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Expression Rate and *PAX5* Gene Methylation in the Blood of People Suffering from Gastric Cancer

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Abstract

BACKGROUND: Gastric cancer is one of the most important health issues in the world. Considering the lack of plenty of pre-awarenesses, the survival of gastric cancer is still quite disappointing. Methylation of *PAX5* gene promoter is observed in most cancer cells of a human. A study has shown that PAX5 is a new tumoral suppressor in gastric cancer and methylation of the *PAX5* promoter is associated with the survival rate of gastric cancer.

AIM: The present research seeks to study the expression rate and methylation of the *PAX5* gene in the blood of patients who have gastric cancer to be used as a biomarker in this type of cancer.

MATERIAL AND METHODS: Real-time PCR technique was used to assess expression of *PAX5* gene, while the methylation status of *PAX5* gene promoter in the blood samples of people who have gastric cancer versus blood samples obtained from normal Iranian population was studied using MS PCR technique.

RESULTS: The final results pointed to the fact that expression of PAX5 in blood samples obtained from those who have gastric cancer is much less than what is observed in normal blood samples. A significant correlation was also observed between expression of this gene and age and promoter methylation rate. The results of methylation also indicated that 28% of *PAX5* gene promoters among patients were methylated, while all normal samples were non-methylated.

CONCLUSION: Studying the decrease observed in *PAX5* gene expression and the rise in promoter methylation can be utilised as a biomarker to enhance pre-awareness of gastric cancer.

Introduction

Gastric cancer is the result of cancer cell formation on the interior lining of the stomach [1]. The most common form of this cancer is known as Adenocarcinoma [2]. Throughout the world, the gastric cancer is the fifth main cause of cancer and the third major reason for death [3]. Lack of pre-awareness factors and proper diagnosis are the main problems in treating gastric cancer [3]. *PAX* genes (paired box) are a family of genes encoding tissue-specific transcription factors that play a major role in adjusting developmental and evolutional programs [4]. *Pax1, Pax2, Pax3, Pax4, Pax5, Pax6, Pax7, Pax8,* and *Pax9* are the members of *PAX* gene. *PAX5* gene encodes a family of transcription factors that regulate development that play a major and central role in cell differentiation and tissue development [4]. Inappropriate expression of PAX5 has been reported in various types of human tumours [4]. Researchers believe that inappropriate expression of *PAX5* contributes to Carcinogenesis and malignant progression of human cancers such as gastric cancer [5]. *PAX5* suppression is closely linked with the hypermethylation status of the promoter, and it can be reconstructed through demethylation treatments [6]. By knocking down *PAX5* trough short hairpin RNA's, the survivability and reproducibility of cells rise [5]. As a result, PAX5 is a useful tumour suppressor in gastric carcinoma and diagnosis of methylated *PAX5* can be considered an independent pre-awareness factor in gastric cancer.

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The present research seeks to study the expression rate and methylation of *PAX5* gene in the blood of individuals who have gastric cancer and compare it against normal blood samples and study their role as pre-awareness and diagonal factors in gastric cancer. We also seek to study the possible link between changes in the expression of this gene and gender, age, and methylation status.

Materials and Method

As many as 70 venous blood samples (35 healthy samples and 35 samples obtained from those with gastric cancer) were obtained from Masoud medical diagnostic laboratory in Tehran, Iran. 18 samples were females, and 52 samples were males.

With due observation of the standard protocol of the kit, RNA extraction kit made by Sinagen Iran Co. (MR7713C) was used to extract Total RNA. Then the quality of purified RNA was studied using a spectrophotometer, and the optical absorption difference of 260 to 280 was taken into consideration. To synthesise cDNA, dNTP 10 Mm (1 μl), Random Hexamer (40 Mm) (1 μl), and Oligo dt (1 μl) obtained from Sinagen Co. and, finally, 10 μl purified RNA was added to the microtube. The resulting mixture was exposed to a temperature of 65℃ for 5 minutes; then it was exposed to a temperature of 4℃ for 1 minute. Next, MMULV 10 X buffer (2 μl) and MMULV (200 U/μl) (0.5 μl) were added to this tube, and the total volume was increased to 20 μl using DEPC water. The tube was exposed to a temperature of 42℃ for 1 hour, and then it was exposed to a temperature of 80℃ for 5 minutes.

To conduct SYBR Green-based Real-Time PCR on cDNA samples, the required primers for *PAX5* and *GAPDH* gene (as internal controls) were designed using the sequences that exist in NCBI and using Annealing Primer Express software. The results are presented in Table 1.

