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

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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 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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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

<table>
<thead>
<tr>
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<th>PCa</th>
<th>BPH</th>
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<tbody>
<tr>
<td>Patients, n</td>
<td>91</td>
<td>94</td>
</tr>
<tr>
<td>Age, yrs, median</td>
<td>68 (40-74)</td>
<td>64 (50-79)</td>
</tr>
<tr>
<td>(range)</td>
<td></td>
<td></td>
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<tr>
<td>PSA, ng/ml, median</td>
<td>9.34 (3.11-48.3)</td>
<td>5.63 (0.79-32.5)</td>
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<tr>
<td>(range)</td>
<td></td>
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<tr>
<td>Gleason score, median</td>
<td>7 (4-9)</td>
<td></td>
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<tr>
<td>(range)</td>
<td></td>
<td></td>
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<tr>
<td>Stage, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2a</td>
<td>16 (7.38)</td>
<td></td>
</tr>
<tr>
<td>pT2b</td>
<td>37 (40.65)</td>
<td></td>
</tr>
<tr>
<td>pT3a</td>
<td>22 (24.17)</td>
<td></td>
</tr>
<tr>
<td>pT3b</td>
<td>16 (17.61)</td>
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</tbody>
</table>
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: 95ºC for 10 minutes followed by 45 cycles of 95ºC for 15 minutes and 60ºC 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 χ² 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).

*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 β2 (RARβ2), Ras association domain family 1A (RASSF1A), caveolin-1. Methylation of CpG islands in some of these genes (GSTP1, RASSF1A and RARβ2), 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 RARβ2 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 RARβ2 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 accumulation of epigenetic and genetic events during prostate carcinogenesis. The presence of RARβ2 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 RARβ2 methylation levels correlated with the pathological tumor stage. In PCa patients with pathologic T3 tumor stage we observed significantly increased methylation levels of gene RARβ2, 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 intermittently 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 RARβ2 gene can be used as a novel tumor biomarker to aid to current investigation methods for early PCa detection by noninvasive methods.

References


