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Retinoic Acid Receptor β2 (RARβ2): Nonivasive Biomarker for Distinguishing Malignant versus Benign Prostate Lesions from Bodily Fluids

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Rezumat

Receptorul β2 al acidului retinoic (RARβ2): biomarker neinvaziv în diferențierea leziunilor prostatice maligne de cele benigne în lichidele biologice

Alterări ale caracteristicilor promoterului insulelor CpG, au fost asociate cu inhibiția transcripției genelor în numeroase cancere umane, inclusiv în cancerul de prostată (CaP).
Obiective: Scopul acestui studiu a fost acela de a evalua valoarea diagnostică a hipermetilării promoterului genei receptorului acidului retinoic β2 (RARβ2), din ADN-ul genomic obținut din serul pacienților cu CaP şi hipertrofie benignă de prostată (HBP), ca un nou biomarker care poate diferenția leziunile prostatice maligne de cele benigne prin metode neinvazive.
Material şi metode: Hipermetilarea genei RARβ2 a fost investigată din ADN-ul genomic al 91 pacienți cu CaP şi 94 cu HBP, prin metoda cantitativă a metilării specifice reacției de polimerizare în lanț (QMSP).
Rezultate: Hipermetilarea genei RARβ2 a fost detectată la 89 din 91 (92.7%) pacienți cu CaP şi în cazul a 10 din 94 (10.7%) pacienți cu HBP.
Concluzii: RARβ2 reprezintă un nou biomarker tumoral care poate fi utilizat în diferențierea leziunilor prostatice maligne de cele benigne prin metode neinvazive.

Cuvinte cheie: cancer de prostată (CaP); hipertrofie benignă de prostată (HBP); receptorul β2 al acidului retinoic (RARβ2)

Abstract

Alterations in the methylation patterns of promoter CpG islands have been associated with the transcriptional inhibition of genes in many human cancers, including prostate cancer (PCa).
Objectives: The aim of our study was to evaluate the diagnostic value of aberrant promoter hypermethylation of retinoic acid receptor β2 (RARβ2) gene in serum DNA samples from patients with the diagnosis of PCa and benign prostatic hyperplasia (BPH), as a new epigenetic biomarker in distinguishing between malignant and non-malignant lesions.
Materials and methods: Aberrant promoter hypermethylation was investigated in genomic DNA isolated from the serum of 91 patients diagnosed with of PCa and 94 with BPH (control subjects). In order to evaluate the methylation status of the RARβ2 gene we used the quantitative methylation-specific PCR (QMSP) method.
Results: Promoter hypermethylation of RARβ2 gene was detected in serum samples from 89 of 91 (92.86%) patients with PCa, and in 10 of the 94 (10.7%) patients with BPH.
Conclusions: RARβ2 represents a promising molecular biomarker which may be used in discriminating between malignant and benign prostatic diseases by noninvasive methods.

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Key words: prostate cancer (PCa); benign prostatic hyperplasia (BPH); quantitative methylation-specific polymerase chain reaction (QMSP); retinoic acid receptor $\beta 2$ (RAR$\beta 2$)

Introduction

Prostate cancer (PCa) represents an increasing threat throughout the world. As a result of a demographic shift in population, the number of men at risk for developing prostate cancer is growing rapidly. It affects men which are in the 6th decade of life and is associated with increased morbidity and mortality (1). Currently, measurement of serum prostate-specific antigen (PSA) levels the commonly used method to detect men who might have PCa, although it presents limitations in the ability to discriminate between adenocarcinoma and benign lesions. Also, digital rectal examination (DRE) and imagistic investigations are limited in PCa detection in early stages.

The biomarkers found in body fluids present the potential to detect different types of tumors in early stages, or metastases which are spread throughtout the body. In the class of fluid biomarkers we include components in blood, serum, plasma, urine, or other bodily fluids that can reflect the presence of the tumor in the body. They include circulating tumor cells (CTC), or macromolecules such as: lipids, proteins, RNA, microRNA and DNA that originates from the tumor cells (2) Epigenetic changes represent changes in gene expression, which are not caused by alterations in the primary sequence of the nucleotides that compose the gene. It has been demonstrated that, one of the most common epigenetic change and the most frequent molecular alteration in human cancers is DNA hypermethylation (3). Analyses of bodily fluids, such as: whole blood, serum, plasma, or urine of patients with diagnosis of PCa have revealed DNA hypermethylation as a new, noninvasive biomarker for early detection, and recurrence monitoring in PCa (4).