Table 1: The primers used in our research

Primer	Sequence	Tm℃	Amplicon size(bp)
PAX5 (F)	GGACCAGCAGGACAGGTATT	59.02	118bp
PAX5 (R)	TTGGCGTTTATATTCAGCGA	59.31	118bp
GAPDH (F)	ATGGAGAAGGCTGGGGCT	62.05	124bp
GAPDH (R)	ATCTTGAGGCTGTTGTCATACTTCTC	61.62	124bp

The reaction mixture consisted of 10 μl master mix obtained from Sinagen Co., 1 μl gene sample, 0.5 μl forward primer (0.4 millimolar), 0.5 μl reverse primer (0.4 millimolar) and 8 μl distilled water. The following temperature was required in this experiment: 95℃ (1 cycle lasting 10 minutes), 95℃ (40 cycles each lasting 15 seconds), 58℃ (1 minute) for *PAX5* and 59℃ (1 minute) for *GAPDH* gene. Finally, the analysis of the data resulting from Real-time PCR was conducted based on the threshold cycle obtained for the target and reference genes. To study the specificity of primers and make sure about the proliferation of exclusive components, separate melting curve graphs were drawn for *PAX5* and *GAPDH* genes using Step One Real-Time PCR Systems-Applied Biosystems. The RQ of samples were calculated by the device, and the chart of results was drawn by Graph pad software (P-value < 0.0001).

To study the presence of CPG islands in *PAX5* gene promoter and its effect on gastric cancer, methylation-specific PCR (MS PCR) technique was used. Using DNA extraction kit made by Sinagen Co. (DN8118C) and the standard protocol of the kit, the genomic DNA was extracted from the blood of participants. The kit made by Thermo Scientific Co. (EPIJET BISULFITE CONVERSION K1461) was used to modify DNA. Two pairs of primers were designed for MS PCR reaction. One pair was used for the areas containing methylated cytosines beginning with G, and the other pair was used for non-methylated cytosines beginning with A (Table 2).

Table 2: The primers designed for methylated and non-methylated areas in MS PCR reaction

Primer	Sequence	Tm℃	Amplicon Size(bp)
PAX5 MET (F)	TTCGCGTATAGCGTAGAGGGTCG	63	bp 250
PAX5 MET (R)	AAACGTAACGAACCCGACCCG	62/2	bp 250
PAX5 UNMET (F)	TTTGTGTATAGTGTAGAGGGTTG	54/7	bp 250
PAX5 UNMET (R)	AAATGTAATGAACCTGACCTG	52/8	bp 250

MS PCR reaction was carried out by adding 12 μl master mix, 1 μl forward primer (0.4 millimolar), 1 μl of reverse primer (0.4 millimolar), 2 μl of modified DNA sample (10 ng), and 9 μl of distilled water to the microtube. The following plan was defined for reaction: exposure to a temperature of 93℃ for 3 minutes for the initial denaturation, forty 30-second cycles of exposure to a temperature of 93℃, exposure to a temperature of 66℃ for 30 seconds, exposure to a temperature of 72℃ for 30 seconds, and, finally, exposure to a temperature of 72℃ for 5 minutes. To validate the product of PCR reaction, as much as 10 μl of it was run in 1% Agarose gel.

Results

A group of 39 samples belonged to people older than 50 years old, while the remaining 31 samples were obtained from people younger than 50.

Having extracted the RNA, the quality of extracted RNA was reported 1.7 by comparing the wavelength of 260 to 280.

Normal samples are used as references to compare changes in the expression rate of each gene compared to ill samples (Figure 1 and 2).
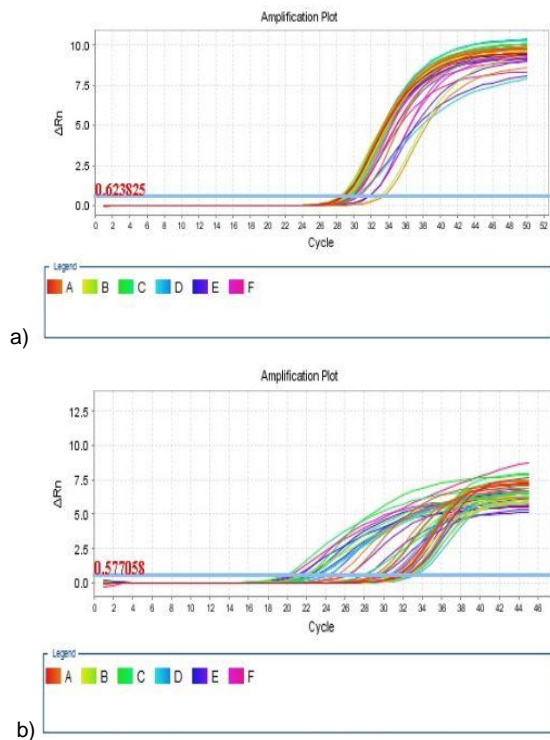
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a)

b)

