MT1G Hypermethylation Is Associated with Higher Tumor Stage in Prostate Cancer

Rui Henrique,1,2 Carmen Jerónimo,1,3,5 Mohammad O. Hoque,1 Shuji Nomoto,1 André L. Carvalho,1 Vera L. Costa,3 Jorge Oliveira,4 Manuel R. Teixeira,3 Carlos Lopes,2 and David Sidransky1

1Department of Otolaryngology–Head and Neck Surgery, Head and Neck Cancer Research Division, Johns Hopkins University School of Medicine, Baltimore, Maryland; Departments of 2Pathology, 3Genetics, and 4Urology, Portuguese Oncology Institute; and 5Fernando Pessoa University School of Health Sciences, Porto, Portugal

Abstract

Purpose: Zinc is involved in several physiologic processes, including cell growth and proliferation. Although in normal prostate tissue zinc levels are high, there is a marked decrease in prostate cancer. Metallothioneins control the bioavailability of zinc and one isoform, MT1G, was reported down-regulated in prostate cancer. Here, we investigated whether promoter methylation might cause MT1G silencing in prostate cancer.

Patients and Methods: The MT1G promoter was assessed by quantitative methylation-specific PCR on prospectively collected tissue samples from 121 patients with prostate cancer, 39 paired high-grade prostatic intraepithelial neoplasias (HGPIN), 29 patients with benign prostatic hyperplasia, 13 normal prostate tissue samples from cystoprostatectomy specimens, and prostate cancer cell lines. The methylation levels were calculated and were correlated with clinical and pathologic variables. Reverse transcription-PCR was done in cell lines to assess MT1G mRNA expression before and after demethylating treatment.

Results: MT1G promoter hypermethylation was found in 29 of 121 prostate cancer, 5 of 39 HGPIN, 3 of 29 benign prostatic hyperplasia, and 0 of 13 normal prostate tissue samples. No significant differences in methylation frequencies or levels were found (P = 0.057, for both). Methylation levels were found to correlate with tumor stage but not with Gleason grade. MT1G hypermethylation was more frequent in prostate cancer that spread beyond the prostate capsule. All prostate cancer cell lines tested showed MT1G promoter methylation, but no differences in expression were apparent after demethylation.

Conclusions: Our findings suggest that MT1G promoter methylation is associated with tumor aggressiveness in prostate cancer and it might be a marker of locally advanced disease. (Cancer Epidemiol Biomarkers Prev 2005;14(5):1274–8)

Introduction

Zinc is an essential trace element as a component of several metalloenzymes involved in critical physiologic processes, including cell growth and proliferation, osteogenesis, immunity, and antioxidant activity (1). In a normal prostate gland, zinc levels are high and heterogeneously distributed (2). Because these levels are significantly reduced in malignant prostate tissue (2, 3), a putative prostate cancer–protective effect has been credited to zinc supplements, although no definitive evidence has been provided thus far (1). Indeed, an association between supplementary doses of zinc over 100 mg/d and increased risk for advanced prostate cancer has been recently reported (4). The bioavailability of zinc is controlled by metallothioneins, a class of low molecular weight proteins with metal-binding and antioxidant properties (5). Human metallothioneins are encoded by a family of genes located at chromosome 16q13 (6, 7) and some of the isoforms seem expressed in an organ-dependent manner (8). In several human cancers, metallothionein expression was found to correlate with cell proliferation, tumor progression, and drug resistance (9). In prostate cancer, an association between tumor grade and expression of metallothioneins has been reported, using immunohistochemical analysis (10). However, the antibody used cross-reacts with different isoforms not allowing for a discriminative expression analysis. Interestingly, the gene transcribing for one of those isoforms, MT1G, was found down-regulated in high-grade (Gleason grade 4 and 5) prostate adenocarcinomas (11), but the mechanism of gene silencing remains elusive. Remarkably, in papillary thyroid carcinoma, MT1G down-regulation was reported mediated by promoter methylation (12). Because hypermethylation of cancer-related genes is a common event in prostate cancer (13, 14), we hypothesized that this epigenetic alteration might be responsible for MT1G down-regulation in prostate adenocarcinoma. Herein, we sought to characterize quantitatively the promoter methylation status of MT1G in tissue specimens from primary prostate cancer, paired high-grade prostatic intraepithelial neoplasia (HGPIN) lesions, benign prostatic hyperplasia (BPH), and normal prostate tissue. Relationships between methylation levels and frequencies and clinicopathologic variables were further assessed. Additionally, prostate cancer cell lines were used to test whether MT1G promoter methylation was associated with altered expression.

