Featured Article

Quantitative RARβ2 Hypermethylation: A Promising Prostate Cancer Marker

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ABSTRACT

Retinoic acid receptor β2 (RARβ2) is a tumor suppressor gene frequently hypermethylated in several human neoplasms. To further characterize this epigenetic alteration in prostate cancer progression, we examined tumor tissue from 118 patients with prostate carcinoma (PCa), 38 paired high-grade prostatic intraepithelial neoplasias (HGPIN), and non-neoplastic prostate tissue from 30 patients with benign prostate hyperplasia (BPH), using quantitative methylation-specific PCR. We found RARβ2 hypermethylation in 97.5% of PCa, 94.7% of HGPIN, and 23.3% of BPH. Methylation levels were significantly higher in PCa compared with HGPIN and BPH (P < 0.00001). By establishing an empiric cutoff value, we were able to discriminate between neoplastic and non-neoplastic tissue, with 94.9% sensitivity and 100% specificity. Moreover, RARβ2 methylation levels correlated with higher pathological stage (r = 0.30, P = 0.0009). This quantitative assay represents a novel and promising molecular marker that may augment current approaches for prostate cancer detection.

INTRODUCTION

Prostate cancer is the most commonly diagnosed noncutaneous neoplasia in Western countries. The disease predominantly affects men after the 6th decade of life and is associated with considerable morbidity and mortality (1). Curative treatment entails radical prostatectomy or radiotherapy, and the best outcome is seen in patients with the earliest stage disease (2, 3). Patients with locally advanced or systemic disease carry a poor long-term prognosis because of the notable lack of curative therapy (4). Serum prostate-specific antigen (PSA) level measurement is currently the most commonly used detection method, although its effectiveness is substantially impaired by the inability to discriminate between carcinoma and benign lesions. Likewise, digital rectal examination and imaging techniques are of limited usefulness in early (i.e., organ confined) disease detection. The emergence of new molecular tests is expected to improve the identification of neoplastic cells and increment diagnosis of prostate malignancy in the earliest stages of disease.

We demonstrated previously that quantitation of glutathione S-transferase P1 (GSTP1) promoter methylation is able to distinguish prostate carcinoma (PCa) from benign prostate lesions, improving standard histological diagnosis in sextant biopsies (5–7). This quantitative assay permitted the detection of approximately 80–90% of prostate adenocarcinomas with perfect specificity. The adduction of other molecular markers may help to further perfect the latter assay. The retinoic acid receptor β2 (RARβ2) is expressed in most tissues and has been shown to function as a tumor suppressor gene in lung, breast, and gynecological neoplasia (8–10). RARβ2 was mapped to chromosomal region 3p24 and found to harbor a CpG-rich region in its promoter (11). Moreover, RARβ2 was shown recently to be hypermethylated in several primary human neoplasms, including prostate (12–15). Nonetheless, RARβ2-methylated alleles were also found in 3 to 17% of nonmalignant prostate tissues by conventional methylation-specific PCR (MSP; Ref. 14, 15).

In the present study, RARβ2 was found to be hypermethylated in the vast majority of prostate adenocarcinomas, high-grade prostatic intraepithelial neoplasia (HGPIN), and a non-negligible number of benign prostate hyperplasia (BPH) lesions. Strikingly, we were able to clearly discriminate between neoplastic and non-neoplastic tissue using a quantitative methylation PCR (Q MSP) assay. Thus, quantitation of RARβ2 methylation represents an additional promising molecular marker for prostate cancer detection.

MATERIALS AND METHODS

Patients, Sample Collection, and DNA Extraction. Primary tumors from 118 patients with clinically localized prostate adenocarcinoma (stages T1c and T2, according to the Tumor-Node-Metastasis staging system; Ref. 16), consecutively diagnosed and primarily treated with radical prostatectomy at the...
Portuguese Oncology Institute–Porto, Portugal, were prospectively collected. Paired HGPIN lesions were identified in 38 of these specimens and were also collected for further analysis. Non-neoplastic prostate tissue samples were further obtained from 30 randomly selected patients with BPH that underwent transurethral resection of the prostate and used as controls. All tissue specimens were promptly frozen immediately after surgery and stored at −80°C for further analysis. Five-μm thick sections were cut from frozen tissue fragments to identify areas of HGPIN and PCa in the radical prostatectomy specimens and to identify BPH in the control tissue samples. These tissues were then carefully microdissected from 12-μm thick sections for cellular enrichment. DNA was extracted from all samples with phenol/chloroform and precipitated with ethanol (17).

