Inactivation of Cyclin D2 Gene in Prostate Cancers by Aberrant Promoter Methylation


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ABSTRACT

Purpose: Loss or abnormal expression of Cyclin D2, a crucial cell cycle-regulatory gene, has been described in human cancers; however, data for prostate tumors are lacking. We investigated the epigenetic silencing of Cyclin D2 gene in prostate cancers and correlated the data with clinicopathological features.

Experimental Design: Cyclin D2 promoter methylation was analyzed in 101 prostate cancer samples by methylation-specific PCR. In addition, we analyzed 32 nonmalignant prostate tissue samples, which included 24 samples of benign disease, benign prostatic hypertrophy, or prostatitis and 7 normal tissues adjacent to cancer. The methylation status of Cyclin D2 was correlated with the methylation of nine other tumor suppressor genes published previously from our laboratory on the same set of samples (R. Maruyama et al., Clin. Cancer Res., 8: 514–519, 2002). The methylation index was determined as a reflection of the methylated fraction of the genes examined.

Results: The frequency of methylation of Cyclin D2 promoter was significantly higher in prostate cancers (32%) than in nonmalignant prostate tissues (6%; P = 0.004), and it was not age related. Aberrant methylation was present at insignificant levels in peripheral blood lymphocytes (8%). We also compared methylation of cyclin D2 with methylation of nine tumor suppressor genes [published previously from our laboratory (R. Maruyama et al., Clin. Cancer Res., 8: 514–519, 2002)] studied in the same set of samples. The concordances between methylation of Cyclin D2 and the methylation of RARβ, GSTP1, CDH13, RASSF1A, and APC were statistically significant, whereas methylation of P16, DAPK, FHIT, and CDH1 were not significant. The differences in methylation index between malignant and nonmalignant tissues for all 10 genes were statistically significant (P < 0.0001). Among clinicopathological correlations, the high Gleason score group had significantly greater methylation frequency of Cyclin D2 (42%; P = 0.004). Although the high preoperative serum prostate-specific antigen (PSA) group did not have significantly greater methylation frequency, methylation of Cyclin D2 had higher mean PSA value. Also, the prostate cancers in the high Gleason score group had high mean values of PSA.

Conclusions: Our results indicate that methylation of Cyclin D2 in prostate cancers correlates with clinicopathological features of poor prognosis. These findings are of biological and potential clinical importance.

INTRODUCTION

In mammalian cells, progression through the cell cycle is governed by a family of Cdk's, whose activity is regulated by phosphorylation, activated by binding of cyclins, and inhibited by Cdk inhibitors (1). The D-type cyclins (cyclins D1, D2, and D3) are involved in the regulation of transition from G1 to S during the cell cycle (2, 3). The INK4 family of cell cycle inhibitors (P16INK4a, P15INK4b, P18INK4c, and P19INK4d) negatively regulates the activity of Cdk4/6 by preventing cyclin D binding. Cdk's are protein kinases that require association with D-type cyclins and phosphorylation for their activity. One of their substrates is believed to be the retinoblastoma gene product, pRb, and a critical negative regulator of the G1-S transition. Phosphorylation of pRb by Cdk's inactivates it and allows cells to enter S phase (2).

Transcription and protein synthesis of D-type cyclin mRNAs are highest in middle-late G1 and lowest during S phase (4). Because they play a critical role in cell cycle regulation, their abnormal or untimely expression or loss of expression could disrupt the cell cycle and therefore render them oncogenes or TSGs. Cyclin D1 has been proposed as a proto-oncogene because its derangement contributes to the uncontrolled cell growth characteristic of tumors (5). Overexpression and rearrangement of the Cyclin D1 gene have been reported to be associated with tumor progression and/or poor prognosis in...
Cyclin D2 has been noted in human ovarian granulosa cell tumors and testicular germ cell tumor cell lines (11). It has been reported that Cyclin D2 is a direct target of Myc and that accumulation of Cyclin D2 contributes to sequestration of the cell cycle inhibitor p27 and to cell cycle entry (12, 13). Overexpression of Cyclin D2 has been reported in gastric cancer and was shown to correlate with disease progression and poor prognosis (4, 14).

