 µL. The reactions are laid out on the AB17500 in parallel. In each reaction, 10 µM SYBER TM (2X) and 10 µM Reverse and forward primer and c DNA template were used at a concentration of 2 µg. The thermal reaction is 1 cycle at 95 c° for 10 minutes and 40 cycles with 2 times 15 seconds and 56 seconds at 95 c° and 54.5 c° and 1 cycle for 3 times 15 seconds, 1 minute and 15 seconds at 95, 54 and 95 c° temperatures, amplification curve analysis was used to confirm the replicated fragment and to ensure the absence of non-specific product, dimer primer and contamination. After optimization, the RNA of all samples was extracted and after confirmation of the quality of the obtained RNAs and cDNA synthesis was performed on the samples. Ct of the samples were calculated by the setting after the proliferation reaction and converted to RQ or expression level, and then the measurement of the gene expression was performed using the ΔΔct method. The expression level of patient samples was expressed in comparison with normal samples. After the reaction, the raw data was extracted from ct by the setting and the expression rate was measured using the ΔΔct method. Then, the gene expression chart was drawn up using the Graph pad software.

DNA extraction: The DNA of the samples were optimized by the protocol and was obtained DNG solution. After extraction, the quality of DNA was evaluated by spectrophotometer.

PCR: For proliferation of the fragment containing rs10490920 were used PTEN RFLP F TCAAGAAGTCCAAGAGCATT / PTEN RFLP R AGACAAGACAAGCCACCTAA primers [23]. First, 12.5 µL Master Mix was mixed with 1 µL Reverse Primer and 1 µL Forward Primer and 5.5 µL DDW and 5 µL of the template DNA. The thermal reaction was consist of 1 cycle at 93 c° for 3 minutes and 40 cycles 3 times 30 seconds at 93, 59±5, 72 c° and 1 cycle and 72 c° for 5 minutes, and the agarose gel 1.5% was placed for ensuring that the PCR was performed correctly. To perform the RFLP reaction, the NOCI restriction enzyme was added to products. First, 2µL 10X Buffer Tango were mixed with 2 µl NOCI restriction enzyme and 10 µl PCR product and 18 µl of DW. The thermal reaction was consist of 16 hours incubation at 65 c° and 20 minutes of inactivation at 80 c°. After RFLP, the agarose gel 2.5% was placed for investigating the presence of polymorphism in samples. The relationship between rs10490920 polymorphism and gene expression was measured by 1wayANOVA test.

Results:
In this study, in order to investigate the specificity of primers and color of fluorescence (syber green) and to ensure the replication of specific fragment and to verify the absence of non-specific components in the PCR product, the melting curve diagram for the *PTEN* gene and *GAPDH* (Diagram 1) were drawn separately by the Real Time PCR (ABI7500), which confirms the correct binding of the primers to the *PTEN* gene and the product was obtained that is desired for the gene. After the replication reaction, ct of the samples were calculated by the setting and converted to (RQ) Relative Quantization and the expression level was measured by ΔΔct method. The expression level of the patient samples was compared with normal samples. RQ of the samples was removed from the setting and the results were plotted by Graph pad software (Diagram 2). The comparison of *PTEN* gene expression level in patient and normal samples (9 normal samples have been converted to a sample in average in this diagram). Most of the expressions belongs to samples of 7, 10, 11 and 30. 1, 12, 14, and 24 samples have the lowest expression, which is likely to be at high stage of cancer. The RQ shown in the chart is the RQ mean of the patient and control groups. As shown in Diagram 3, the expression level of the gene in the patient group is reduced in comparison with the control group (Diagram 3). Also the mean of *PTEN* gene expression in men is higher than women (Diagram 4). The *PTEN* gene expression level in the 55-year-old group is lower than the 55-year-old group (Diagram 5). To ensure the quality and purity of the extracted DNA, the samples were electrophoresed in the agarose gel 1.5% (Fig. 1). The PCR product was prolonged using the primers used for 334bp, which was confirmed in the agarose gel. After enzyme treatment, homozygous CC samples with molecular weights of 334bp have 3 bands, heterozygote CT specimens with molecular weight of 194bp have 2 bands, and homogeneous TT samples with molecular weight of 174bp have 2 bands. As shown in Fig. 2. NOCI restriction enzyme were added to the PCR products and all samples were electrophoresed in agarose gel 2.5%. Homozygote CC samples with molecular weight of 334bp have 3 bands, heterozygote CT