Histological slides from formalin-fixed, paraffin-embedded tissue fragments were obtained from the same surgical specimen and assessed for Gleason grade (18) and Tumor-Node-Metastasis stage (16). Relevant clinical data were abstracted from the clinical records. The institutional review board of Portuguese Oncology Institute–Porto, approved these studies.

**Bisulfite Treatment and QMSP.** Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patient tissue samples was performed as described previously (19). Four μg of DNA were used for the chemical treatment. DNA samples were then purified using the Wizard purification resin (Promega, Madison, WI), treated again with sodium hydroxide, precipitated with ethanol, and resuspended in 200 μl of water and stored at −80°C.

The modified DNA was used as a template for real-time fluorogenic MSP. The primers and probes used for the target gene (RARβ2) and the internal reference gene (β actin, ACTB) were as follows, respectively: sense, 5′-CGAGACGCGAGC-GATCC-3′; antisense, 5′-CAAACTTACCTGACCAATCC-AACC-3′; and probe, 6-carboxyfluorescein-5′-TCGGAACG-TATTGCGAGGTTCATTGAACTTT-3′-6-carboxyteretetra-methylrhodamine and sense, 5′-TGGTATGAGGAGGTTT-TAGTAAGT-3′; antisense, 5′-AACCAATAAACCTACTC-CTCCCTTAA-3′; and probe, 6-carboxyfluorescein-5′-ACC-ACCCCAACACACAAATAACAAACACA-3′-6-carboxyteretetra-methylrhodamine. To determine the relative levels of methylated promotor DNA in each sample, the values of the target gene were compared with the values of the internal reference gene to obtain a ratio that was then multiplied by 1000 for easier tabulation (RARβ2/ACTB × 1000).

Fluorogenic quantitative MSP assays were carried out in a reaction volume of 20 μl in 384-well plates in an Applied Biosystems 7900 Sequence Detector (Perkin-Elmer, Foster City, CA). PCR was performed in separate wells for each primer/probe set, and each sample was run in triplicate. The final reaction mixture consisted of 600 nM each primer (Invitrogen, Carlsbad, CA); 200 nM probe (Applied Biosystems, Foster City, CA); 0.75 unit of platinum Taq polymerase (Invitrogen); 200 μM each of dATP, dCTP, dGTP, and dTTP; 16.6 μM ammonium sulfate; 67 μM Trizma; 6.7 μM magnesium chloride (2.5 mM for p16); 10 μM mercaptoethanol; 0.1% DMSO, and 3 μl of bisulfite-converted genomic DNA. PCR was performed using the following conditions: 95°C for 2 min, followed by 50 cycles at 95°C for 15 s and 60°C for 1 min. Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the calibration curve on each plate. Leukocyte DNA collected from healthy individuals was used as a negative control. The same leukocyte DNA was methylated in vitro with SssI bacterial methyltransferase (New England Biolabs Inc., Beverly, MA) and used as positive control for all studied genes.

**Statistical Analysis.** The median and interquartile range of the methylation ratios for each group of samples was determined and analyzed using nonparametric tests (i.e., the Kruskal-Wallis one-way ANOVA), followed by the Bonferroni-adjusted Mann-Whitney U test when appropriate. For this comparison test among three groups of tissue samples, the nonadjusted statistical level of significance of P < 0.05 corresponds to a Bonferroni-adjusted statistical significance of P < 0.0167. Differences in methylation frequencies among PCa, HGPIN, and BPH were examined using the χ² or Fisher’s exact test, as appropriate. The Mann-Whitney U test was used to compare age and PSA levels between patients with BPH or prostate adenocarcinoma. The correlations between the methylation ratios on the one hand, and age, PSA level, Gleason score, and pathological stage, on the other, were determined by calculating a Spearman’s correlation coefficient. All statistical tests were two-sided. Statistical analyses were performed using a computer-assisted program (Statistica for Windows, version 6.0, StatSoft, Tulsa, OK).

