Original Article

Prognostic Value of Promoter Hypermethylation of *Retinoic Acid Receptor Beta (RARB)* and *CDKN2* (*p16/MTS1*) in Prostate Cancer

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ABSTRACT

Objective: The molecular mechanism of prostate cancer is poorly understood. The aim of the study was to investigate the prevalence and prognostic value of promoter hypermethylation of *retinoic acid receptor beta (RARB)* and *p16* among benign prostatic hyperplasia (BPH) and prostate cancer patients.

Methods: In this case-control study, 63 patients were included in three groups; 21 with BPH as the control group, 21 with prostate cancer and good prognostic factors (based on prostate-specific antigen, Gleason score and stage) as good prognosis group, and 21 with prostate cancer and poor prognostic features as poor prognosis group. The prostate biopsy specimen of each individual was examined for hypermethylation of *RARB* and *p16* promoters by methylation specific PCR (MSPCR).

Results: Seven (33.3%) patients with good prognosis and 15 (71.4%) patients with poor prognosis were positive for *RARB* methylation, which were significantly higher than controls (P <0.0001). *p16* promoter methylation was shown in 19.0% and 47.6% patients with good and poor prognosis, respectively. The *RARB* and *p16* promoter methylation in the poor prognosis group was significantly higher than that in the good prognosis group (P =0.02 for *RARB* and P<0.0001 for *p16*).

Conclusion: Hypermethylation of *RARB* and *p16* promoters may predict prognosis in prostate cancer.

Key words: Prostate cancer; CpG island; DNA methylation; *Retinoic acid receptor beta* (*RARB*); *CDKN2 (p16/MTS1)*; Methylation Specific PCR

INTRODUCTION

As the most commonly diagnosed cancer (excluding skin cancer) among men, prostate cancer is a global public health problem. Previous studies showed about 27% of prostate cancer had poor prognosis and prostate cancer is the second leading cause of cancer-related deaths among men in North America and Western/Northern Europe[1-6]. While many prostate cancer patients present with advanced disease, early detection with highly specific and sensitive methods might be one of the main approaches of reducing mortality[5-7].

Currently, prostate-specific antigen (PSA), histological grade [Gleason score (GS)] and stage of disease are widely used to predict the prognosis[8-10], but the tumor behavior is not always predictable using these factors. Genetic mechanisms of malignant changes are the pioneer events during disease process, long before the clinical factors appear[11]. To date, more than 50 genes have been shown to be inactivated by promoter CpG island hypermethylation in prostate cancer[6].

In the recent years, a new group of cancer markers based on the characterization of epigenetic alterations are introduced to evaluate the tumors[12-15]. Investigations on these epigenetic markers in tissue samples are mainly restricted to the evaluation of retrospective series of just a fraction of all prostate tumors. Amongst them, promoter CpG island methylation is mainly emerged as a putative prognostic assessment tool to seek some biological markers for aggressive prostate cancer[5,16-21].

Retinoic acid receptor beta (*RARB,* OMIM: 180220), and *cyclin-dependent kinase inhibitor 2a* (*CDKN2a*, *p16/Cdkn2*^{ink4}*/Mts1,* OMIM: 600160) were widely reported to be hypermethylated in prostate cancer and some suggested their potential as diagnostic and prognostic markers[22-28].

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Given the importance of finding a reliable prognostic marker, we investigated prognostic value of promoter hypermethylation of *RARB* and *p16* genes in Iranian patients with prostate cancer with good and poor prognosis, in comparison to the patients affected with benign prostatic hyperplasia (BPH).

MATERIALS AND METHODS

Patients and Samples

In this case-control study, all participants were enrolled from men referred to the Radiotherapy-Oncology Ward in Imam Hossein Hospital, Urology Ward in Shahid Modarres Hospital, and Shahid Labbafinezhad Hospital due to clinically suspected prostate cancer from 2007 to 2008.

Prostate biopsy specimens were collected by surgery. If the pathologic studies confirmed the diagnosis of prostate cancer, the patient was included in the study. Included patients signed the informed consent form if they were candidates for prostatectomy. A 5 ml blood sample was taken to measure PSA level through routine lab procedures before surgery. Histologic slides from formalin-fixed and paraffin-embedded tissue fragments were reviewed to confirm BPH or prostate cancer or to reassess GS of cancer cases by an expert in prostate diseases at the Department of Pathology, Shahid Labbafinezhad Hospital. Relevant clinical data, such as age, serum PSA level at diagnosis, and clinical stage of disease, were obtained from medical records.