specimens with molecular weight of 194bp have 2 bands, and homogeneous TT samples with molecular weight of 174bp have 2 bands. As shown in Diagram 6, the expression of the CT genotype decreased compared to other two genotypes. The CC normal genotype shows a higher expression level (Diagram 7). As shown in Diagram 8, the CT heterozygote genotype has been reduced to the other genotypes.
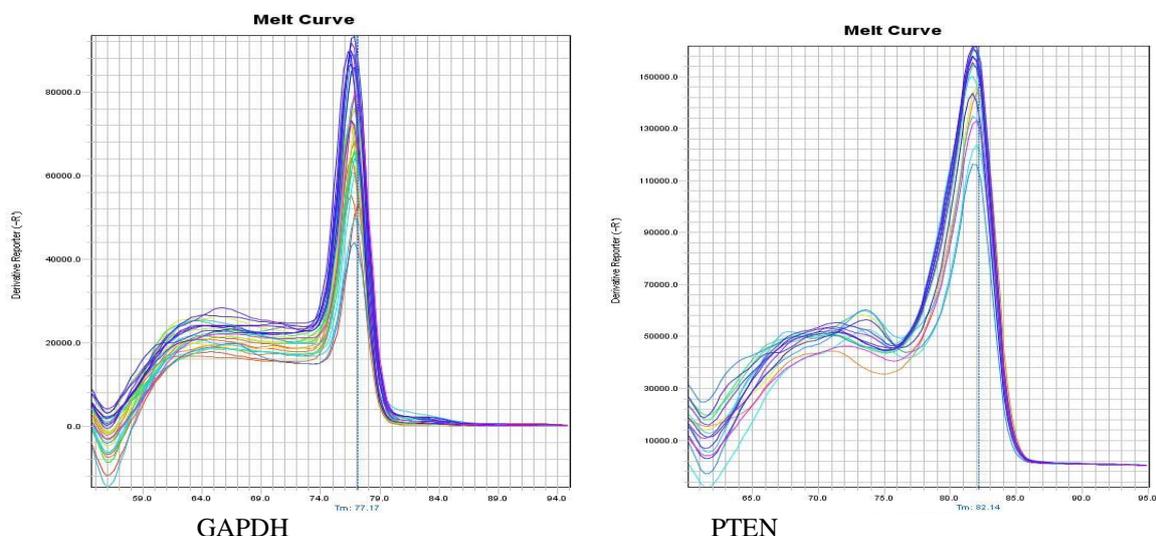

GAPDH   PTEN
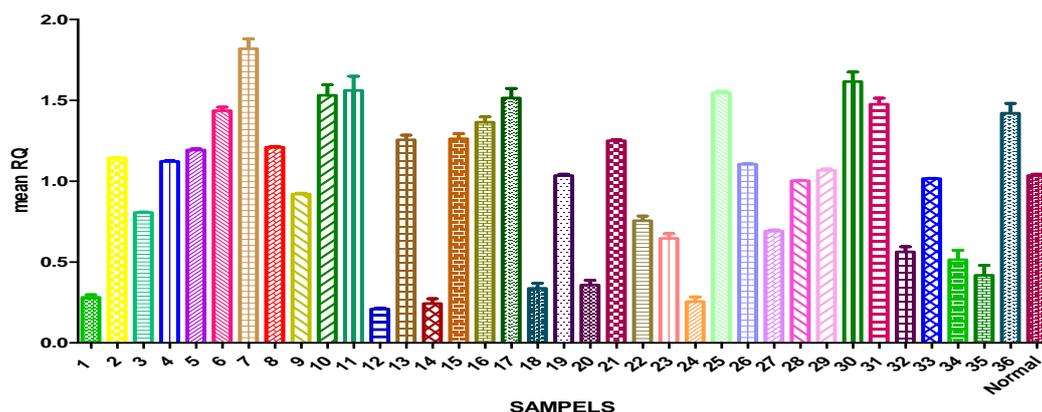
Diagram 1. Analysis of the melting curve of *PTEN* and *GAPDH* genes