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Note: R. Henrique and C. Jerónimo contributed equally to this study and should be regarded as joint first authors.

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Requests for reprints: Carmen Jerónimo, Department of Genetics, Portuguese Oncology Institute, Porto, Rua Dr. Antonio Bernardino Almeida, 4200-072 Porto, Portugal. Phone: 351-225/89400 ext. 5602; Fax: 351-225/894016. E-mail: cjeronimo@hotmail.com

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Materials and Methods

Patients, Sample Collection, and DNA Extraction. Primary tumors from 121 patients with clinically localized prostate adenocarcinoma (stages T1c and T2, according to the tumor-node-metastasis staging system; ref. 15), consecutively diagnosed and primarily treated with radical prostatectomy at the Portuguese Oncology Institute, Porto, Portugal, were prospectively collected. In 39 radical prostatectomy specimens with prostate cancer, a HGPIN lesion was identified and collected for further analysis. For control purposes, nonneoplastic prostate tissue samples were obtained from 29 randomly selected patients with BPH that underwent transurethral resection of the prostate and also from 13 prostatectomy tissue samples of bladder cancer patients (normal prostate tissue). All tissue specimens were promptly frozen immediately after surgery and stored at −80°C for further analysis. Five-micrometer-thick sections were cut and stained for the identification of the areas of prostate cancer (i.e., the index or dominant tumor), HGPIN, BPH, and normal tissue. Then, the tissue block was trimmed to maximize the yield of target cells (>70% of target cells). Subsequently, an average of fifty micrometer-thick sections were cut and every fifth section was stained to ensure a uniform percentage of target cells and to exclude contamination from neoplastic cells in normal and BPH tissue samples. DNA was extracted from all samples with phenol/chloroform and precipitated with ethanol (16).

Histologic slides from formalin-fixed, paraffin-embedded tissue fragments were obtained from the same surgical specimens and assessed for Gleason grade (17) and tumor-node-metastasis stage (15) by two pathologists (R.H. and C.L.) blinded to clinical and methylation analysis data. Relevant specimens and assessed for Gleason grade (17) and tumor-node-metastasis stage (15) by two pathologists (R.H. and C.L.) blinded to clinical and methylation analysis data. Relevant clinical data were abstracted from the clinical records. The Institutional Review Board of Portuguese Oncology Institute, Porto approved these studies and permission to test these samples without identifiers was also granted by exemption from the Institutional Review Board of Johns Hopkins University.

Cell Culture Conditions and Demethylating Treatment. Human prostate cancer cell lines LNCaP, DU-145, PC-3, and 22RV1 were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum for isolation of DNA and RNA. The cells were split to low density (5 × 10³ per T-25 flask) 12 to 24 hours before treatment. Then, cells were treated at days 1 and 4 with 5 μmol/L 5-aza-2′-deoxycytidine (Sigma, St. Louis, MO) from 100 μmol/L of 50% acetic acid–dissolved stock. Cells were harvested at day 5 and processed for DNA and RNA extraction.

Bisulfite Treatment and Quantitative Methylation-Specific PCR. Sodium bisulfite conversion of unmethylated (but not methylated) cytosine residues to uracil of genomic DNA obtained from patient tissue samples and cell lines was done as described previously (18). Four micrograms 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, resuspended in 200 μL of water, and stored at −80°C.

The modified DNA was used as a template for real-time fluorogenic quantitative methylation-specific PCR (Q MSP). The primers and probes used for the target gene (MT1G, Genbank accession no. J03910), and the internal reference gene (β-actin, ACTB) were, respectively, (sense) 5′-TGCGAAGG GGTTGTTTGGC-3′, (antisense) 5′-CCACGGCTTAACTGCGCCAA-3′, (probe) 6FAM-5′-GC ATCCGCACCTAACCTAGC-3′, (antisense) 5′-TGGTATGGAGGGATTGTAAGT-3′, and a fluorogenic probe for 5′-ACAAAATACCCTACTCCTCCCTTAA-3′, (probe) 6FAM-5′-ACACCACCACCAACACAAATAACACACA-3′-TAMRA. To determine the relative levels of methylated promoter 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 1,000 for easier tabulation (MT1G/ACTB × 1,000).