Figure 1: The linear curve of PAX5 gene proliferation in healthy and ill samples (a); the linear curve of GAPDH gene proliferation in healthy and ill samples (b)

The expression rate of sick samples was compared against normal samples. The results represent the expression of the same gene compared to what was observed in the blood of normal people (Figure 3).
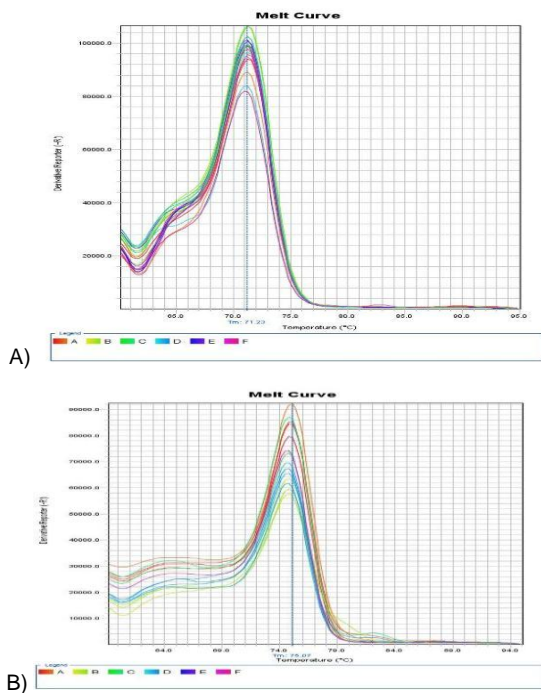

A)

B)

Figure 2: PAX5 gene melting curve in normal and sick samples A); GAPDH gene melting curve in normal and sick samples B)
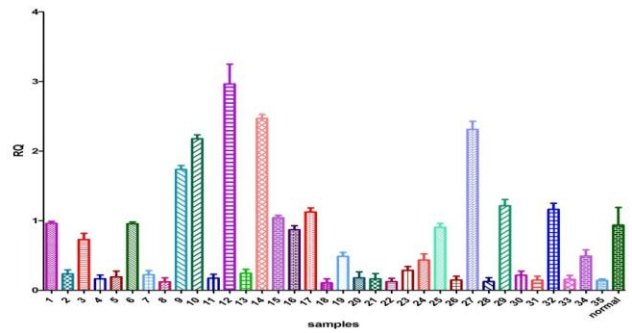

Figure 3: An analysis of PAX5 gene expression in sick samples compared to controls (P-value < 0.0001)

As the chart shows, most samples obtained from patients indicate less expression compared to normal samples. The normal samples are based upon the analysis made by the device, and all samples are compared against the normal samples. The least expression rates are observed in number 2, 4, 5, 7, 8, 11, 13, 18, 20, 21, 22, 26, 28, 31, 33, and 35 samples. As it is expected from PAX5 as a tumour suppressor in gastric cancer, the average expression of this rate in normal samples is more than all samples.

Further analysis showed that the mean expression rate of the _PAX5_ gene among patients is less than what is observed among normal participants. What this means for patients is 0.2, while it was 0.55 among normal people. The greater expression may vary based upon gender, age, and methylation status (P-value = 0.0378) (Figure 4).
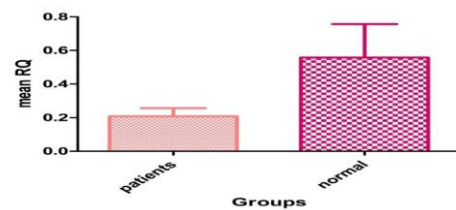

Figure 4: Mean expression of PAX5 gene in among patients and normal participants (P-value = 0.0378)

An analysis of results has shown that the mean expression rate of _PAX5_ gene among patients compared to normal participants younger than 50 years old is 1.44, while this value for those older than 50 years old was 0.54 (P-value = 0.0159) (Figure 5).
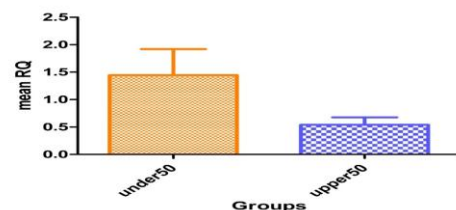

Figure 5: Mean expression rate of the PAX5 gene based upon age (P-value = 0.0159)

