



Original Article

Comparison of Matrix Metalloproteinases2 mRNA Expression in Prostatic Adenocarcinoma and Benign Prostatic Hyperplasia

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ABSTRACT

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Key words

Matrix Metalloproteinase2

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Background and Aims: Prostate cancer is the second most common cancer in men worldwide in men. Matrix metalloproteinase-2 (MMP2) has a role in the invasion and destruction of the basement membrane and the extra-cellular matrix and facilitating the process of tumor cell invasion. The present study was conducted to compare the expression of MMP2 gene in prostate cancer (PCa) and benign prostatic hyperplasia (BPH).

Materials and Methods: 48 samples of PCa and 48 samples of BPH (as controls) were examined. RNA was extracted from paraffin tissue blocks and then used for cDNA synthesis. The real time polymerase chain reaction method was used to quantify the expression of MMP2 gene. The level of gene expression was assessed using participants' clinicopathological characteristics.

Results: The expression of MMP2 gene increased in the PCa samples compared with the BPH samples ($p=0.003$). MMP2 gene increased in all the grades of this group compared with the controls. The highest expression, however, was observed in the moderately differentiated grade. No significant correlations or relationships were observed in the PCa samples between prostate specific antigen and the other study variables such as age, MMP2 expression, disease grade and the Gleason score; however a positive correlation was observed between prostate specific antigen and the samples' pathological stage.

Conclusions: The expression of MMP2 increased in the PCa samples compared with the BPH samples, with the highest expression occurring in the samples with a Gleason score of 6. Further tests and studies conducted with larger sample sizes may help to use this marker in differentiating malignant from benign samples.

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Introduction

Prostate cancer is the second most common (after skin cancer) and the second most fatal (after lung cancer) cancer in men in developed countries. One in every six men is affected by this cancer [1]. The highest prevalence of prostate cancer has been reported in Australia, New Zealand, North America and Western Europe, with a moderate prevalence reported in Eastern Europe and Africa and the lowest prevalence in Asia (an annual of 3-8 new cases per every 100,000 men) [2]. The prevalence of this cancer, thus varies across the world depending on race, age and geographical region and is associated with genetic and hormonal factors [3]. In Iran, prostate cancer is the eighth leading cause of death and has been reported as one of the ten most common cancers in northern regions of the country in recent years. Prostate cancer leads to a relatively high rate of mortality compared to other cancers [4]. Most prostate tumors are adenocarcinomas and a hereditary history is considered an important factor in developing this cancer. Hereditary factors are involved in 10% of all the cases of prostate cancer and are often associated with an early onset too [2]. A special protein called prostate specific antigen (PSA) is produced by the prostate that is a tumor marker for diagnosing prostate cancer [2]. PSA is measured in screening tests used for prostate disease and is usually high in the patients with prostate cancer. It is worth noting that blood PSA levels measured on a test are not indicative of prostate cancer in and by themselves. In some cases, infection or benign

prostatic hyperplasia (BPH) enlargement can lead to increased blood PSA levels [5]. The matrix metalloproteinase (MMP) includes more than 20 zinc-dependent proteinases that destroy extracellular compounds such as collagens, proteoglycans and glycoproteins [6, 7]. With their destructive function of extracellular compounds, these proteins cause the spread of cancer and metastasis. Metalloproteinase also causes the release of growth factors, which are themselves involved in the stimulation of tumor growth and advanced tumor invasion [6- 9]. Thus, MMP family is targeted in the treatment of cancer. Metalloproteinases are very similar to each other in structure and have a major role in the migration of lymphoid and myeloid cells, the healing of wounds and the physiological reconstruction of tissues, including the processes of normal growth, fetal development [10]. These enzymes often have a low expression and their transcription is regulated and controlled by factors such as cytokines, interleukins 1, 4, 6 and growth factors such as transforming growth factor beta, hepatocyte growth factor, epidermal growth factor, and steroid hormones [11]. Due to the tendency to bind to its substrate, matrix metalloproteinase2 is referred to as gelatinase-A, with repeating domains (Fibronectin-link) immediately before binding to the zinc atom in its catalytic domain, which facilitates its binding to the substrate [12].

Since PSA is not an ideal marker for the diagnosis of prostate cancer and since its

serum levels can be affected by various factors such as race, androgenic and non-androgenic factors and dietary regimens, and since they increase even in benign prostate tumors, the present study was conducted to determine the expression levels of MMP2 gene as an effective marker in differentiating PCa from BPH and to also assess the association between MMP2 expression and the patients' clinicopathological data. This is the first study describing the MMP2 expression in Iranian men.

