

Original Article

Evaluation of *c-Myc* mRNA Expression Level in Benign Prostatic Hyperplasia and Prostatic Adenocarcinoma Tissues and Its Correlation with Clinicopathological Characteristics

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Background and Aims: Prostate cancer (PCa) is one of the most common cancers among men in Iran. Since changes in the regulation of proto-oncogenes expression are the main causes of most human cancers, including PCa, evaluating the expression of marker genes can be helpful for early diagnosis of cancer and better understanding of its etiology. The present study compared *c-Myc* expression level in prostatic adenocarcinoma and benign prostatic hyperplasia (BPH).

Material and Methods: Paraffin-embedded prostatic tissues from patients with prostate adenocarcinoma (n=38) and BPH (n=38) were selected. The samples were included only if the patients underwent radical prostatectomy and had no history of hormone therapy, chemotherapy, or radiotherapy. After RNA extraction and cDNA synthesis, *c-Myc* expression in the samples was compared using SYBR green-based real-time polymerase chain reaction.

Results: Significantly higher *c-Myc* mRNA expression was observed in adenocarcinoma samples than in BPH group (p=0.001). No significant correlation was observed between *c-Myc* expression and Gleason Score (p>0.05). There were no significant correlations between *c-Myc* expression and prostate-specific antigen levels and age (p>0.05).

Conclusions: The *c-Myc* mRNA expression increased in the PCa samples compared with the BPH group. It seems that *c-Myc* expression can be introduced as a prognostic marker for determination of the invasive potential of tumor cells. Further tests and studies conducted with larger sample sizes may help to use this marker in differentiating malignant from benign samples.

Introduction

Prostate cancer (PCa) is the second most common cancer after skin cancer and the second leading cause of cancer deaths after lung cancer in men. This type of cancer, occurring in one out of every six men, is one of the most prevalent malignancies all over the world [1, 2]. The prevalence rate of PCa differs in different ethnicities and communities and the highest rates have been reported from Australia, New Zealand, North America, and Western Europe [3]. While the rates are lower in Eastern Europe and Africa, the lowest prevalence of the disease (three-eight per 100,000 men per year) is seen in Asia [4-6]. PCa is also a major cause of death and the third most common cancer, after skin and gastric cancers, accounting for 9.4% of all cancers in Iranian men [7].

Prostatitis, or the inflammation of the prostate gland, is a disease of the prostate gland caused by various factors such as bacterial infections. While different types of proliferative lesions are found in the peripheral, central, median, and periurethral zones of the parenchyma of prostate, hyperplastic lesions and carcinomas generally present in the central and peripheral zones, respectively. As another prostatic disease, Nodular prostatic hyperplasia is characterized by the proliferation of stromal and epithelial cells and enlargement of the prostate gland and may sometimes induce urinary tract obstruction. The enlarged prostate of the patients may weigh up to 300 g in severe cases and contain clearly identified nodules in its cross-section [8].

Although the exact causes of PCa are not known, some factors, including positive family history, increased age, and environmental factors (e.g. diet) are believed to increase its risk [9, 10]. Moreover, 9% of patients with advanced PCa experience bone metastasis [11, 12]. Since most prostate tumors are adenocarcinomas, they have similar characteristics to common epithelial cancers such as breast and colon cancers [13].

The prostate gland produces a specific protein, called the prostate-specific antigen (PSA), which enters the semen and increases sperm motility [14]. As PSA levels are generally raised in patients with PCa, they are routinely measured in prostate disease screening. However, elevations in blood levels of PSA do not necessarily indicate prostate cancer and can be caused by various infections and benign prostatic hyperplasia (BPH) [15]. While a PSA cutoff point of 4 ng/ml is usually considered, some men with $PSA \leq 4$ ng/ml may also have histological evidence of PCa [16].

According to epidemiological research, genetic factors are responsible for 10% of PCa cases [1, 2]. Familial aggregation of prostate cancer, mainly caused by the inheritance of the involved genes, has been reported by several studies [5, 6]. Changes in a number of molecular and genetic factors including *c-Myc*, are involved in the incidence and progression of PCa [17]. Despite its different behaviors in different tumors, abnormal expression of *c-Myc* seems to always contribute to the growth of tumor cells. A correlation has been reported

between the overexpression of this gene and tumor invasion and metastasis [18].