Diagram 2: The level of *PTEN* gene expression in the patient and control groups. RQ shows the *PTEN* gene expression level in comparison with the normal sample. The numbers represent the sample number (P=0.7587).
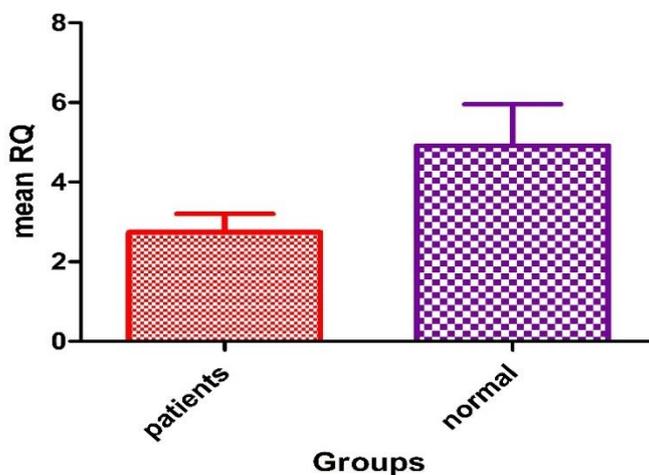

Diagram 3. Comparison of the gene expression in the patient and control groups. The mean of expression level in patient and normal groups were 2.74 and 4.91, respectively (P= 0.04).
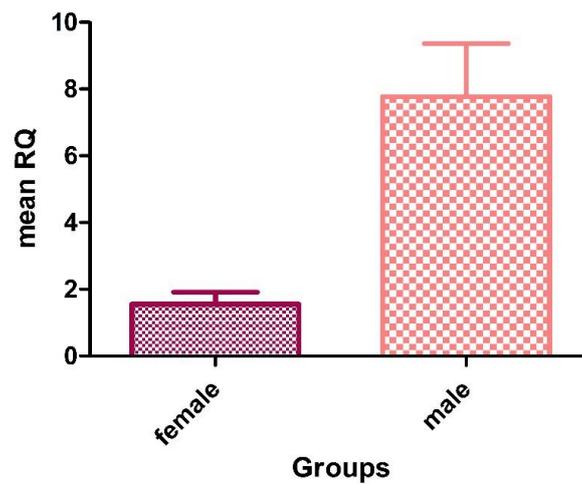
Diagram 4. Comparison of mean gene expression with sex: The average of expression level in women's and men's group were 1.55 and 7.77, respectively (P=0.04).
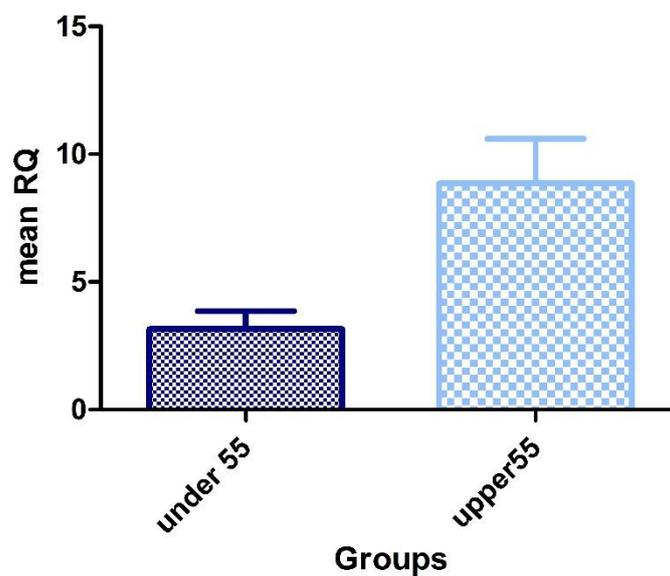

Diagram 5. Comparison of *PTEN* gene expression level in different age groups: The mean of gene expression rate in the group under 55 years of age and the group over 55 years old were 3.15 and 8.85, respectively (P=0.012).
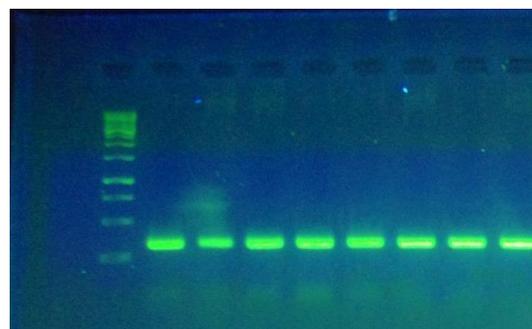