**RESULTS AND DISCUSSION**

We examined the frequency and distribution of RARβ2 methylation levels in 118 patients with clinically localized prostate adenocarcinoma and 30 patients with benign prostatic hyperplasia (Table 1). Additionally, we also tested the same target gene by QMSP in paired 38 HGPIN lesions collected from the same 118 radical prostatectomy specimens. The primers and probe were designed to include a CpG island in the RARβ2 promoter, the hypermethylation of which was shown to be correlated with a lack of protein expression (12). The RARβ2 methylation frequencies in PCa, HGPIN, and BPH were 97.5%, 94.7%, and 23.3%, respectively (Table 2). Statistical analysis

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Demographic characteristics of patient populations</th>
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<tbody>
<tr>
<td></td>
<td>PCa</td>
</tr>
<tr>
<td>Patients, n</td>
<td>118</td>
</tr>
<tr>
<td>Age, yrs, median (range)</td>
<td>64 (40–74)</td>
</tr>
<tr>
<td>PSA, ng/ml, median (range)</td>
<td>9.34 (3.11–48.3)</td>
</tr>
<tr>
<td>Gleason score, median (range)</td>
<td>7 (4–9)</td>
</tr>
<tr>
<td>Stage, n (%)</td>
<td>NA</td>
</tr>
</tbody>
</table>

PCA, prostate carcinoma; BPH, benign prostatic hyperplasia; PSA, prostate-specific antigen; NA, not applicable.

\(^{a}\) P = 0.0022.

\(^{b}\) P = 0.0018 (Mann-Whitney U test).
showed that PCa and HGPIN frequencies differed significantly from BPH ($P < 0.00001$) but not between PCa and HGPIN. Previous studies based on conventional MSP also found significant differences in $RAR\beta$2 methylation frequencies between malignant and nonmalignant prostate tissues (14, 15). However, the reported values were generally lower than ours, ranging from 53 to 78% in PCa, 20% for HGPIN, and 0 to 3% in BPH (14, 15). Although these dissimilarities may reflect differences in the patient population, we used different PCR conditions and a quantitative methodology that under certain conditions is likely to be more sensitive than conventional MSP. Moreover, $RAR\beta$2 mRNA and protein expression was reported to be frequently decreased or absent in prostate cancer glands and nonbasal cells of HGPIN (20, 21). Because promoter methylation is a widely recognized mechanism for gene silencing, this observation is consistent with the high frequency of $RAR\beta$2 methylation in PCa and HGPIN demonstrated in our study.

Importantly, $RAR\beta$2 methylation levels (Table 2; Figs. 1 and 2) were found to be significantly different among the three groups of lesions (Kruskall-Wallis test, $P < 0.00001$). Using the Mann-Whitney $U$ test with Bonferroni’s correction, we found the methylation ratios to statistically differ between PCa and HGPIN ($P = 0.00002$), PCa and BPH ($P < 0.00001$), and HGPIN and BPH ($P < 0.00001$). This finding parallels our previous observations on $GSTP1$ methylation in the same type of prostate lesions, although the frequencies of $RAR\beta$2 methylation exceed those reported for $GSTP1$ in PCa and HGPIN (5). Interestingly, all paired PCa of methylated HGPIN lesions (i.e., collected from the same radical prostatectomy specimen) were also consistently methylated. Moreover, we and others have shown that HGPIN displays intermediate methylation frequencies between nonmalignant prostate tissue and invasive adenocarcinoma for several genes (15, 22, 23). These observations are indicative of a progressive acquisition of epigenetic events in prostate carcinogenesis, in addition to the more common accumulation of genetic alterations (24).

On the basis of the high frequency of $RAR\beta$2 methylation in PCa and the significant difference in methylation levels between PCa and BPH, we further investigated whether $RAR\beta$2 methylation quantitation might prove useful for prostate cancer molecular detection. Because all BPH lesions displayed methylation ratios $< 1$ (Fig. 2), we set this empirical value as the cutoff to exclude false-positive samples. Using this cutoff value, the sensitivity of prostate cancer detection by QMSP was 94.9%, because only 6 of 118 PCa displayed $RAR\beta$2 methylation levels $< 1$. Because all radical prostatectomy specimens harbored adenocarcinoma, the true specificity, positive and negative predictive values could not be determined. However, these parameters could be estimated considering the methylation levels observed in the BPH samples. Accordingly, the estimated specificity and positive predictive value of the assay were 100%, and the negative predictive value was 83.3%. Hence, $RAR\beta$2 methylation quantitation compares favorably with the molecular detection rate of prostate carcinoma obtained with the quantitative $GSTP1$ methylation assay (5). However, these results need validation in a large series of prospectively collected biopsy samples to further assess the proficiency of this molecular assay, as done for $GSTP1$ (6, 7).