C-Myc is located on the long arm of chromosome 8 and its expression increases in a variety of cancers including breast and colon cancers. Among the members of the *Myc* family of transcription factors (*l-Myc*, *n-Myc*, *b-Myc*, *s-Myc*, and *c-Myc*), *l-Myc*, *n-Myc*, and *c-Myc* are the most important ones and possess the neoplastic potential [19]. The helix-loop-helix domain of *Myc* genes affects its DNA binding ability. Based on evolutionary studies, L-*Myc* and N-*Myc* are actually produced following *c-Myc* duplication [20]. Yang et al. demonstrated that the increased expression of *c-Myc* increases the activity of a group of gene promoters and leads to extensive changes in the regulation of gene expression in cancer cells [21]. According to Johnson et al., an induced increase in *c-Myc* expression affects the inhibition of main gene promoters involved in cell proliferation (thus increased their expression) and causes various types of cancer including Burkitt lymphoma, lung carcinoma, glioblastoma multiform, and multiple myeloma [22].

This study evaluated the *c-Myc* expression level as an efficient marker for differentiation between prostate adenocarcinoma and BPH. This is the first study describing the *c-Myc* expression in Iranian men.

Materials and Methods

Patients

All Ethical considerations were considered in this study. Paraffin-embedded prostatic tissues from 48-85-year-old patients with prostate

adenocarcinoma (n=38) and 38 BPH specimens (as a control group) were studied after obtaining their written consent. The samples were referred to the pathology laboratory of Modarres Hospital (Tehran, Iran) and were examined after a confirmed diagnosis by the pathologist. The samples were included only if the patients had undergone 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. The study was approved by the Committee of Islamic Azad University, Parand Branch, Parand, Iran (2983051792205).

RNA extraction from paraffin-embedded tissues

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 xylene and maintained at 56°C for five 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 µl of protease buffer and 20 µl of proteinase K 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 µl 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 µl of chloroform were added to the samples and the samples were vortexed for 15 seconds and placed on ice for five 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 eight 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 was added to the samples and the samples were maintained at -20°C [23]. 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 synthesis

CDNA was synthesized using a commercial kit (Fermentas, 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 five minutes and then placed in ice immediately. Afterward, they were kept at 42°C for one hour. In the present study, glyceraldehyde phosphate dehydrogenase (*GAPDH*) was considered as the housekeeping gene because of its permanent expression in most cells and tissues.

Real-time polymerase chain reaction (PCR)

The sequence of specific primers for *c-Myc* and *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 of *c-Myc* and *GAPDH* (as the control at mRNA level). A Step one 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 Company), 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. 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 $\Delta\Delta ct$, and

converted into relative quantity. The present case-control study was conducted after obtaining written informed consent from qualified patients interested in participating in the study.

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

Name		Tm	Amplicon Size
<i>c-Myc</i> F	CTCGGATTCTCTGCTCTCCT	58.72	114 bp
<i>c-Myc</i> R	TCTTGTCCTCCTCAGAGTCG	59.58	
<i>GAPDH</i> F	CCCACACACATGCACTTACC	60	85 bp
<i>GAPDH</i> R	TGCCTGTCCTTCCTAGCTCT	60	

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

Sample Characteristics	Number of Samples	
	PCa (N=38)	BPH (N=38)
Age		
<60 years	10 (26%)	16 (10%)
≥60years	28 (74%)	22 (90%)
PSA Level (ng/ml)	14.31±15.59	9.36±10.23
Gleason Score		
GS 5	1 (2.63%)	
GS 6	6 (15.78%)	
GS 7	26 (68.42%)	
GS 8	4 (10.52%)	-
GS 9	1 (2.63%)	
Pathological Stage		
PT2	24 (63.15%)	
PT3	14 (36.84%)	-

Pca= Prostate cancer; BPH= benign prostatic hyperplasia; PSA= prostate-specific antigen

Statistical analysis

The results of this study were obtained in three replicates. T-tests and correlation analyses were applied to analyze the data. All analyses were performed using Graph Pad6 ver. 6 (La Jolla, California, USA) and SPSS 19.0 (SPSS Inc., Chicago, IL, USA) at a significance level of $p < 0.05$.