Material and Methods

Patients

48 Paraffin-embedded prostatic tissues from the patients with prostate adenocarcinoma and 48 BPH samples as a control group were studied. All Ethical considerations are considered in this study. The study was approved by the Ethics Committee of Islamic Azad University, Parand Branch, Parand, Iran. The samples were referred to the pathology laboratory of Modarres Hospital (Tehran, Iran) during 2011-2014 and were examined after a confirmed diagnosis by the pathologist. The samples were included only if they were underwent radical prostatectomy at Modarres Hospital and had no history of hormone therapy, chemotherapy, or radiotherapy. Gleason grade and pathological TNM 2002 stage were used as prognostic factors. Gleason score was classified as low grade (Gleason score ≤ 6) and high grade (Gleason score ≥ 7). The patients were grouped according to the prognostic parameters pT2 (n=30) and pT3

(n=18), Gleason score ≤ 6 (n=9), and Gleason score ≥ 7 (n=39).

RNA extraction and cDNA synthesis

The histological sections (10 microns in thickness) were cut from each block using a microtome (Leica. RM 2125). In order to deparaffinize the sections, the samples were first added with 1000 ml of xylose and maintained at 56°C for 5 minutes. They were then centrifuged at 13000 g and 24°C for five minutes and the supernatant was removed. In the next stage, 1 ml of cold 100% ethanol was added to the samples and the samples were inverted 10 times. They were centrifuged at 13000 g and 4°C for five minutes and the sediment was removed. Afterward, 100 μ l of protease buffer and 20 μ l of proteinase K (Fermentas Co., USA) were added to the samples. The samples were vortexed for 10 seconds and then incubated for 15 minutes at 56°C and for another 15 minutes at 85°C. After adding 500 μ l of RNX-plus (Cinnagen Co., Iran), the samples were inverted 10 times and vortexed for five seconds. The microtubes were kept at room temperature for five minutes. Then, 10-200 μ l of chloroform were added to the samples and the samples were vortexed for 15 seconds and placed on ice for 5 minutes. They were then centrifuged at 12000 g for 15 minutes. The aqueous phase (supernatant) was transferred to another tube and the same volume of cold isopropanol was added to the tube. The microtubes were inverted 10 times and then maintained at -20°C for one hour (or overnight). They were then centrifuged at 12000 g for 15 minutes and

the supernatant was removed. After adding 1000 µl of cold 70% ethanol to the samples, the microtubes were inverted 15 times and then centrifuged at 7500 g for 8 minutes. The supernatant was removed and the microtubes were then kept at room temperature to allow the evaporation of alcohol. Finally, 30 µl of diethylpyrocarbonate (DEPC) -treated water were added to the samples and the samples were maintained at -20°C [13].

The NanoDrop™ ND-2000 was used to examine the quantity and quality of the extracted RNA. The 260/280 ratio represents the nucleic acid to protein ratio (i.e. the purity of the extracted RNA). While ratios of 1.8-2 suggest the favorable quality of RNA, lower ratios indicate higher contamination with proteins or aromatic matters such as phenol. cDNA was synthesized using a commercial kit (Fermentas Co., USA). Each microtube was added with 1 µl of random hexamer (5 µM), 1 µl of oligo (dT) primer (5 µM), 1 µl of deoxynucleotide (dNTP) (10 mM), 5 µl of RNA, 0.5 µl of Moloney murine leukemia virus (MMLV) reverse transcriptase, 2 µl of MMLV buffer, and 9.5 µl of DEPC-treated water. The total volume of the final mixture was expected to reach 20 µl. The samples were maintained at 65°C for 5 minutes and then placed in ice

immediately. Afterward, they were kept at 42°C for one hour.

Real-time polymerase chain reaction (PCR)

The sequence of specific primers for MMP2 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was retrieved from the National Center for Biotechnology Information (NCBI) website. The specific primers of these two genes were designed using the Primer Express Software and their specificity was blasted in the NCBI. Table 1 presents the sequence of the primers used in this study. Real-time PCR was used to measure the expression levels of MMP2 and GAPDH (as the control at mRNA level). A StepOne real-time PCR system was used for relative quantification through the measurement of fluorescence increase following the application of SYBR Green. The real-time PCR reaction was optimized at the final volume of 20 µl. The reactants included 10 µl of SYBR™ (2X) Master Mix (Takara Co., Korea), 10 µM of the reverse and forward primers (Takapoo Zist Co.), 7 µl of deionized water, and 2 µl of the cDNA template. The temperature program of the device was optimized as follows: pre-denaturation at 95°C for 10 seconds; 50 cycles of denaturation at 95°C for five seconds; and binding and expanding at 60°C for 34 seconds.

