Correlation between methylation of the E-Cadherin gene and malignancy of prostate cancer

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Received August 8, 2015
Accepted November 26, 2015
Published July 15, 2016
DOI http://dx.doi.org/10.4238/gmr.15028046

ABSTRACT. Prostate cancer is a common malignant tumor in males with an unclear pathogenic mechanism. As one epigenetic regulation mechanism, DNA methylation of the whole genome and specific gene(s) plays critical roles in pathogenesis, progression, diagnosis, and treatment of prostate cancer. The E-Cadherin gene is involved in cell metabolism and has been suggested to be related with malignancy of multiple tumors. This study investigated the correlation between E-Cadherin methylation and malignancy of prostate cancer. Gradient concentrations of 5-Aza-CdR (5, 10, and 20 μM) were used to treat the prostate cancer cell line (LNCaP), and mRNA level of E-Cadherin was detected by reverse transcription-polymerase chain reaction (RT-PCR). A total of 82 prostate cancer patients were recruited to detect the methylation status of the promoter region of the E-Cadherin gene by pyrophosphate sequencing. Real-time fluorescent quantitative PCR
(qRT-PCR) was employed to determine mRNA levels of E-Cadherin. Methylation and mRNA levels of E-Cadherin were analyzed by the SPSS software. With elevated concentrations of 5-Aza-CdR, mRNA levels of E-Cadherin gradually increased. DNA methylation levels of tumor tissues were significantly elevated with increased Gleason score (P < 0.05) and tumor-node-metastasis stage (P < 0.05) but were not related to age, smoking habits, or alcohol consumption (P > 0.05). DNA methylation level was negatively correlated with mRNA expression of the E-Cadherin gene. Methylation in tumor tissues was significantly higher than that in tumor adjacent tissues (P < 0.05). DNA methylation level of the E-Cadherin gene could be an important predictive index for malignancy of prostate cancer.

Key words: DNA methylation; Prostate cancer; E-Cadherin gene

INTRODUCTION

Prostate cancer is a malignant tumor that severely affects male health and it most commonly occurs in older males over 60 years old. African Americans had a higher incidence of prostate cancer than Asian men, but there is an increasing trend of incidence in most countries (Ferlay et al., 2010). Current diagnostic methods for prostate cancer include digital rectal examination and serum prostate-specific antigen (PSA) analysis. However, while most prostate hypertrophy patients exhibit a PSA level change, ~25% had no significant change in PSA. This results in difficulties in diagnosis and requires the development of alternative early diagnostic methods that are both convenient and reliable.

DNA methylation is a common epigenetic mechanism and has been found to be closely related to tumor pathogenesis, thus it could be a potential biological marker for tumors. In tumor cells, hypermethylation of specific tumor suppressor genes and hypomethylation of the whole genome frequently occur. DNA methylation of promoter regions is one important process for suppressing or silencing target genes. The methylation of tumor suppressor genes usually occurs at the early stage of cancer (Yang and Park, 2012). Therefore, alteration of DNA methylation may be one potential biological marker reflecting early tumor pathogenesis and malignancy grades.

E-Cadherin, or LDH1, is a critical protein for cell-to-cell and cell-to-matrix adhesion. Its abnormal expression can break the connections in the cytoskeleton, leading to tumor proliferation, infiltration, and metastasis. Various studies have been performed regarding the methylation level of E-Cadherin in prostate cancer cells but inconsistent results (0 to 77%) have been obtained (Li et al., 2001; Florl et al., 2004; Schwarzenbach et al., 2011). Such differences might be caused by different promoter regions or tissues selected or variability in test methods. 5-Aza-CdR is a methyltransferase inhibitor that can restore gene expression by demethylation (Klar et al., 2015). A previous study found restoration of E-Cadherin expression in a prostate cancer cell line after treatment with 5-Aza-CdR (Li et al., 2001), further suggesting the regulation of E-Cadherin expression by promoter methylation. Pyrophosphate sequencing is a new high-output, fully automatic sequencing approach for short DNA fragments. It can analyze up to 96 samples simultaneously with precise quantitative determination of methylation levels of specific gene regions within short time periods (Tost et al., 2006). Therefore, this study...
selected prostate cancer tissues with different stages of malignancy and analyzed their DNA methylation level of the E-Cadherin gene by pyrophosphate sequencing in order to provide evidence for early diagnosis and staging of prostate cancer.