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The mean expression rate of *PAX5* gene among male patients has been much more than what was observed among the female peers. This average of RQ among men was 73%, while it was 66.8% among women. We may conclude that the expression rate of *PAX5* among male patients was much more than what was observed among female patients (P-value=0.8721) (Figure 6).
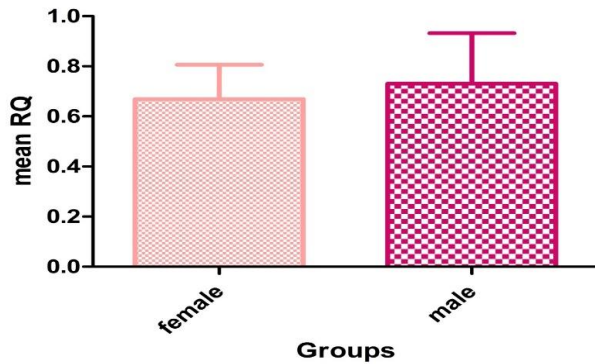

Figure 6: Mean RQ of PAX5 gene based upon gender (P-value = 0.8721)

By conducting MS PCR using methylated primers on 70 samples to study the methylation status of *PAX5* gene promoter, the band was observed in 4, 5, 31, 33, 34, 35, 39, 41, 43, 48 that it indicates methylation of *PAX5* gene promoter in these samples. The band sizes are in the range of 250 bp (Figure 7).
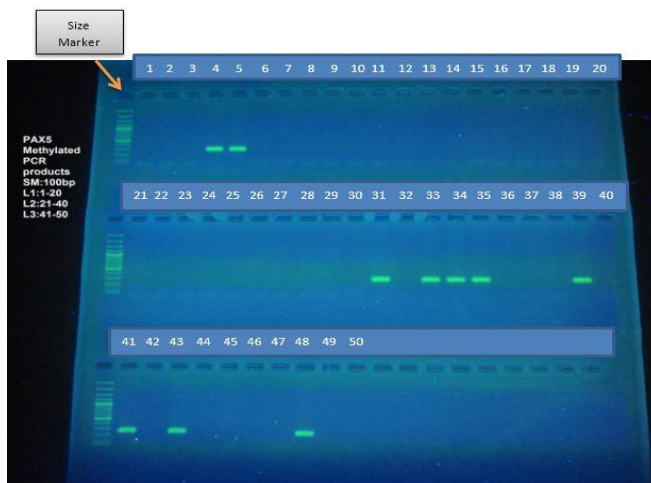

Figure 7: A sample of MS PCR results using methylated primers. Presence of bands with the size of 250 bp indicates methylation of PAX5 gene promoter

By conducting MS PCR using non-methylated primers on 70 samples to study the methylation status of *PAX5* gene promoter, the band was observed in 60 samples out of 70 samples which points to the fact that *PAX5* gene promoter is non-methylated in these samples (Figure 8).

Figure 8: A sample of the results of MS PCR using non-methylated primers. Presence of bands with a size of 250 bp indicates the fact that PAX5 gene promoter is non-methylated

Considering the results of MS PCR test for *PAX5* gene, it was shown that only 10 DNA samples (of the whole 35 DNA samples) of patients are methylated (28%) but all normal samples are non-methylated. Consequently, 28% of samples for *PAX5* gene promoter are methylated. The average expression rate of the *PAX5* gene in non-methylated samples was 99%, while this value in methylated samples is 17%. As a result, the mean expression rate of the *PAX5* gene in non-methylated samples is higher than methylated samples (Figure 9).
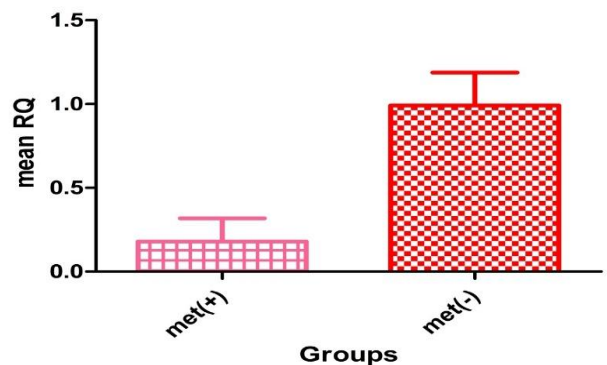
Figure 9: Mean RQ of the PAX5 gene based upon methylation status (P-value = 0.0409)

The P-values obtained through analysis of changes in expression of *PAX5* gene based upon gender, age, and methylation status are 0.8721, 0.0159, and 0.0409 which are respectively insignificant, significant and significant. As a result, no significant correlation is observed between less expression of *PAX5* and gender, but the correlation between less expression of *PAX5* and age and level of promoter methylation is significant.