Table 1. The sequence and characteristics of the specific primers of real-time polymerase chain reaction

Name		Tm	Aplicon size
MMP2 F	CCTGAGATCTGCAAACAGGA	58.96	84pb
MMP2 R	AATGAACCGGTCCTTGAAGA	59.53	
GAPDH F	CCCACACACATGCACTTACC	60	85pb
GAPDH R	TGCCTGTCCTTCCTAGCTCT	60	

The melting curve was drawn through measuring the changes in the fluorescence level at different

times using the real-time PCR device. After the amplification reaction using the relative

quantitative real-time PCR, the raw data in the form of ct values were drawn out of the device, calculated through $\Delta\Delta\text{ct}$, and converted into relative quantity. In order to measure the decrease or increase in the expression of a target gene, its expression is generally compared to that of housekeeping genes. In the present study, glyceraldehyde phosphate dehydrogenase (GAPDH) was considered as the housekeeping gene because of its permanent expression in most cells and tissues.

Statistical analysis

The results of this study were obtained in three replicates. T-test and correlation analysis were used to compare MMP2 expression between two groups and applied to analyze the association between MMP2 expression and patients' clinicopathological data. All analyses were performed using Graph Pad6 and SPSS 19.0 (SPSS Inc., Chicago, IL, USA) at a significance level of $P < 0.05$.

Results

Clinicopathological characteristics of patients

Samples from 48 patients with PCa (mean age: 64.57 ± 7.94 years; range: 48-85 years) and 48 patients with BPH (mean age: 63.56 range: 54-80 years) were examined in this study. The patients with PCa and BPH had no significant differences in terms of mean age.

Out of 48 patients with PCa, 13 individuals aged less than 60 years and 35 subjects aged over 60 years. Poorly differentiated, moderately differentiated, and moderately-poorly differentiated and moderately-well differentiated adenocarcinoma was detected in 6, 8, 33 and one samples, respectively. Gleason score of 6 or less and 7 or greater were detected in 9 and 39 samples, respectively. Table 2 shows the summary of clinical and clinicopathological characteristics of the studied samples.

Table 2. The clinical and clinicopathological characteristics of the studied samples

Sample Characteristics	Number of Samples	
	Adenocarcinoma N=48	Benign Prostatic Hyperplasia N=48
Age		
<60 Years	13 (27.08%)	17 (35.42%)
≥60 Years	35 (72.91%)	31 (64.58%)
Tumor Grade		
Moderate Well	1 (2.08%)	
Moderate	8 (16.67%)	
Moderate- Poorly	33 (68.75%)	
Poorly	6 (12.5%)	
Gleason Score		
6 or Less	9 (18.75%)	
7 or Greater	39 (81.25%)	
Pathological Stage		
PT2	30 (62.5%)	
PT3	18 (37.5%)	

Quality assessment of RNA extraction, cDNA synthesis, and fidelity of real-time PCR

The quantitative and qualitative assessment of the extracted RNA with a spectrophotometer showed a high degree of purity and the absence of phenolic contaminants. Likewise, the results of spectrophotometry suggested the favorable quantity and quality of the produced cDNA for the real-time PCR. In order to examine the specificity of the primers and the fluorescent dye (SYBR Green), ensure the amplification of specific components, and examine the absence of nonspecific components in PCR products, melting curves were separately drawn for MMP2 and GAPDH using the real-time PCR device (StepOne). The curve confirmed the proper and specific binding of the primers to binding sites on MMP2 and GAPDH. The presence of only one peak for each gene fragment (at its own melting temperature) confirmed the specificity of the product (Fig. 1).

Once the proper performance of the primers was ensured, real-time PCR was performed. The output was an amplification plot for both genes in BPH and adenocarcinoma samples. Figure 2 shows the amplification plot for MMP2.