**MATERIAL AND METHODS**

**Reagents and equipment**

The LNCaP cell line was provided by Meilian BioTech (China). Whole genome extraction kit and Trizol RNA extraction kit were purchased from Invitrogen (USA). Bisulfite modification kit, polymerase chain reaction (PCR) amplification kits, pyrophosphate sequencing reagent, and PyroMark Q96 ID sequencer were all products of Qiagen (USA). Real-time fluorescent kit was produced by TaKaRa (Japan). Gel imaging system and ViiA7 fluorescent quantitative PCR cycler were produced by ABI (USA).

**Clinical information**

A total of 82 prostate cancer patients who received surgery in the Department of Urology in Jining First People’s Hospital from January 2014 to July 2015 were recruited to this study. Gleason score was employed to classify cancer tissues. Adjacent normal tissues (>2 cm from the edge of the tumor) were also collected during the surgery. Tissue samples were kept in -80°C for further use. The PSA assay was performed on an automatic chemiluminescence immunoassay. The study protocol was approved by the Research Ethics Committee of Jining First People’s Hospital, and all patients gave their informed consent before study commencement.

**Cell culture and 5-Aza-CdR treatment**

LNCaP prostate cancer cells were cultured in RPMI1640 medium containing 10% fetal bovine serum in a humidified chamber with 5% CO₂ at 37°C. 5-Aza-CdR was dissolved in DMSO and was used to treat cells at 5, 10, and 20 μM concentrations, with a DMSO-treated control group in parallel. Culture medium was changed every 24 h. After 72 h, cells were collected by trypsin digestion. Reverse transcription-PCR (RT-PCR) reagent kit was used to detect the expression level of mRNA in cells. Products were separated by agarose gel electrophoresis.

**mRNA extraction and real-time fluorescent quantitative PCR**

Cancer tissue samples were homogenized in liquid nitrogen. Total RNA was extracted by Trizol reagent, and purity and concentration were determined using a spectrophotometer. The integrity of RNA was identified using 1% agarose gel electrophoresis. RNA (1 μg) was used as the template for synthesizing cDNA by reverse transcription. Real-time fluorescent quantitative PCR was then performed using cDNA as the template under the following conditions: 95°C pre-denaturation for 5 min, followed by 40 cycles of 95°C denaturation for 15 s and 60°C annealing for 60 s. Experiments were repeated in triplicate and were performed on a ViiA7 fluorescent quantitative PCR cycler using β-actin as the internal reference.
Primers were: E-Cadherin-F, 5'-ATTGCTCACATTCCCAACTC-3'; E-Cadherin-R, 5'-GTCACCTTCAGCCATCCT-3'; β-actin-F, 5'-AAACTGGAACGGTGAAGGTG-3'; and β-actin-R, 5'-AGTGGGGTGGCTTTTAGGAT-3'. Relative levels of mRNA were determined by the 2^{-∆∆Ct} method using Ct values in adjacent tissues as the control group.

DNA extraction and whole genome bisulfite modification

A whole genome extraction kit was used to collect DNA from both cancer and adjacent tissues following the manufacturer instructions. An ultraviolet spectrometer was employed to detect concentration and purity of DNA, and those with A_{260}/A_{280} ratios between 1.7 and 1.9 were considered adequate for further experiments. DNA (1 µg) was used for bisulfite modification using the test kit. Human genomic DNA with SssI methylation and bisulfite sodium treatment was used as the positive control. DNA after modification and purification was stored at -20°C for further experiments.

Pyrophosphate sequencing for gene methylation level

DNA after bisulfite modification and purification was amplified for the E-Cadherin gene using the PCR kit. Amplification products were separated by gel electrophoresis to obtain a single band. Those products with biotin labels were mixed with microbeads carrying streptavidin. Those single-stranded DNA with biotin labels were separated with unlabeled strands. Single-stranded DNA was then mixed with sequencing primers for E-Cadherin. The methylation status of the E-Cadherin gene promoter was tested in the Pyromark Q 96 ID pyrophosphate sequencing analyzer.

Statistical methods

The SPSS13.0 software (Chicago, IL, USA) was used to process all collected data. The Student t-test was used to compare two independent samples and enumeration data were compared by the chi-square test. A P value less than 0.05 was considered significant.