Prostate cancer cases were divided into good and poor prognosis with respect to the serum PSA level, GS, and staging. PSA value more than 9, or GS summation equal or more than 7, or stage III and more, was considered as poor prognosis group, the rest with GS ≤6 and stage II, and PSA <10 were labeled as the good prognosis group.

Methods

The paraffin wax-embedded blocks, consisting of 42 cancerous and 21 BPH without cancer were prepared for methylation specific PCR (MSPCR) in our genetic laboratory to measure the degree of methylation. The genetic laboratory members were not informed of the prognostic situation of patients to which the paraffin blocks belong.

First, the DNA was extracted from tissue samples using the classical method of phenol/chloroform/isoamyl alcohol. Purified DNA samples were subjected to treatment with sodium bisulfite, which reacts with cytosine (C) bases in preference to methylated cytosine (5-mC) bases, facilitating the deamination of C to produce uracil (U) while 5-mC remains unchanged. Consequently, differences in DNA methylation become apparent as differences in DNA sequence. PCR primers specific for target sequences resulting from bisulfate modification of 5-mCpG-containing DNA are used for PCR to detect target methylated CpG island[29,30].

In brief, 40 μl of DNA (2 μg) was denatured at 97°C for 10 min, centrifuged briefly, and chilled on ice. Ten microlitres of 1 mol/L NaOH was then added and the mixture was stored at room temperature for 15 min. Then 500 μl of 3.5 mol/L sodium bisulphate and 1 mmol/L hydroquinone mixture was added to the denatured DNA, and stored at 55°C for 16 h. The treated DNA was purified using Wizard DNA purification resin (DNA clean up kit, Promega, Madison, WI, USA) according to the manufactures' instruction and desulphonated with 0.3 mol/L NaOH at room temperature for 10 min. After adding 2.5 volumes of 100% cold ethanol and a two-thirds volume of 7.5 mol/L ammonium acetate and storing at −20°C for 12 h, the precipitated DNA was centrifuged. After washing in 70% ethanol and drying, DNA was dissolved in 10 mmol/L Tris buffer. This process was performed twice for each sample in order to increase the amount of remaining DNA after bisulfit treatment.

There were 63 paraffin blocks treated with bisulfit before MSPCR. Subsequent to purification of modified DNA, methylation specific amplification was performed for evaluating methylation of *p16* and *RARB* using bisulphate-modified DNA (30–50 ng), primers specific for methylated and non-methylated cytosines (10 pmol each), dNTPs (each at 1 mmol/L), and 1× buffer [16.6 mmol/L $(NH4)_2SO_4$/67 mmol/L Tris/pH 8.8/6.7 mmol/L $MgCl_2$/10 mmol/L β-mercaptoethanol] in a volume of 25 μl.

MSPCR

For each biopsy specimen, 6 PCRs were run (using methylation specific primers, non-methylation specific primers, and housekeeping primers) (although it was not required to use any housekeeping specific primers, it was used just in case that needed further studies). The products of all PCRs (7 μl) were electrophoresed on 2% agarose gels and visualized under UV illumination after staining with ethidium bromide.

The results were reported as positive or negative for *p16* and *RARB* methylation. To confirm the positive results, the procedure was repeated one more time.

Statistical Analysis

To find out any correlation between *p16* and *RARB* methylation and prognosis of prostate cancer, Chi-square (χ^2) and Fisher exact tests were used to examine the results by SPSS software (version 11.5). $P<0.05$ was considered significant.

The whole study protocol was approved by the Ethic committee of Shahid Beheshty University of Medical Sciences.

RESULTS

We investigated the methylation profile of 63 subjects in three groups of controls (BPH), good and poor prognosis prostatic cancer, each consisted of 21 individuals. Data are provided in Tables 1 and 2.

The mean age was 61.5±5.5 in control group, 64.3±5.5 and 61.2±8.4 in good and poor prognosis groups respectively, without any significant difference. The mean PSA value was 6.14±2.48 and 17.77±9.46 in good and poor prognosis groups, respectively.

Table 3 shows *RARB* hypermethylation distribution and the two by two comparisons between the control, good, and poor prognosis groups. There was no *RARB* methylation positive subject in control group. In good prognosis group 33.3% were positive for *RARB* methylation, which was