Determination of MMP2 expression in BPH and PCa specimens and the correlation between MMP2 expression and clinicopathological data

According to our findings, MMP2 expression was 2.18 ± 1.09 higher in adenocarcinoma samples than in BPH samples ($p=0.003$) (Fig. 3). The adenocarcinoma samples were

categorized into four grades, namely poorly differentiated, moderately-poorly differentiated, moderately differentiated, and moderately-well differentiated. These samples scored six-nine on the Gleason grading system. There were 33 patients with moderately-poorly differentiated adenocarcinoma (mean age: 63.56 years). The minimum and maximum PSA levels in this group were 1 and 82 ng/ml, respectively. The mean of PSA level in adenocarcinoma samples was 14.68 ± 15.94 ng/ml. All samples of this group scored seven on the Gleason grading system and their mean MMP2 expression was 2.33 ($p=0.0001$). Moreover, all the 8 patients with moderately differentiated adenocarcinoma (mean age: 64.87 years) obtained a Gleason score of 6. The mean MMP2 expression and PSA range of this group were 2.40 and 5.9-10.3 ng/ml, respectively. Six patients had poorly differentiated adenocarcinoma. Their mean age, mean MMP2 expression, and PSA range was 67.40 years, 2.07, and 7.4-40 ng/ml, respectively. Five samples of this group scored eight and one sample scored nine on the Gleason grading system. Only one patient, who aged 82 years, had moderately-well differentiated adenocarcinoma (Gleason score of 5; PSA level=8.2 ng/ml; mean MMP2 expression=1.15). Comparison of MMP2 expression according to Gleason Score of GS 6 ($p=0.0005$), GS 7 ($p=0.0001$), GS 8 ($p=0.0005$) was significant (Fig. 4).

There was no correlation between PSA levels and MMP2 expression. While MMP2 expression was 2.18 in the patients over 60 years and 1.99 in those under 60 years, there

was not found any significant correlation between age and gene expression in the studied samples. Increments in Gleason score were associated with reductions in the MMP2 expression, i.e. minimum and maximum increase in the mean MMP2 expression was observed at Gleason scores of 9 and 6, respectively. All samples with Gleason scores of 6 were in the stage two of the disease (moderately- poorly differentiated adenocarcinoma). In cases of PCa there were no correlations between PSA

and other factors, including age, MMP2 expression, disease grade, and Gleason score. However, there was a positive correlation between PSA levels and pathological stage of the disease, i.e. higher PSA levels were observed in more advanced stages of the disease ($r=0.363$; $P=0.015$). No correlation was found between the mean age of the patients and MMP2 expression in adenocarcinoma samples.

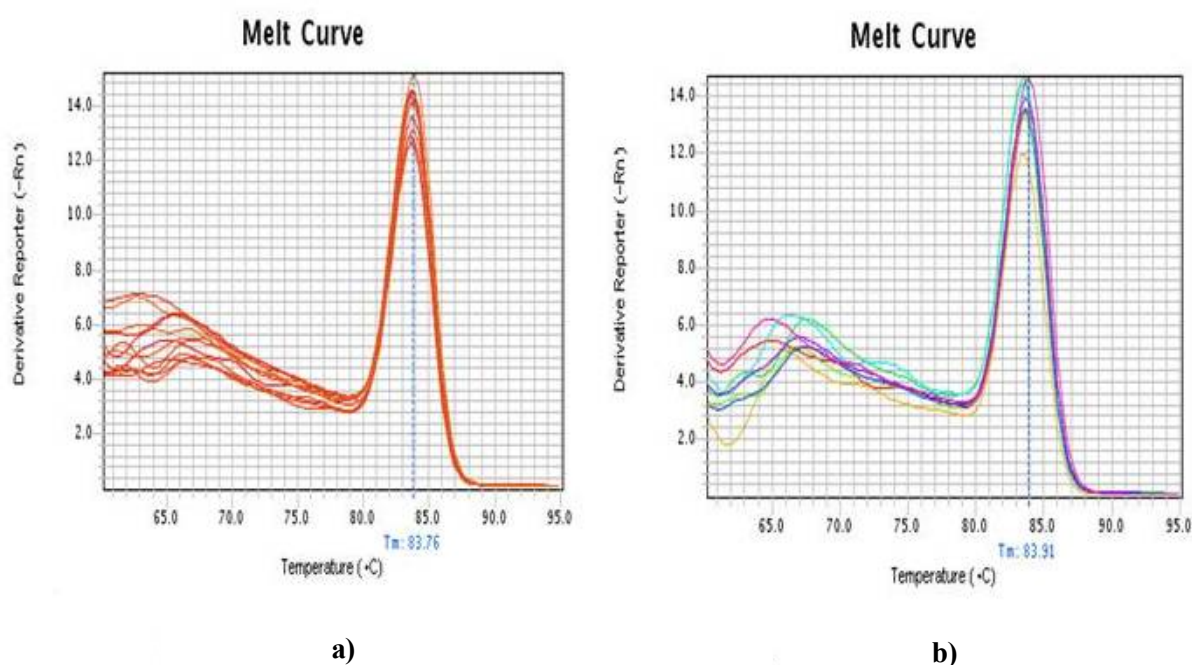


Fig. 1: The melting curve for primers of MMP2 (a) and GAPDH (b).