Results

Clinical and clinicopathological characteristics of the studied samples

Samples from 38 patients with PCa (mean age: 64.57 ± 8.08 years; range: 48-85 years) and 38 patients with BPH (mean age: 68.5 ± 5.03 years; age range: 54-80 years) were studied in this

research. The summary of clinical-pathological features of the studied samples is presented in table 2. In the patients group, 10 subjects were under 60 and 28 others over 60 years. Gleason scores five-nine were found in 1, 6, 26, 4 and one samples, respectively. Gleason score 7 was recorded in 68.42% of patients.

Quality assessment of the extracted RNA, cDNA synthesis, and *c-Myc* expression

The quantitative and qualitative assessment of the extracted RNA with a spectrophotometer revealed 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 *c-Myc* and *GAPDH* using the real-time PCR device (Step One). The curve confirmed the proper and specific binding of the primers to binding sites on *c-Myc* and *GAPDH*. The presence of only one peak for each gene fragment (at its own melting temperature) confirmed the specificity of the product (Figure 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.

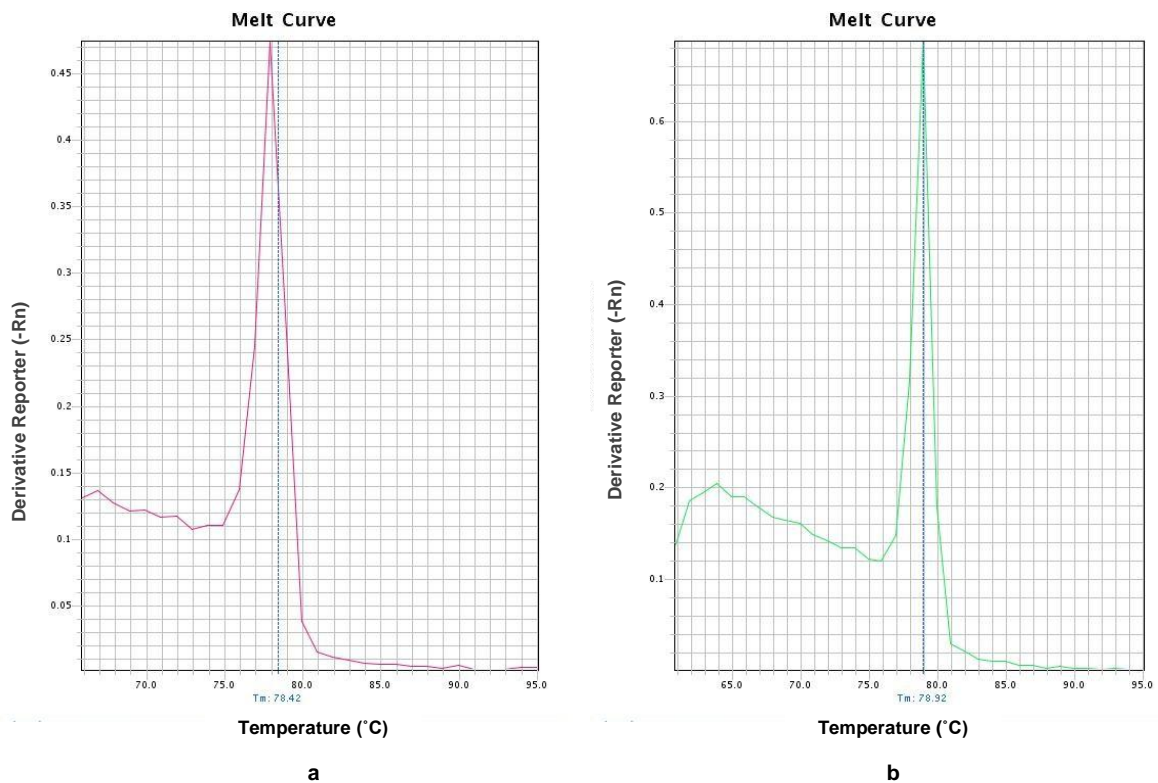


Fig. 1. The melting curve for primers of *c-Myc* (a) and *GAPDH* (b).