The retinoic acid receptor $\beta 2$ (RAR$\beta 2$) has been demonstrated to be localized on chromosomal region 3p24, and found to harbor a CpG region in its promoter region (5). RAR$\beta 2$ has been recently shown to be expressed in most tissues and to have a function of tumor suppressor gene in many types of malignancies, such as: lung, breast, prostate or ovarian cancers. Recently, it has been demonstrated that RAR$\beta 2$ is hypermethylated in primary human cancers, including prostate (6). Today there is no established pretreatment laboratory test available to distinguish by non-invasive methods between men with localized PCa and those with benign prostatic lesions. In the present study, using quantitative methylation-specific polymerase chain reaction (QMSP) assay, we examined RAR$\beta 2$ hypermethylation in the serum of men with localized PCa and BPH as controls, and found that RAR$\beta 2$ hypermethylation discriminates between neoplastic and non-neoplastic lesions.

Materials and Methods

Patients, Sample Collection and DNA extraction

Blood was prospectively collected from 91 patients with clinically localized prostate adenocarcinoma, who underwent radical prostatectomy at the Department of Urology, Clinical County Emergency Hospital, Timișoara, between January 2008 and January 2010. Non-neoplastic blood samples were obtained from 94 patients with benign prostatic hyperplasia (BPH), which underwent transurethral resection of the prostate, and used as controls. All blood samples have been collected before the surgical interventions. At the time of arrival none of the patients had pelvic lymph node involvement or clinical information of distant metastases. The demographic characteristics of the patients included in our study are presented in *Table 1*.

5 milliliters of blood were drawn and collected in a serum separator tube containing clot activator and gel (Vacutainer, Becton Dickinson, USA). Tubes were inverted 8 times and centrifuged within 2 hours of collection for 10 minutes at 1500 X g. Using ZR Serum DNA (Zymo Research, U.S.A) we extracted DNA from 1 ml serum following the manufacturer's protocol, and stored it at - 80°C until further analysis.

Bisulfite treatment and quantitative methylation-specific polymerase chain reaction (QMSP)

Sodium bisulfite conversion of non-methylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patients serum samples was performed as previously described. We used the bisulfite modified DNA as a template for the fluorescence-based real-time polymerase chain reaction (PCR). The primers and probes have been designed to amplify the bisulfite-converted promoter of the gene of interest. Fluorescence-based real-time PCR assays were carried out in a reaction volume of 25 μL consisting of 0.25 μL of each primer, 12.5 μL qPCR Master Mix (Fermentas, Vilnius, Lithuania), 3 μL of bisulfite-converted DNA, 0.5μL of probe, 8 μL distilled water. PCR was performed in separate wells for each primer/probe set. Each sample was run in triplicate.

Table 1. *Demographic characteristics of patient populations*

	PCa	BPH
Patients, n	91	94
Age, yrs, median (range)	68 (40-74)	64 (50-79)
PSA, ng/ml, median (range)	9.34 (3.11-48.3)	5.63 (0.79-32.5)
Gleason score, median (range)	7(4-9)	
Stage, n (%)		
pT2a	16 (17.58)	
pT2b	37 (40.65)	
pT3a	22 (24.17)	
pT3b	16(17.6)	

Leukocyte DNA collected from healthy individuals was methylated in vitro using bacterial Sss I methyltransferase (New England Bio Labs. Inc., Beverly, MA), and was included in each assay as a positive control (methylated control). All amplifications were carried out in a 96 -well plates (Applied Biosystems, N.J) on a 7500 Real-Time PCR system (Applied Biosystems, N.J) under the following conditions: 95°C for 10 minutes followed by 45 cycles of 95°C for 15 minutes and 60°C for 1 minute. (*Fig. 1*)

Ethics

The study was conducted in accordance with The World Medical Association Declaration of Helsinki 2008, statements and written informed consent was obtained from each patient by signature on the specific form provided by the Ethical Committee.