Fluorogenic QMSP 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 done in separate wells for each primer/probe set and each sample was run in triplicate. The final reaction mixture consisted of 600 mmol/L of each primer (Invitrogen); 200 mmol/L of each primer (Applied Biosystems, Foster City, CA); 0.75 unit of platinum Taq polymerase (Invitrogen); 200 μmol/L of each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma; 6.7 mmol/L magnesium chloride; 10 mmol/L mercaptoethanol; 0.1% DMSO; and 3 μL bisulfite-converted genomic DNA. PCR was done using the following conditions: 95°C for 2 minutes followed by 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each plate included multiple water blanks, a negative control, and serial dilutions of a positive control for constructing the calibration curve on each plate. Leucocyte DNA collected from healthy individuals was used as negative control. The same leucocyte DNA was methylated in vitro with SssI bacterial methyltransferase (New England Biolabs, Inc., Beverly, MA) and used as positive control.

A given sample was considered positive for MT1G hypermethylation when amplification was detected in at least two of the triplicates of the respective QMSP analysis. The QMSP threshold was determined for each run adjusting the best fit of the slope and R² using the calibration curve.

RNA Extraction and Conventional Reverse Transcription-PCR. Total RNA was extracted from all prostate cancer cell lines using RNeasy kits (Qiagen, Valencia, CA) and it was reverse transcribed by random primers (Invitrogen) according to manufacturers’ protocol. cDNA was amplified by PCR with primers spanning several exons to avoid amplification of DNA: for MT1G, 5′-GCCAGCTCTGCAAGTGGCAATGCAAA-3′ (sense) and 5′-TCTCCGATGGGCTTACCAG-3′ (antisense), which amplify a 119-bp product; for glyceraldehyde-3-phosphate dehydrogenase, 5′-TCTTCCAGGACGAGATGACC-3′ (antisense), which amplify a 274-bp product. Reverse transcription-PCR for glyceraldehyde-3-phosphate dehydrogenase served as a positive control. Each cDNA sample (1 μL) was used as a template for PCR reaction, done as previously described (19). In brief, one cycle at 94°C for 5 minutes, 30 cycles at 94°C for 30 seconds, 58°C for 20 seconds, and 72°C for 30 seconds, with final extension of 5 minutes at 72°C. Electrophoresis of the PCR products was carried out on 2% agarose gels, stained with ethidium bromide, and visualized by UV fluorescence. Nontemplate control served as negative control. To confirm the efficiency of the demethylating treatment, the expression of TIG-1 was analyzed before and after treatment with 5-aza-2′-deoxycytidine (20).

Statistical Analysis. The Shapiro-Wilk’s W test allowed for the examination of the appropriateness of a normal distribution assumption for each of the variables (data not shown). Then, 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 four groups of tissue samples, the nonadjusted statistical level of significance of P < 0.05 corresponds to a Bonferroni-adjusted statistical significance of P < 0.0125. Differences in methylation frequencies among prostate cancer, HGPIN, BPH, and normal
Prostate tissue were examined using the \( \chi^2 \) or Fisher’s exact test, as appropriate. The Mann-Whitney \( U \) test was used to compare age and prostate-specific antigen levels between patients with BPH or prostate adenocarcinoma. The Kruskal-Wallis one-way ANOVA followed by the Bonferroni-adjusted Mann-Whitney \( U \) test where used to compare the age distribution among the three patient populations (prostate cancer, BPH, and normal prostate tissue). All statistical tests were two sided. Statistical analyses were done using a computer-assisted program (Statistica for Windows, version 6.0, StatSoft, Tulsa, OK).

**Results**

Clinical and Pathologic Data. Tissue samples from 121 prostate adenocarcinomas, 39 HGPIN lesions, 29 BPH, and 13 normal tissue samples were tested. The clinical and pathologic characteristics of the patients are depicted in Table 1. Although prostate-specific antigen levels were higher in patients with prostate cancer (\( P = 0.004 \)), there was considerable overlap with BPH cases. Statistically significant differences in patient’s age were detected among the three groups of patients (\( P = 0.003 \)). The Mann-Whitney \( U \) test disclosed a significant difference only between the median age of BPH and prostate cancer patients (\( P = 0.0006 \)).

MT1G Promoter Methylation in Prostatic Tissues and Clinicopathologic Correlations. The frequency of MT1G promoter methylation in normal prostate tissue and prostate lesions is displayed in Table 2. Although the frequency of this epigenetic alteration was higher in prostate cancer, no statistically significant differences among the four groups of tissue samples were noted (\( P = 0.057 \)).