Previous studies have reported that Cyclin D2 mRNA and protein were absent in almost all breast cancer cell lines examined, whereas cultured normal breast epithelial cells had abundant expression (15–18). In tumorigenesis, multiple mechanisms of inactivating TSGs such as loss of heterozygosity, point mutations, homozygous deletions, and aberrant promoter methylation have been reported (19). Recently, it has been reported that promoter methylation is a mechanism for the loss of Cyclin D2 expression in breast cancers (20). Inactivation of many TSGs by epigenetic phenomenon has been reported in prostate cancers (21). To investigate whether Cyclin D2 is silenced by epigenetic phenomenon in prostate cancers, we studied the methylation status of Cyclin D2 promoter in prostate cancers and nonmalignant tissue samples. We report here that inactivation of Cyclin D2 in prostate cancers is associated with the hypermethylation of the Cyclin D2 promoter.

**MATERIALS AND METHODS**

**Clinical Samples.** The 101 prostate samples used in this study were collected from patients with prostate cancer. Among these samples, seven of them had adjacent nonmalignant tissue from the same patients. We also obtained nonmalignant tissues from 24 patients with BPH or prostatitis. The patients underwent radical prostatectomy or transurethral resection at University of Texas Southwestern Medical Center-affiliated hospitals in Dallas, Texas between 1994 and 2000, after Institutional Review Board approval and signed consent were obtained. The tissues were maintained frozen in the urology tissue bank at −70°C until use. The histological grading was performed according to GS (22), and the stage of the disease was assessed by using the clinical tumor-node-metastasis (TNM) classification of the American Joint Committee on Cancer (23). The clinicopathological features of cancer patients are described elsewhere (21).

Briefly, the median age was 63 years (range, 43–81 years), and there were mainly white (n = 69), black (n = 15), and Hispanic (n = 5) races. Median ages (and the range in years) of patients from whom malignant and nonmalignant prostate tissue samples were collected are presented in Table 1. The TNM staging consisted mainly of three groups (I, n = 12; II, n = 14; and III, n = 25). Peripheral blood lymphocytes from 13 healthy volunteers were also obtained.

**DNA Extraction.** Genomic DNA was extracted from malignant and nonmalignant tissue samples with proteinase K (Life Technologies, Inc.) for 1 day at 50°C, followed by two extractions with phenol:chloroform (1:1; Ref. 24).

**MSP.** The MSP uses an initial bisulfite reaction to modify the DNA as described previously (25). Briefly, 1 μg of DNA was denatured by incubation with 0.2 M NaOH for 15 min at 37°C. Aliquots of 10 mM hydroquinone (30 μl; Sigma Chemical Co., St. Louis, MO) and 3 mM sodium bisulfite (pH 5.0; 520 μl; Sigma Chemical Co.) were added, and the solution was incubated at 52°C for 16 h. Treated DNA was purified by use of a Wizard DNA Purification System (Promega Corp., Madison, WI). Modified DNA was stored at −80°C until use. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which is then converted to thymidine during subsequent PCR (25). Thus, after bisulfite treatment, alleles that were originally methylated have DNA sequences different from those of their corresponding unmethylated alleles, and these differences can be used to design PCR primers that are specific for methylated or unmethylated alleles. PCR was performed using primer sequences essentially as described previously (20). The primers used for amplification of the methylated form of the Cyclin D2 promoter were 5′-TACGTGTAGGTCGATCG-3′ (sense; −1427 to −1409) and 5′-CGAAATATCTACGCTAAACG-3′ (antisense; −1152 to −1171), which yielded a 276-bp PCR product (20). Reactions were hot started at 94°C for 12 min, and temperature conditions for PCR were as follows: 5 cycles of 94°C for 20 s, 55°C for 55 s, and 72°C for 20 s; and 30 cycles of 90°C for 20 s, 55°C for 40 s, and 72°C for 40 s; followed by 1 cycle of 72°C for 4 min. P16 unmethylated primer was used as control in MSP to check the integrity of bisulfite-treated DNA in tissue samples (25). DNA from peripheral blood lymphocytes (n = 13) was used as negative controls for MSP assays. DNA from lymphocytes of healthy volunteers treated with 5-azacytidine (New England Biolabs, Beverly, MA) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks and PCR mixtures (without template) were used as negative controls in each assay. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