Statistical Analysis

Differences in methylation frequencies among PCa and BPH patients were examined using the χ^2 test. To compare age and serum PSA levels between patients with prostate adenocarcinoma and BPH, we used the Mann-Whitney U test. The correlations between the methylation levels and age, serum PSA, Gleason score and pathological stage were determined using the Spearmann's correlation coefficient. The receiver operating characteristic (ROC) curve and the area under the curve (AUC) were calculated for RARβ2 gene to distinguish patients with PCa from BPH patients (7).

Results

In this study, using the QMSP analysis we measured the RARβ2 methylation levels in serum samples from 91 patients with clinically localized prostate adenocarcinoma who underwent radical prostatectomy, and 94 patients with histologically BPH who underwent transurethral resection of the prostate. The RARβ2 methylation frequencies in PCa and BPH were 97.8% and 10.6%, respectively. The sensitivity and specificity of RARβ2 methylation levels in discriminating PCa patients from BPH, were determined by receiver operating curve (ROC) analysis. RARβ2 gene had a sensitivity of 98%, a specificity of 89% and yielded an area under the curve (AUC) of 0.936 (95 CI 0.895 to 0.977; $p < 0.001$), as presented in *Fig. 2*. Furthermore, significant correlations were found between age and serum PSA levels and methylation levels of RARβ2 gene in PCa and BPH patients. Using the Spearman rank-correlation, a significant correlation was found between serum PSA levels and RARβ2 methylation in PCa patients, but not in BPH patients ($r = 0.831$; $p < 0.001$).

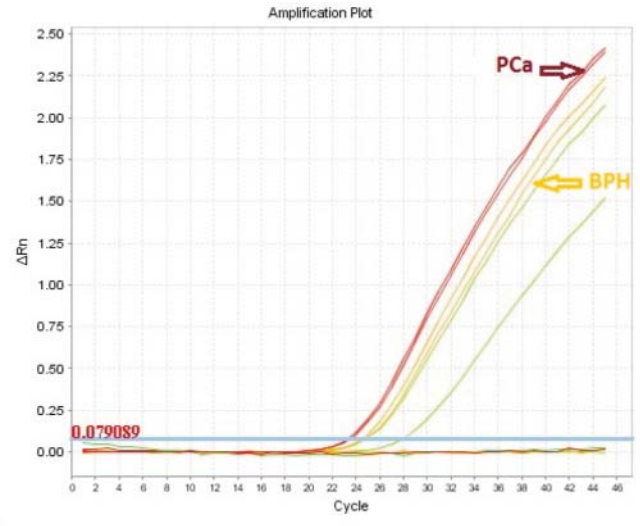

Figure 1. *Quantitative methylation-specific polymerase chain reaction (QMSP) amplification plots for RARβ2 from PCa (91 cases) and BPH (94 cases). The figure shows that PCa cases present a stronger amplification of the target methylation gene, compared to BPH cases*

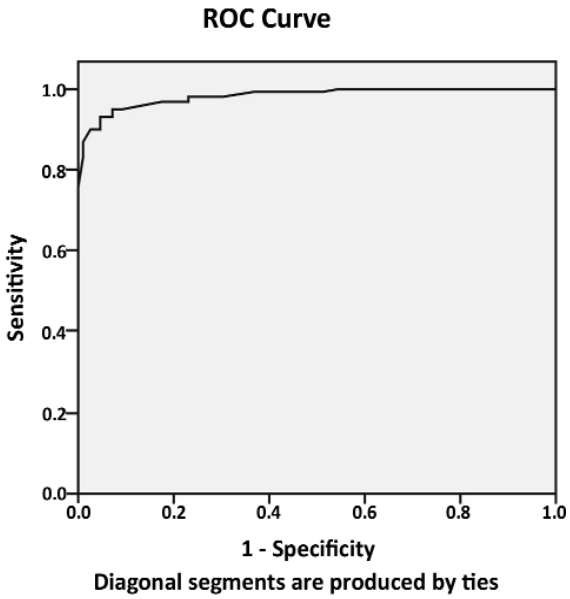

Figure 2. *Receiver operating curve (ROC) analysis of GSTP1 gene hypermethylation levels in prostate cancer (PCa) patients*

Discussions

Inactivation of some tumor suppressor genes (TSG), classified in class I, which is mediated by genetic mechanisms such as loss of function mutations, gene rearrangements, gene deletions or loss of heterozygosity. Class II of TSG includes those in which the wild type remains intact, but its loss of functions results due to mutations or deletions, which occur in different genes (8). Methylation of the promoter regions have been associated with suppression of gene transcription and gene expression. In PCa, over the last years, DNA methylation has received attention because of the increase in number of new TSG, which were found to be regulated by this mechanism. Due to the inherent stability of of the DNA molecule compared

to the RNA molecule, DNA methylation is considered a powerful biomarker in PCa detection (9).