Comparison of *c-Myc* expression in BPH and adenocarcinoma samples and the correlation between *c-Myc* expression and clinicopathological data

According to our findings, *c-Myc* expression was 2.35 ± 1.12 higher in adenocarcinoma samples than in BPH ones ($p=0.001$) (Fig. 2). The mean age of the patients with PCa was 64.57 ± 8.08 years. The patients with PCa and BPH had no significant difference in terms of mean age ($p>0.05$).

The adenocarcinoma samples were categorized and scored according to Gleason Grading system (GS). These samples obtained score five-nine on the Gleason grading. Only one patient with GS 5 was observed. PSA level, age and mean *c-Myc* expression was 8.2 ng/ml, 82 years and 1.04-fold, respectively. The mean age of six patients with Gleason score 6 was 67.33 years. The mean *c-Myc* expression and PSA

range of this group were 2.58-fold and 5.9-10.3 ng/ml, respectively. Twenty six patients were grouped in GS 7 (mean age: 62.73 years). The minimum and maximum PSA levels in this group were 1 and 82 ng/ml, respectively. The mean *c-Myc* mRNA expression was 2.37-fold. Four patients had GS 8. Their PSA range mean age, and mean *c-Myc* expression were 7.4-40 ng/ml, 67.40 years and 2.20-fold, respectively. Also one patient was grouped in GS 9. PSA level, age and mean *c-Myc* expression was 11 ng/ml, 79 years and 1.33-fold respectively. There was no correlation between PSA levels and *c-Myc* expression ($r=-0.214$, $p=0.198$). While *c-Myc* expression was 2.31-fold in patients over 60 years and 2.01-fold in those less than 60 years, there was no significant correlation between age and gene expression in the studies samples ($r=-0.1$, $p=0.552$).

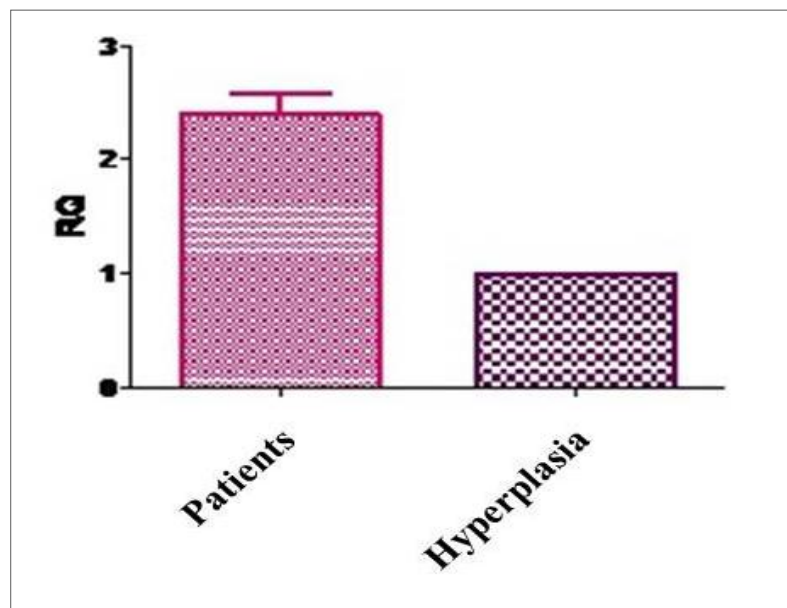


Fig. 2. Comparison of *c-Myc* Expression in Adenocarcinoma and benign prostatic hyperplasia tissues.

The mean PSA levels in adenocarcinoma and BPH samples were 14.31 ± 15.59 and 9.36 ± 10.23 ng/ml, respectively. In cases of PCa, there were no correlations between PSA and other factors, including age ($r=0.189$, $p=0.256$), disease grade ($r=0.076$, $p=0.649$), and Gleason score ($r=0.250$, $p=0.130$). 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 ($r=0.427$; $p=0.019$).