**RESULTS**

**Table 1** Frequency of Cyclin D2 promoter methylation in prostate cancers and control tissues

<table>
<thead>
<tr>
<th>Sample designation</th>
<th>Median age (range) (yrs)</th>
<th>Sample size (n)</th>
<th>Methylation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate cancers&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63 (43–81)</td>
<td>101</td>
<td>32 (32)</td>
</tr>
<tr>
<td>Nonmalignant prostate tissues&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67 (49–86)</td>
<td>24</td>
<td>1 (4)</td>
</tr>
<tr>
<td>BPH&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Prostatitis&lt;sup&gt;b&lt;/sup&gt;</td>
<td>65 (54–76)</td>
<td>7</td>
<td>1 (14)</td>
</tr>
<tr>
<td>Adherent to cancer&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37 (32–65)</td>
<td>13</td>
<td>1 (8)</td>
</tr>
</tbody>
</table>

<sup>a</sup> The median age differences by Mann-Whitney nonparametric U statistical test were insignificant (P = 0.061).<br>
<sup>b</sup> From patients without cancer.<br>
<sup>c</sup> NA, not applicable.

**Data Analysis.** The frequencies of methylation between two groups were compared using χ² test and Fisher’s exact test with continuity correction. To compare the overall extent of methylation for the panel of genes examined, we calculated the MI. The MI is defined as the total number of genes methylated divided by the total number of genes analyzed. The MI of different groups was compared using the Mann-Whitney non-parametric test.
parametric \( U \) test. For all of the tests, \( P < 0.05 \) was considered statistically significant. All of the statistical tests were two sided. All data were analyzed using Stat View program.

RESULTS

Promoter Methylation of Cyclin D2 in Prostatic Tissues.

To examine the methylation pattern of Cyclin D2 promoter in prostatic tissues, we used the MSP assay as described in “Materials and Methods.” Results of aberrant methylation in malignant prostatic tissues and control tissues are detailed in Table 1, and representative examples are illustrated in Fig. 1. The unmethylated form of \( P16 \) run as a control for DNA integrity, was present in all samples. The frequency of methylation of Cyclin D2 promoter was significantly higher in prostate cancers (32 of 101, 32%) than in nonmalignant prostate tissues (2 of 32, 6%; \( P = 0.004 \)). The percentage of methylation in BPH (1 of 24, 4%), prostatitis (0 of 1, 0%), and samples from tissues adjacent to cancer (1 of 7, 14%) were found to be at very low frequency. Aberrant methylation was present at low levels in peripheral blood lymphocytes (1 of 13, 8%). We also compared methylation of Cyclin D2 with methylation of 9 TSGs (published previously from our laboratory; Ref. 21) studied in the same set of samples, and the concordances are presented in the Table 2. The concordances between methylation of Cyclin D2 and the methylation of \( RAR\beta \) (67 of 101, 66%; \( P = 0.0001 \)), \( GSTP1 \) (83 of 101, 82%; \( P < 0.0001 \)), \( CDH13 \) (70 of 101, 69%; \( P = 0.004 \)), \( RASSF1A \) (65 of 101, 64%; \( P = 0.0007 \)), and \( APC \) (68 of 101, 67%; \( P = 0.032 \)) were significant, whereas the methylation of \( P16, DAPK, FHIT, \) and \( CDH1 \) were not significant. The differences in MI between malignant and nonmalignant tissues for all 10 genes were statistically significant (\( P < 0.0001 \)).

Correlation between Methylation of Cyclin D2 and Clinicopathological Features. We compared the methylation frequency of Cyclin D2 with the GS, the preoperative serum PSA, and the tumor stage, three known clinicopathological features that were available for prostate cancer patients. It was reported that prostatic tumors with GS values of 5–6 have a significantly better clinical course than those with values of \( \geq 7 \) (26). Hence we divided our tumors into those with values of \( \leq 6 \) (low GS group; \( n = 36 \)) and those with values of \( \geq 7 \) (high GS group; \( n = 65 \)). The median value of preoperative serum PSA in our cancer patients was 7.5 ng/ml. To obtain an approximately equal number of patients in each category, we divided our patients into a “low PSA” group (\( \leq 8 \) ng/ml; \( n = 47 \)), and a “high PSA” group (\( > 8 \) ng/ml; \( n = 45 \)). The median values of PSA in the low and high PSA groups were 5.6 and 14.2 ng/ml, respectively. Tumor staging information was available for 60 of the patients. Because of the relatively small numbers in each of the four grades, we pooled patients into low stage (stages I and II; \( n = 26 \)) or high stage (stages III and IV; \( n = 34 \)) groups.