In PCa, different degrees of hypermethylation have been reported for many genes, such as: androgen receptor, E-cadherin, glutathione S-transferase P1 (GSTP1), retinoic acid receptor $\beta 2$ (RAR$\beta 2$), Ras association domain family 1A (RASSF1A), caveolin-1. Methylations of CpG islands in some of these genes (GSTP1, RASSF1A and RAR$\beta 2$), have been found at early stages of carcinogenesis such as premalignant lesion prostatic intraepithelial neoplasia (PIN), whereas hypermethylation levels of other genes, have been found only in prostate adenocarcinoma (10). In one of their studies using QMSP assay, Jeronimo et al., found increased RAR$\beta 2$ hypermethylation levels in 97.5% men with PCa , 94.7% with high-grade PIN (HGPIN) and in 23.3% BPH men (11).

Also, previous studies using the conventional methylation-specific PCR (MSP), Yamanaka et al. found significant differences between RAR$\beta 2$ methylation frequencies in PCa and BPH. These differences could be due to the fact that we used different PCR conditions, and the QMSP methodology, that is likely to be more sensitive than conventional MSP.

De Marzo et al., have revealed that BPH displays intermediate methylation frequencies between nonmalignant and malignant lesions for several genes .All these observations reveal a progressive acquisition of epigenetic and genetic events during prostate carcinogenesis. The presence of RAR$\beta 2$ methylation levels in 10 (10.6%) of the 94 BPH patients is not surprising, because in this case we cannot exclude the idea that they might harbor an occult microscopic foci of PCa in the context of BPH, that has been omitted by prostatic biopsy. We found that RAR$\beta 2$ methylation levels correlated with the pathological tumor stage. In PCa patients with pathologic T3 tumor stage we observed significantly increased methylation levels of gene RAR$\beta 2$, when compared with pathologic T2 tumor stage ($p < 0.001$; Mann-Whitney test). Also, according to the Spearman rank-correlation test, a significant correlation between GS and the pathological stage exists ($r = 0.749$; $p < 0.001$). The results obtained results suggest a role for increasing methylation levels during prostate carcinogenesis and therefore appear to represent an important link between methylation levels of a gene and clinical-pathological parameters.

The results obtained by us should also be considered in further studies regarding the role of circulating methylated DNA as therapeutic targets. Previous studies have shown that all-trans-retinoic acid (ATRA) was effective in controlling symptomatic PCa patients during Phase II of a clinical trial, which consisted in intermittenly administering of ATRA for hormone refractory PCa (12). Also, there is an established relationship between RA and PCa. Expression of RAR has been shown to be elevated in androgen receptor (AR) positive cells and a good correlation between RAR levels, tumor grade, and proliferation in clinical primary prostate carcinoma was revealed (13,14). On the other hand, detection of DNA hypermethylation in bodily fluids is technically possible and, it might aid in improving current screening and diagnosis methods of PCa and in establishing indications regarding the surveillance or repeat biopsy (15,16).

The limitations of the study include the small number of patients and the lack of long-term follow-up. Further studies of our group in the area of noninvasive detection of PCa, will include the disease recurrence monitoring from the preoperative serum samples of men following radical prostatectomy, using a panel of biomarkers by QMSP analysis.

Conclusions

In summary, the evaluation of serum samples obtained from PCa patients presents some advantages because, unlike tissue biopsy or imagistics, blood sampling is a minimally invasive method which does not present the risk of morbidity, and can be repeated to monitor the changes which occur during disease progression or to detect the recurrence of the disease.

In our study we have demonstrated that RAR$\beta 2$ gene can be used as a novel tumor biomarker to aid to current investigation methods for early PCa detection by noninvasive methods.

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