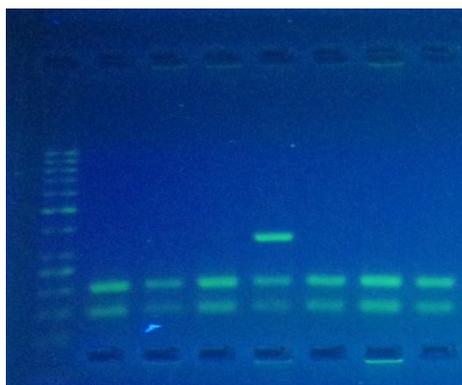**Figure 1.** Agarose gel electrophoresis 1.5%. Ladder (50bp fermentas)

Figure 2. Agarose gel electrophoresis of PTEN gene. 1: ladder (50bp fermentas) 5: PCR product of patient sample, 6: PCR product control sample
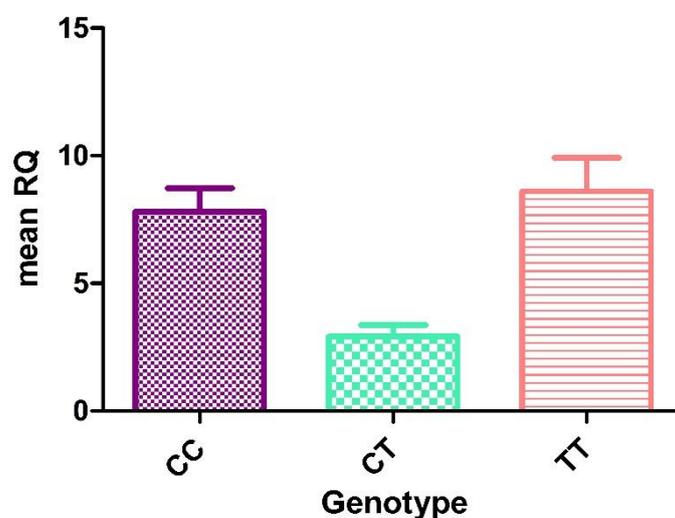

Diagram 6. Comparison of the expression level in 3 genotypes: The mean of expression rate in CC genotype and CT genotype ct: 2.91 and TT genotype are 7.80, 2.91 and 8.60, respectively (P=0.00).

Diagram 7. Comparison of *PTEN* gene expression with 3 above genotypes for people under 55 years old: The expression level average in the TT genotype, CC genotype and CT genotype are 4.66, 6.49 and 2.42, respectively (P=0.01).
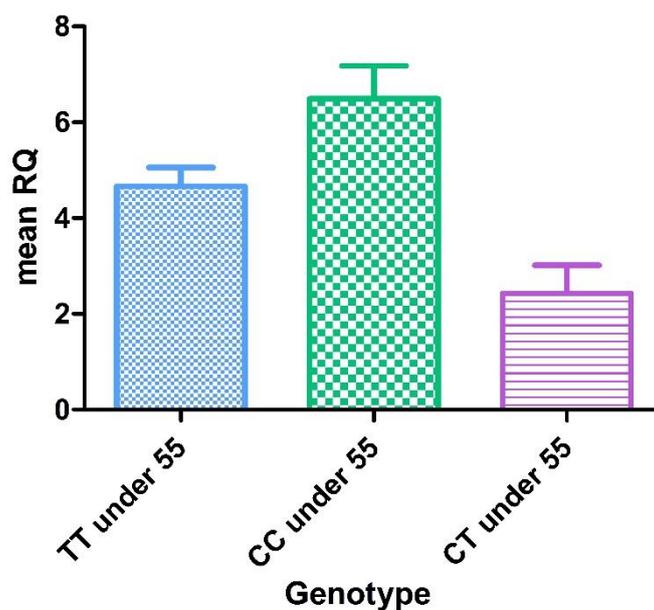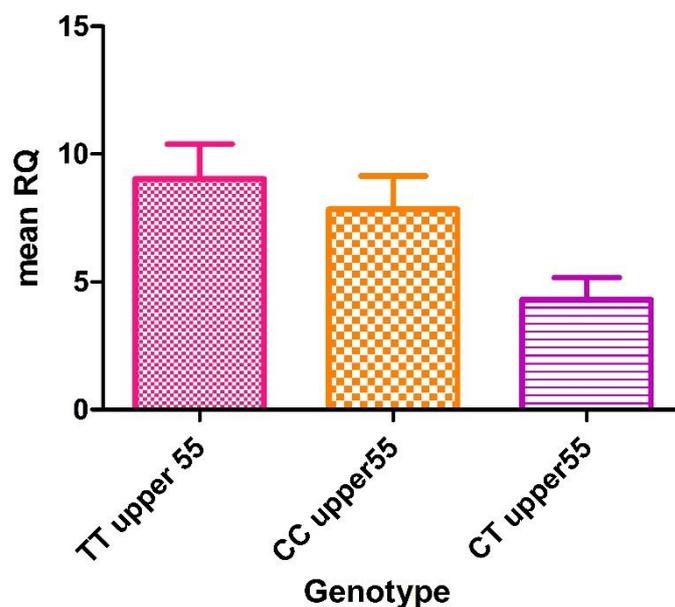
Diagram 8. Comparison of PTEN gene expression with 3 above genotypes for people above 55 years old: The expression level average in the TT genotype, CC genotype and CT genotype are 9.02, 7.84 and 4.30, respectively (P=0.02).