We further analyzed the methylation levels of MT1G in all tissue samples. The distributions of MT1G methylation levels in BPH, HGPIN, and prostate cancer are depicted in Fig. 1. Median methylation levels and respective interquartile ranges were 0 (0-0) for prostate cancer, HGPIN, BPH, and normal prostate tissue and significant differences were not found among the four groups of tissue samples (\( P = 0.057 \)). In three HGPIN lesions with MT1G promoter methylation, the respective prostate cancer was also methylated; whereas in the remaining two cases of methylated HGPIN, the paired normal prostate tissue was unmethylated.

In prostate cancer tissue samples, no correlation was found between age or prostate-specific antigen levels and MT1G methylation levels. However, a significant, although not statistically significant, correlation (\( r = 0.19, P = .035 \)) was apparent. The demethylating treatment was effective as demethylation, the respective prostate cancer was also methylated; whereas in the remaining two cases of methylated HGPIN, the paired normal prostate tissue was unmethylated.

In prostate cancer tissue samples, no correlation was found between age or prostate-specific antigen levels and MT1G methylation levels. However, a significant, although weak positive correlation was found between MT1G methylation levels and tumor stage (\( r = 0.19, P = .035 \)). In addition, the frequency of methylated tumors at the MT1G promoter was significantly higher in cases with extraprostatic extension (pT3 and pT4) than in localized tumors (pT2; \( P = 0.049 \)).

**Discussion**

The aim of this study was to determine the promoter methylation status of MT1G in tissue specimens from primary prostate cancer, paired HGPIN lesions, BPH, and normal prostate tissue using a highly sensitive and specific method.

**Table 1. Demographic characteristics of patient populations**

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>Prostate cancer</th>
<th>BPH</th>
<th>Normal prostate tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, ( n )</td>
<td>121</td>
<td>29</td>
<td>13</td>
</tr>
<tr>
<td>Age (y), median (range)</td>
<td>64 (40-74)*</td>
<td>68 (54-79)*</td>
<td>61 (49-80)*</td>
</tr>
<tr>
<td>Prostate-specific antigen (ng/mL), median (range)</td>
<td>9.34 (3.1-48.3)*</td>
<td>5.75 (0.8-32.5)*</td>
<td>NA</td>
</tr>
<tr>
<td>Gleason score, median (range)</td>
<td>7 (4-9)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Stage, ( n ) (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>63 (52.1)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pT3 + pT4</td>
<td>58 (47.9)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Abbreviation: NA, not applicable.

\( *P = 0.003 \) (Kruskal-Wallis ANOVA).

\( P = 0.004 \) (Mann-Whitney U test).

**Table 2. Frequency of MT1G promoter methylation in normal prostate tissue and prostate lesions, and correlation with histopathologic variables**

<table>
<thead>
<tr>
<th>Tissue sample</th>
<th>MT1G promoter methylation status</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylated, ( n ) (%)</td>
<td>Unmethylated, ( n ) (%)</td>
</tr>
<tr>
<td>Normal</td>
<td>0 (0)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>BPH</td>
<td>3 (10.3)</td>
<td>26 (89.7)</td>
</tr>
<tr>
<td>HGPIN</td>
<td>5 (12.8)</td>
<td>34 (87.2)</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>29 (24)</td>
<td>92 (76)</td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-6</td>
<td>11 (20.4)</td>
<td>43 (79.6)</td>
</tr>
<tr>
<td>7</td>
<td>16 (26.7)</td>
<td>44 (73.3)</td>
</tr>
<tr>
<td>8-10</td>
<td>2 (28.6)</td>
<td>5 (71.4)</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT2</td>
<td>10 (15.9)</td>
<td>53 (84.1)</td>
</tr>
<tr>
<td>pT3 + pT4</td>
<td>19 (32.8)</td>
<td>39 (67.2)</td>
</tr>
</tbody>
</table>

*\( \chi^2 \) or Fisher’s exact test, as appropriate.
In our series, MT1G promoter methylation was observed in 24% of prostate cancer cases and less frequently in HGPIN (9, 10). Because promoter methylation is recognized as an alternative mechanism for gene silencing (23) and MT1G promoter methylation was shown to correlate with decreased expression in papillary thyroid carcinoma (12), we did QMSP and reverse transcription-PCR in prostate cancer cell lines to ascertain whether the same event occurred in prostate cancer. The promoter region of MT1G is 70% G-C rich, displaying a 361-bp CpG island (12). The primers and probe used in our study were designed to encompass the CpG sites found methylated in thyroid tumors exhibiting MT1G underexpression by microarray analysis (12). MT1G transcripts were identified in all cell lines, although all of them were found methylated at the MT1G locus. However, methylation levels were low in most cell lines, meaning that a large proportion of cells are indeed unmethylated at that locus. This result might explain the lack of significant differences in gene expression before and after treatment with 5-aza-2′deoxycytidine (Aza-C). Interestingly, MT1G methylation levels and frequencies correlated with higher pathologic stage. Finally, nonquantitative reverse transcription-PCR showed that all prostate cancer cell lines analyzed expressed MT1G mRNA, although MT1G promoter methylation was detected in all of them.