Discussion

Gastric cancer is still the second and the most common reason of death caused by cancer throughout the world [6]. In Iran, gastric cancer has the greatest prevalence of all cancers among men [7]. Various transcriptional and regulatory factors that play a tumor suppressive role have been diagnosed in gastric cancer. For instance, PAX transcription factor has been diagnosed as a tumor suppressor in gastric cancer [8]. Other researches have pointed to the little or no expression of PAX5 in human cancers which shows PAX5 may act as a potential tumor suppressor in carcinogenesis [8]. Many researches have reported that methylation of the DNA promoter of *PAX5* gene will result in the shutdown of *PAX5* diagnosed in several malignancies [9]. *PAX5* has recently been used in diagnostic predictions of gastric cancer [10]. Using PCR technique for proliferation of methylated areas, Palmisano et al showed that activation of *PAX5* gene can help neoplastic development by suppressing growth regulators through affecting expression of CD19 gene [11]. They suggested that *PAX5* gene methylation is observed in tumors and their surrounding tissues and rarely in natural epithelial cells (assessments confirm them as intermediate markers in breast and lung cancers used to improve sensitivity and exclusiveness for development of risk models in order to diagnose these types of cancer) [11]. Using MSPCR technique and bisulfate genomic sequence, Liu et al., (2011) showed that *PAX5* is a functional tumor-suppressing gene and plays a major role in liver carcinogenesis by direct regulation of P53 signaling path which is usually deactivated through promoter methylation in Primary hepatocellular carcinoma tissues [12]. Using genome-wide methylation screening, Li et al., (2012) reported that suppression *PAX5* is closely associated with promoter hypermethylation and improper expression of *PAX5* in the shutdown cell lines of GC (AGS, BGC823) suppresses formation of colony and survivability of cells, stops cell cycle, induces apoptosis, suppresses cell immigration and invasion, and suppresses tumorigenesis in NUDE mice [13]. Using real-time PCR technique, a study was conducted by Liu et al and the results showed that *PAX5* is suppressed or shut down in most HCC cell lines and primary tumors [12]. In 2014, Deng et al studied the expression and methylation of *PAX5* gene in gastric tumor tissue and normal tissue for the first time. According to this research, mRNA expression of *PAX5* gene was diagnosed in 25 cancerous tissues of the stomach (of the whole 460 cancerous tissues) and 25 healthy mucosal gastric tissues through RT-PCR techniques [14]. Important differences in mRNA expression of *PAX5* gene were found in 25 cancerous gastric tissues. The mean value of the relative expression of mRNA in *PAX5* gene in 25 cancerous gastric tissues was 0.836 ± 0.357, while the mean value of the relative expression of mRNA in *PAX5* gene in 25 normal gastric mucosal tissues was 1.759 ± 0.821. The mean value of the relative expression of mRNA in *PAX5* gene in 25 cancerous gastric tissues was less than the 25 normal gastric mucosal tissues [14]. Considering the importance of *PAX5* gene, the present research studied expression of this gene in the blood samples of people suffering from gastric cancer in Iranian population. This is the first comprehensive research which presents statistical data of *PAX5* gene expression in the blood samples obtained from people suffering from gastric cancer in Iran. Using Real-Time PCR technique, the expression of this gene was compared accurately across the healthy participants and patients. Contrary to other studies and further to investigating the expression rate of *PAX5* gene, the present research studied the methylation rate of *PAX5* gene promoter and the correlation between methylation level and *PAX5* gene expression simultaneously. As the data resulting from this research indicate, the expression rate of *PAX5* in the blood samples obtained from patients is less than what is observed in normal samples. This data is in line with the results of a research by Deng et al., indicating that *PAX5* gene in gastric cancer acts as a tumor-suppressing gene plus less expression of *PAX5* gene in tumor samples obtained from stomach [14]. A review of methylation status of *PAX5* gene promoter showed that 28% of the DNA obtained from patients is methylated and all normal samples were non-methylated. These results are in line with the results of researches conducted on tumor samples of gastric tissue [15]. What's more, there is a significant correlation between the mean expression rate of *PAX5* and age and methylation status of promoter; however, no significant correlation was observed between average expression rate of *PAX5* gene and gender. All these researches were conducted on paraffined tissue samples; however, gastric cancer is not usually diagnosable in its early phases.

As a result, studying the expression and methylation of the *PAX5* gene in the blood of people who have gastric cancer shortly can be used as a diagnostic biomarker for gastric cancer.

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