DISCUSSION:

The PTEN protein in its catalytic region contains a very similar sequence to cellular skeletal proteins such as Tensin and Auxilin, which led to the hypothesis that *PTEN* also regulates tumor invasion and metastasis [9]. *PTEN* is one of the most common targets for mutation in scattered human cancers, which in terms of mutation frequency is equal and compete with P53 and , so that after the discovery of P53 in 1985, no other genes have been remarkable except *PTEN* [8]. *PTEN* homozygote removal is observed in 10% of cancers. *PTEN* deactivating mutations have been reported in 33-83% in uterus cancer and 20-55% in uterine hyperplasia, suggesting that the gene is also effective at the early stages of tumor formation. WT et al. in 2014 identified the deactivation of the *PTEN* tumor inhibitor gene in various gastric cancer. One of the factors that causes the gene to be inactivated include gene mutation, promoter hypermethylation, post-translation phosphorylation and ineffective heterozygote [10]. In 2013, Canbey et al. examined the association between *PTENIVS4* polymorphism and gastric cancer in a Turkish population (with 93 patients and 113 healthy subjects). They found that *PTENIVS4* genotype polymorphism increased the risk of gastric cancer compared to control subjects significantly [11]. Xiang et al in 2015 found that increased expression of FAS (fatty acid synthase) and decreased *PTEN* expression may include the spread of peritoneal, which is involved in the progression of gastric cancer. In 2004, Zhoa et al. examined the expression of *PTEN / MMAC* (1) / *TEP* (1) and *VEGF*, as well as their role in angiogenesis and their association with gastric cancer, which observed deactivation of *PTEN* gene and increased the expression of *VEGF* in gastric cancer. Finally, it was suggested that *PTEN* and *VEGF* can show the biological behavior of the tumor and be used as biomarkers [12]. GUO et al. (2006) studied the protein expression with the PCR-SSCP-DNA sequence method in discovering the *PTEN* gene mutation and its value of gastric cancer, in the detection of protein was examined by immunohistochemical isolation in cancerous and non-cancerous tissues. According to their data, the overall positive expression of *PTEN* protein in the gastric cancer tissue was 66%, which was significantly lower of non-cancerous tissues. Finally, it was suggested that *PTEN* gene mutation may play an important role in the incidence of gastric cancer. In 2008, GeH et al investigated the *PTEN* polymorphisms and the incidence of esophageal and gastric cancer in China, suggesting that 2 common IVS4 (-/+) and -9 (C/G) polymorphisms could alter the sensitivity of the disease. This investigation of *PTEN* polymorphisms was genotyped by PCR-RFLP. The results showed that the risk of esophageal and gastric cancer increased significantly after adjusting based on age and sex and smoking in people with a positive family history of upper gastrointestinal cancers [13]. The current study as well as past reports suggest that the prediction of the outcome in patients with cancer is a complicated and complex problem. In addition, the progression of the disease is associated with an increased risk of death from the disease. The results of this study showed that *PTEN* gene expression can play a role

in the early diagnosis of gastric cancer as a biological marker. This content provides basic information on the role of *PTEN*, and more extensive studies are needed to confirm the precise role of this factor.

CONCLUSION:
According to this study , reduced PTEN gene expression is a possible etiology of stomach cancer and its progression

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