Despite the controversial role of zinc in prostate carcinogenesis, it is acknowledged that zinc levels are significantly lower in prostate cancerous tissue compared with normal or hyperplastic tissue (2, 3). Because metallothionein isoforms are expressed in a critical role in zinc uptake, distribution, storage, and release, they are likely involved in deregulation of zinc homeostasis observed in prostate cancer. Previous reports emphasized the correlation between metallothionein expression and variables related to tumor aggressiveness in several human neoplasms, including prostate cancer (9, 10). However, immunohistochemical assessment of metallothionein expression is limited by the cross-reactivity of the antibody with the various isoforms. Indeed, one of such isoforms, MT1G, was found down-regulated in high-grade prostate cancer compared with BPH, with an average 7-fold change (11). Because in prostate cancer mutations, deletions or loss of heterozygosity are infrequent at 16q13 (21, 22), the chromosomal region to which metallothionein isoforms were mapped, we hypothesized that promoter methylation might be responsible for MT1G down-regulation.

In our series, MT1G promoter methylation was observed in 24% of prostate cancer cases and less frequently in HGPIN and BPH. Normal prostate tissue samples from the peripheral zone were unmethylated at that locus. Although these differences did not reach statistical significance, probably due to the small sample size of normal tissue samples, there was a trend for increased frequency of MT1G promoter methylation in prostate cancerous tissues. Furthermore, methylation levels did not significantly differ among the four groups of samples either. However, considering the correlation we found between methylation frequencies and levels, on one hand, and pathologic tumor stage, on the other, it is tempting to speculate whether this epigenetic event is actually related with prostate tumor progression. Remarkably, MT1G down-regulation was reported in Gleason grade 4 and 5 prostate cancer (11), which are the most aggressive prostate neoplasms (i.e., more frequently associated with local invasion and systemic spread). Interestingly, we recently reported similar clinicopathologic correlates concerning methylation levels of other genes (GSTP1, APC, RARβ2, and RASSF1A) in prostate cancer (13, 14). Thus, MT1G joins the growing list of cancer-related genes that are epigenetically altered during prostate tumorigenesis.

Because promoter methylation is recognized as an alternative mechanism for gene silencing (23) and MT1G promoter methylation was shown to correlate with decreased expression in papillary thyroid carcinoma (12), we did QMSP and reverse transcription-PCR in prostate cancer cell lines to ascertain whether the same event occurred in prostate cancer. The promoter region of MT1G is 70% G-C rich, displaying a 361-bp CpG island (12). The primers and probe used in our study were designed to encompass the CpG sites found methylated in thyroid tumors exhibiting MT1G underexpression by microarray analysis (12). MT1G transcripts were identified in all cell lines, although all of them were found methylated at the MT1G locus. However, methylation levels were low in most cell lines, meaning that a large proportion of cells are indeed unmethylated at that locus. This result might explain the lack of significant differences in gene expression before and after treatment with 5-aza-2′deoxycytidine (Aza-C). Interestingly, MT1G methylation levels and frequencies correlated with higher pathologic stage. Finally, nonquantitative reverse transcription-PCR showed that all prostate cancer cell lines analyzed expressed MT1G mRNA, although MT1G promoter methylation was detected in all of them.

Figure 2. Reverse transcription-PCR analysis of MT1G in prostate cancer cell lines LNCaP, PC-3, DU-145, and 22RV1, and of TIG-1 in prostate cancer cell lines LNCaP and PC-3, before (−) and after (+) treatment with 5 μmol/L 5-aza-2′deoxycytidine (Aza-C). TIG-1 reexpression in PC-3 cells was used as a control of the efficiency of the demethylating treatment, whereas LNCaP cell line was not affected by the treatment (20). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA served as a control to ensure the integrity of mRNA and reverse transcription reactions. H2O, negative control; MM, molecular marker.
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