The high GS group had significantly greater methylation frequency of Cyclin D2 (42%; \( P = 0.004 \)), whereas methylation of Cyclin D2 was not significant in the high stage group. Although the high PSA group did not have significantly greater methylation frequency, methylation of Cyclin D2 had higher mean PSA (mean ± SE: 14 ± 3 for methylation-positive samples and 12 ± 1.7 for methylation-negative samples) value. Also, the prostate cancers in high GS group had high mean values of PSA (mean ± SE: 15 ± 2 for high GS group and 8 ± 1 for low GS group).

Patients from whom the nonmalignant prostate tissues were collected had slightly higher median ages and very low level of frequency of methylation compared with those from whom malignant tissues were collected (Table 1). Mann-Whitney non-parametric \( U \) statistical test for the median age differences between malignant and nonmalignant prostate samples revealed that the differences are not statistically significant (\( P = 0.061 \)). Survival data were available for 44 prostate cancer patients with a median follow-up period of 27 months. Although methylation status of Cyclin D2 did not correlate with survival, no deaths were noted until 48 months after surgery. Thus, a lengthy follow-up period will be required to determine whether methylation is a prognostic factor for survival.

DISCUSSION

Lack of growth control is one of the hallmarks of transformed cells (27). In addition to the role of cyclins in cell cycle regulation, the D-type cyclins have been implicated in differen-
tion and neoplastic transformation (2, 20, 28). It has been reported that Cyclin D2 overexpression correlated with gastric cancer progression and prognosis, suggesting that Cyclin D2 is a proto-oncogene (4, 14). However, a recent report on association of loss of Cyclin D2 expression with promoter methylation suggests a different role as a TSG (20). These differences suggest that Cyclin D2 may function as an oncogene or TSG in a tumor type-dependent manner. Thus, we studied the methylation pattern of Cyclin D2 in malignant and nonmalignant prostate tissue samples and correlated the data to clinicopathological features.

Multiple mechanisms of inactivating TSGs such as loss of heterozygosity, point mutations, homozygous deletions, and aberrant promoter methylation have been reported in various types of cancers (19). Aberrant methylation of CpG islands was identified as an epigenetic mechanism for the transcriptional silencing of TSGs in many cancer types, and the number of methylated genes in individual cancers is estimated to be very high (29–31). Inactivation of multiple TSGs by promoter hypermethylation has been reported in prostate cancers (21). Transcriptional silencing of Cyclin D2 gene by promoter methylation has been reported in breast cancers, whereas normal breast epithelium expressed Cyclin D2 (20). Previous studies have shown low frequency of epigenetic inactivation of p16 (21, 32), which negatively controls progression of the cell cycle through the G1 phase of the cell cycle, in prostate cancers. It has been suggested that abrogation of the Rb pathway in prostate cancer is achieved by down-regulation of RB protein (or mutation) rather than by p16 changes (33).

The methylation of Cyclin D2 was significantly higher in malignant prostate samples than in nonmalignant samples (which include BPH and tissues adjacent to cancer) and lymphocytes, indicating that methylation of Cyclin D2 may play a role in tumorigenesis. Tumor suppressor role for Cyclin D2 in breast cancers has been reported previously (20). We also correlated the methylation of Cyclin D2 with clinicopathological features such as GS and preoperative serum PSA and observed that methylation of Cyclin D2 was significantly associated with high GS group and had higher mean PSA value. This might help in prognosis. Cyclin D2 methylation might play a role in increased proliferation and poor differentiation that is reflected by high GS. To address whether the observed methylation frequency for Cyclin D2 in malignant prostate samples is age dependent (i.e., a higher methylation frequency in older people), we compared the median ages (and age range) of patients from whom malignant and nonmalignant prostate tissues were collected. We found that those patients from whom the nonmalignant prostate tissues were collected had slightly higher median ages (and age range) than those from whom the malignant tissues were collected, ruling out the possibility of age-dependent methylation of Cyclin D2 in prostate cancer patients. The differences in median ages between malignant and nonmalignant prostate samples were statistically insignificant. Also, lymphocytes from healthy volunteers had a very low level of methylation frequency, further supporting the observed Cyclin D2 methylation frequency as tumor specific and a potential tumor suppressor function of Cyclin D2. We also compared the methylation of Cyclin D2 with methylation of other TSGs studied in the same set of samples (21) in our laboratory and observed that there was a high degree of concordance with the methylation of APC, RARβ, CDH13, RASSF1A, and GSTP1 genes. Association of methylation of multiple TSGs in the same set of samples may help in using these markers in prognosis.

REFERENCES


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