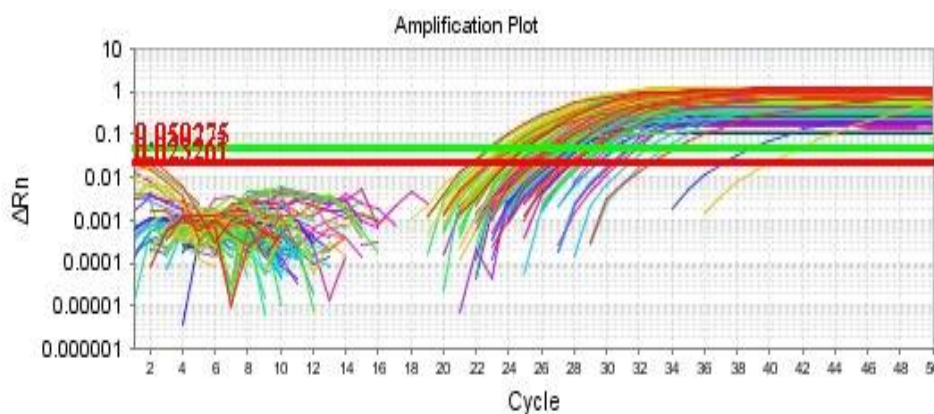


Fig. 2. The amplification plot for MMP2

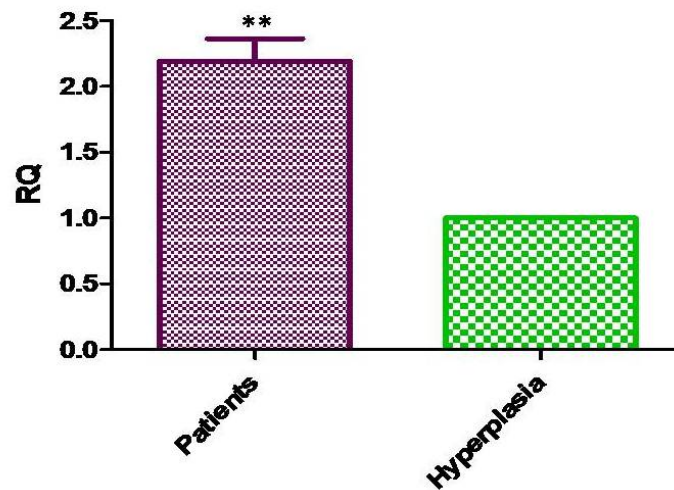


Fig. 3. Comparison of MMP2 Expression in Adenocarcinoma and BPH tissues (** p=0.003).

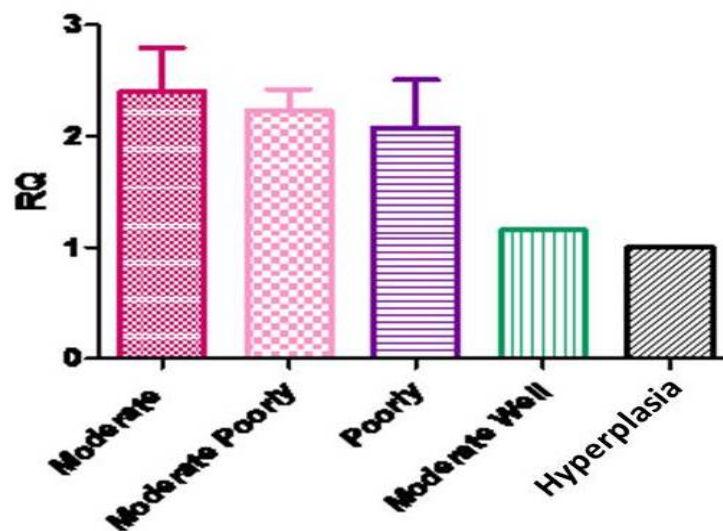


Fig. 4. Comparison of MMP2 expression according to grade of tumor. p value in Moderate, Moderate poorly, Poorly groups (p=0.0002), but in Moderate Well group was not significant.

