

**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 Pathology, Genetics, and Urology, Portuguese Oncology Institute; and 2Fernando 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 prostate intraepithelial neoplasia (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 demethylation treatment.

Results: MT1G promoter hypermethylation was found in 29 of 121 prostate cancer, 5 of 39 HGPIN, 3 of 29 benign prostate 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, 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. Herein, we sought to characterize quantitatively the MT1G promoter methylation status of patients with prostate cancer, paired high-grade prostate intraepithelial neoplasia (HGPIN), 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.

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 prostate intraepithelial neoplasia (HGPIN) lesions, benign prostate 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.
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 prostates that did not harbor prostate cancer collected from cytoprostatectomy specimens 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 12-µm-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 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 × 10⁶ 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-deoxycytidine (Sigma, St. Louis, MO) from 100 mmol/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 (QMSP). 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′-TGCGAAGGGGTGCTGGTTTG-3′, (antisense) 5′-GCCAAGCTTTGCTGGTTTG-3′, (probe) 6FAM-5′-GGATCCCGACTACTAGT-TAMRA and (sense) 5′-GGATCCCGACTACTAGT-TAMRA and (sense) 5′-GGATCCCGACTACTAGT-TAMRA, (antisense) 5′-GGATCCCGACTACTAGT-TAMRA, (probe) 6FAM-5′-GGATCCCGACTACTAGT-TAMRA and (sense) 5′-GGATCCCGACTACTAGT-TAMRA, (antisense) AACCAATTAACCCCTACTCCCTCCCTAA-3′, (probe) 6FAM-5′-ACCCCAACCCCAACACAATAACACAA-3′-TAMRA. 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 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 nmol/L of each primer (Invitrogen); 200 nmol/L of probe (Applied Biosystems, Foster City, CA); 0.75 unit of platinum Taq polymerase (Invitrogen); 200 µmol/L each of dATP, dCTP, dGTP, and dTTP; 16.6 mmol/L ammonium sulfate; 67 mmol/L Trizma base; 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 RNasea 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′-GCCAGCTCCTGCAAGTGAATGCA-3′ (sense) and 5′-TTTCCCAGTTGCTAGTA-3′ (antisense), which amplify a 119-bp product; for glyceraldehyde-3-phosphate dehydrogenase, 5′-TTTCCCAGTTGCTAGTA-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-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 adenocarcinoma 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 $MT1G$ promoter methylation status and tumor stage $(r = 0.19, P = 0.035)$. 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; $P = 0.049$).

MT1G Promoter Methylation and RNA Expression in Prostate Cancer Cell Lines. Prostate cancer cell lines LNCaP, DU-145, PC-3, and 22RV1 were found methylated at the $MT1G$ locus and the methylation levels were 75.6, 61.3, 107.4, and 40, respectively. Reverse transcription-PCR showed that all cell lines expressed $MT1G$ mRNA (Fig. 2). After demethylating treatment, no difference in the size and intensity of the bands was apparent. The demethylating treatment was effective as $TIG-1$ was reexpressed in prostate cancer cell line PC-3 (Fig. 2), as previously reported (20).

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>
<thead>
<tr>
<th>Table 1. Demographic characteristics of patient populations</th>
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<tbody>
<tr>
<td><strong>Prostate cancer</strong></td>
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<tr>
<td>---------------------</td>
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<tr>
<td><strong>Patients, n</strong></td>
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<tr>
<td>Age (y), median (range)</td>
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<tr>
<td>Prostate-specific antigen (ng/mL), median (range)</td>
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<tr>
<td>Gleason score, median (range)</td>
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<td>Stage, n (%)</td>
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<tr>
<td>pT2</td>
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<td>pT3 + pT4</td>
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Abbreviation: NA, not applicable.
* $P = 0.003$ (Kruskall-Wallis ANOVA).
$^1P = 0.004$ (Mann-Whitney U test).

<table>
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<tr>
<th>Table 2. Frequency of $MT1G$ promoter methylation in normal prostate tissue and prostate lesions, and correlation with histopathologic variables</th>
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<tbody>
<tr>
<td><strong>Tissue sample</strong></td>
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<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Normal</td>
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<tr>
<td>BPH</td>
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<tr>
<td>HGPIN</td>
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<tr>
<td>Prostate cancer</td>
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<tr>
<td>Gleason score</td>
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<td>4-6</td>
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<tr>
<td>7</td>
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<tr>
<td>8-10</td>
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<tr>
<td>Tumor stage</td>
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<tr>
<td>pT2</td>
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* $^2$ or Fisher’s exact test, as appropriate.
In conclusion, our data suggests that acquisition of MT1G promoter methylation is associated with tumor aggressiveness. Moreover, this epigenetic change might be a marker of locally advanced disease and could be useful as stage predictor in prostate sextant biopsies.
References

12. Huang Y, de la Chapelle A, Pellegrato NS. Hypermethylation, but not LOH, is associated with the low expression of MT1G and CRABP1 in papillary thyroid carcinoma. Int J Cancer 2003;104:735 – 44.
Stage in Prostate Cancer

Hypermethylation Is Associated with Higher Tumor

MT1G

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