A possible drawback for the use of promoter methylation levels in the detection of prostate malignancy is the overlapping

### Table 2 Number of positive cases and distribution of methylation levels ($RAR\beta$2/$ACTB \times 1000$) among the different tissue samples

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>n (%)</th>
<th>Median (IQR*)</th>
</tr>
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<tbody>
<tr>
<td>BPH</td>
<td>30</td>
<td>7 (23.3)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>HGPIN</td>
<td>38</td>
<td>36 (94.7)</td>
<td>87.6 (19.3-189.32)</td>
</tr>
<tr>
<td>PCa</td>
<td>118</td>
<td>115 (97.5)</td>
<td>234.7 (81.6-407.06)</td>
</tr>
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* IQR, interquartile range; BPH, benign prostatic hyperplasia; HGPIN, high-grade prostatic intraepithelial neoplasia; PCa, prostate carcinoma.

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**Fig. 1** Illustrative QMSP amplification plots for $RAR\beta$2 from PCa (case 72), HGPIN (case 8), and BPH (case 21) tissues. PCa and HGPIN cases show stronger amplification of the target methylated gene compared to BPH. The $RAR\beta$2/$ACTB$ ratios were determined using the cycle number where fluorescence per reaction crossed the threshold (Ct, thick line), which is set to the geometrical phase of PCR amplification above background. $\Delta Rn$ is defined as the cycle-to-cycle change in the reporter fluorescence signal normalized to a passive reference fluorescence signal (log scale). QMSP, quantitative methylation PCR; $RAR\beta$2, retinoic acid receptor $\beta$2; PCa, prostate carcinoma; HGPIN, high-grade prostatic intraepithelial neoplasia; BPH, benign prostatic hyperplasia; Ct, threshold cycle.
values observed for HGPIN and adenocarcinoma (Fig. 2). Although the median methylation levels differ significantly between HGPIN and PCa, if a cutoff value was assigned to distinguish HGPIN from carcinoma with high specificity, the detection rate would drastically decrease and would cease to be of practical use. However, just as QMSP of GSTP1 was shown to augment histopathological assessment (6, 7), the distinction between HGPIN and PCa would not likely be a critical issue. HGPIN alone does not increase serum PSA and, consequently, is not a major reason of subsequent prostate biopsy (25). Moreover, the isolated finding of HGPIN in a prostate biopsy is reportedly low, varying from 0.3 to 2.3% (26). Finally, the finding of HGPIN on needle biopsy is indicative of significant risk for prostate cancer and up to 75% of patients with this finding will show prostate cancer on repeat biopsy (27). Therefore, on the rare occasion that an elevated RARβ2 methylation level is attributable to HGPIN and not PCa in a prostate biopsy, it would be very likely that the patient harbored a synchronous prostate cancer.

Strikingly, we found that RARβ2 methylation levels correlated with pathological tumor stage \((r = 0.30, P = 0.0099)\) but not with Gleason score. Furthermore, no correlation was found between age or serum PSA levels and methylation levels, neither for PCa nor BPH patients. These findings suggest an independent role for increasing methylation levels in prostate cancer progression and therefore represent an important link between methylation levels at a particular gene and standard clinicopathological parameters. This observation may also have important implications for prostate cancer detection and management. Although a high RARβ2 methylation level detected in prostate biopsy may mark clinically relevant disease, it could also be incorporated in predictive models for preoperative prostate cancer staging.

The present results might also have important implications for the use of retinoid-based therapeutics in prostate cancer. Previous studies have shown that all-trans-retinoic acid fails to abrogate cell growth in some strains of LNCaP, a hormone-sensitive prostate cancer cell line with a methylated RARβ2 promoter (28). Moreover, all-trans-retinoic acid was marginally effective in controlling symptomatic prostate cancer patients in Phase II clinical trials (29). Interestingly, the combined treatment with all-trans-retinoic acid and a demethylating agent was shown to induce re-expression of the RARβ2 gene in breast cancer cell lines (13) and also to produce a synergistic anti-neoplastic effect on colon cancer cell lines (30). Hence, it is tempting to speculate whether the combined use of these two agents would not only prove beneficial for prostate cancer treatment but also for chemoprevention because of the prevalent RARβ2 promoter methylation in HGPIN.

We demonstrated that RARβ2 methylation levels are significantly different among PCa, HGPIN, and BPH. The use of this quantitative assay may augment the detection rate of prostate cancer in tissue biopsies, alone or in combination with GSTP1. Furthermore, QMSP for RARβ2 may provide clinically relevant information for prognosis and retinoid-based chemoprevention or treatment, allowing for accurate selection of patients that might benefit from endogenous RARβ2 reactivation and therapy with retinoids (31).

REFERENCES
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