Discussion

The MMPs family includes more than 20 zinc-dependent proteinases that destroy extracellular compounds such as collagens, proteoglycans and glycoproteins [6, 7]; with their destructive function of the extracellular matrix, these proteins cause the spread of

cancer and metastasis. MMPs also causes the release of growth factors, which are themselves involved in the stimulation of tumor growth and advanced tumor invasion and angiogenesis. So, the MMPs family targeted in the treatment of cancer [6- 9]. The overexpression of MMP2 has

been observed in many neoplasms, including ovarian [14-16], urothelial [17-19], cutaneous [20, 21], gastric [22], breasts [23, 24] and the cervical cancers [25].

In a study conducted by Fang et al. the suppression of MMP2 in chondrosarcoma cells was found to have led to the suppression of tumor growth through reducing angiogenesis, showing the importance of MMP2 in micro-metastasis and angiogenesis [26].

Several studies have reported an overexpression of MMP2 in cases of prostate cancer [27-38]. A number of studies have investigated the relationship between MMP2 expression and the progress of prostate cancer and proposed MMP2 to be a prognostic factor for the disease [32-34]. Some studies have proposed an association between MMP2 expression and Gleason Score and the pathological stage of the disease [30, 32, 33]. Wood et al., introduced MMP2 as a prognostic factor independent of the grade and stage of the disease and attributed its expression to stromal cells and not to malignant epithelial cells [32]. Several studies reported the overexpression of MMP2 in both types of prostatic cells [27, 30, 31, 34, 35]. Stearns et al. noted the correlation between MMP2 expression and increased Gleason Score [31].

In one study, Alizadeh et al. MMP2 and PSA activities was compared in the grading of prostate cancer and MMP2 was analyzed in the serum samples of the patients with BPH and PCa in comparison with the control group. The results obtained confirmed that MMP2

can be used as a better marker than PSA in screening for prostate cancer [36].

Berahmer et al. investigated MMP2 and MMP-9 and tissue inhibitors in prostate cancer. They conducted an immunohistochemical investigation and a gene expression analysis and assessed the association between their new data and the available clinical and pathological data and found a significant increase in gene expression in the tumor cells compared to the epithelial prostate cells of the same patients; however, no associations were found between the pathological stage of the disease and the PSA levels [37]. Nevertheless, the present study found a positive correlation between the stage of the disease and the PSA levels.

In immunohistochemical study, Traudel et al. noted the association between MMP2 expression and prostate cancer. In the present study, the expression of MMP2 has increased in malignant prostate tissues and had a positive correlation with the Gleason Score and the pathological stage of the disease; however, They found no association between the variables [35]. Moreover, in contrast to Trudel's study, the present study found no correlations between MMP2 expression and PSA level.

Ogui et al. investigated the relationship between the expression of MMP2 and MMP9 in prostate carcinoma in the main tumor and the marginal tumor cells and showed a higher Gleason Score in tumors with positive marginal cells and also demonstrated the expression of MMP2 not only in the cytoplasm of the tumor cell, but also in the cytoplasm of prostate

stromal cells, the endothelial cells, fibroblasts, macrophages and lymphocytes [38].

Zhang et al., found no significant increase or decrease in the expression of MMP2 in either the carcinoma or the hyperplasia tissues [39]. In the present study, the expression of this gene in the adenocarcinoma samples was 2.18 times higher than in the hyperplasia samples, and the difference was significant ($P < 0.05$).

In the present study, the metastatic adenocarcinoma samples were examined using a Real Time PCR, which is a powerful technique. The results showed an increased expression of the gene in the PCa samples compared to in the BPH samples. Nevertheless, a different result was obtained in relation to a simultaneous increase in both the gene expression and the Gleason Score. In a study conducted by Sauer et al. MMP2 was most active in the advanced stages of the disease (PT2-PT3) and with a $GS > 7$ [40].

In the present study, the highest gene expression was reported in the moderately differentiated group at the PT2 stage and with a Gleason score of 6. The disparity of findings may be due to the difference in the study

populations in terms of genetics, lifestyle, nutrition, effective environmental factors and genetic factors.

Conclusion

As the expression of the gene changes with factors such as race, geographical region and lifestyle, these factors can affect on the results obtained. Certain differences between the present study and other performed studies may have been due to these factors as well as the different techniques used. The MMP2 gene had a greater expression in the adenocarcinoma samples compared with the hyperplasia samples. Further tests and efforts to localize the expression of genetic markers such as MMP2 in prostate cancer appear to help differentiate malignant from benign samples.

Conflict of Interest

There is no conflict of interest regarding this manuscript.

Acknowledgements

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