Discussion

The hereditary nature of PCa and the importance of its early diagnosis emphasize the need for its genetic evaluation. While PSA testing is widely incorporated in PCa screening, numerous factors such as obesity, inflammation of the prostate gland, prostate diseases, and even diet can affect serum PSA levels [24, 25]. Researchers have thus been seeking a more appropriate and accurate substitute for PSA over the recent years. Genetic studies on PCa have hence adopted both quantitative approaches (measurement of gene expression at the RNA and protein levels) and qualitative methods (examining chromosome mutations and changes such as translocation, deletion, and duplication at the DNA level). The duplication of 8q24 is a chromosome change leading to PCa [26]. Studies using antibodies for evaluations at the protein level have also confirmed the findings of studies on chromosomes and have suggested increased *c-Myc* expression in patients with PCa [27]. The present study evaluated *c-Myc* expression in

Iranian patients with PCa in order to differentiate between adenocarcinoma and BPH samples. The comparison of *c-Myc* expression in BPH and adenocarcinoma samples in the present study indicated 2.35-fold higher *c-Myc* expression level in PCa than in BPH tissues. The *c-Myc* expression in samples with GS 6 was 2.58-fold higher than that in patients with BPH. Moreover, *c-Myc* expression in samples with GS 7 and GS 8 was respectively 2.37 and 2.20-fold higher than that in BPH group. In another analysis, for comparison of *c-Myc* mRNA expression of adenocarcinoma samples according to GS grade, samples were categorized in three groups (i.e., $GS < 7$, $n=7$; $GS=7$, $n=26$; $GS > 7$, $n=5$). *C-Myc* expression level in $GS < 7$, $GS=7$ and $GS > 7$ was 2.35, 2.1 and 2.2-fold higher than BPH group. A higher significant *c-Myc* expression was observed in three mentioned GS groups compared with the BPH samples. No significant difference of *c-Myc* expressions was observed between the three different GS groups. Fleming et al. used the Northern blot procedure to examine *c-Myc* expression in seven patients with adenocarcinoma and eleven patients with BPH. They found higher *c-Myc* expression in adenocarcinoma samples than in BPH samples. However, there was no correlation between increased gene expression and PSA levels [28]. Chen et al. also found a significant correlation between MYC gene amplification and high Gleason score in patients with PCa [26]. We evaluated a *c-Myc* expression using a strong technique, i.e. real-time PCR. The results demonstrated a higher *c-Myc* expression in

patients with PCa than in those with BPH. Alike the findings of Fleming et al., there was no correlation between increased *c-Myc* expression and PSA levels in the present research. Yuen et al. examined oncogene expression (*c-Myc*, *c-jun*, and *c-fos*) in patients with hepatocellular carcinoma using no anti-cancer drugs before. They reported *c-Myc* expression in 74% of the patients to be higher than that in normal people [29]. Gurel et al. observed higher gene expression in metastatic prostate adenocarcinoma than in prostatic hyperplasia and normal samples [27]. The current study also showed the greatest level of gene expression in PCa samples.

Hawksworth et al. used real-time PCR to compare *c-Myc* expression in patients with BPH and PCa. They reported higher *c-Myc* expression in patients with PCa than in those with BPH. They also found a correlation between increased *c-Myc* expression and elevated serum PSA levels [30]. However, the present study failed to establish a correlation between the mentioned parameters. Similar to the previous research [26-30], the present study suggested *c-Myc* expression to be higher in patients with PCa than in individuals with BPH.

Conclusions

Higher *c-Myc* expression was observed in PCa

samples than in BPH samples. The administration of complementary tests to localize the expression of genetic markers, such as *c-Myc*, seems to facilitate the differentiation between PCa and BPH samples and may be considered as a possible target for anticancer therapy. Moreover, long-term examinations of the patients and their families can tap not only the causes of changes in gene expression, but also the association between *c-Myc* expression and pathological complications of the disease. Furthermore, the application of gene expression analysis methods to identify other genes involved in PCa can also enhance our understanding of the etiology of the disease and facilitate its prevention. *cMyc* expression can be introduced as a prognostic marker for determination of the invasive potential of tumor cells.

Conflict of interest

The authors declare no conflicts of interest.

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