RESULTS

Clinical data

Based on information collected from patients, no significant difference was found in methylation level of the E-Cadherin gene regarding age, smoking habits, or alcohol consumption (P > 0.05; Table 1). Higher Gleason scores were correlated with higher methylation levels of the E-Cadherin gene (P < 0.05; Table 1).

E-Cadherin mRNA levels are regulated by 5-Aza-CdR

In those cells treated with 5-Aza-CdR, RT-PCR and agarose gel electrophoresis revealed significantly elevated expression levels of the E-Cadherin gene and this expression increased with higher concentrations of 5-Aza-CdR (Figure 1).
Table 1. E-Cadherin gene methylation and clinical indexes.

<table>
<thead>
<tr>
<th>Clinical index</th>
<th>N</th>
<th>E-Cadherin methylation level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>37</td>
<td>62.13 ± 12.24</td>
<td>0.30</td>
</tr>
<tr>
<td>≥60 years</td>
<td>45</td>
<td>58.53 ± 10.29</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>61</td>
<td>63.21 ± 8.09</td>
<td>0.14</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>60.01 ± 9.21</td>
<td></td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>53</td>
<td>61.43 ± 9.87</td>
<td>0.34</td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>59.32 ± 8.54</td>
<td></td>
</tr>
<tr>
<td>Gleason score</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-7</td>
<td>49</td>
<td>54.32 ± 7.34</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>8-10</td>
<td>33</td>
<td>68.34 ± 6.54</td>
<td></td>
</tr>
<tr>
<td>PSA concentration (ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤20</td>
<td>22</td>
<td>56.32 ± 5.43</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>&gt;20</td>
<td>60</td>
<td>67.21 ± 6.89</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. mRNA level of E-Cadherin after 5-Aza-CdR treatment (0-20 µM). β-actin was used as the internal control.

Methylation and mRNA levels of the E-Cadherin gene

Pyrophosphate sequencing and quantitative RT-PCR results showed significantly lowered E-Cadherin mRNA levels in tumor tissues compared to adjacent tissues (P < 0.05; Figure 2). The methylation levels in tumor tissues were also higher than in adjacent tissues (60.21 vs 23.12%; Figure 2).

Correlation between E-Cadherin gene methylation and mRNA level

Pearson correlation analysis was used to analyze the correlation between DNA methylation level and mRNA levels of the E-Cadherin gene in tumor tissues and revealed a significantly negative relationship (r = -0.56, P < 0.01). Therefore, the expression level of E-Cadherin decreases with elevated DNA methylation level, suggesting the possible effect of DNA methylation level on expression of the E-Cadherin gene.
E-Cadherin gene methylation level across different degrees of malignancy

The analysis of E-Cadherin DNA methylation revealed significant differences across different tumor-node-metastasis (TNM) stages. Stage III tumor tissues had significantly higher methylation rates (73.4%) compared to stage I (47.32%) and stage II (65.21%) tumors (Figure 3). Tumors with higher malignancy had elevated methylation levels. Therefore, E-Cadherin gene methylation levels increased with malignancy, further affecting gene expression.

Figure 2. DNA methylation rate (right) and mRNA levels (left) of E-Cadherin. **P < 0.05 compared to adjacent tissue.

Figure 3. DNA methylation in tumor and adjacent tissues across different TNM stages (I, II, or III). **P < 0.05 compared to adjacent tissue.
DISCUSSION

Epigenetic mechanisms include DNA methylation, histone covalent modification, and chromosome remodeling. Abnormal epigenetic control is believed to be closely related to tumor pathogenesis. As increasing research has focused on the effect of abnormal DNA methylation on tumor occurrence, novel biological markers for early diagnosis and treatment of tumors may be developed.

Prostate cancer is the result of the interaction between multiple tumor suppressor genes and oncogenes (Thomas et al., 2008). Adenomatous polyposis coli is one tumor-suppressing gene that modulates the Wnt signaling pathway, with significantly higher methylation levels in prostate tumor tissues compared to hypertrophy tissues (P < 0.001) (Yoon et al., 2013). The ETS-related gene participates in tumor angiogenesis, infiltration and metastasis, and abnormal methylation of its promoter region also occurs in prostate cancer patients (Schwartzman et al., 2011). In addition, retinoic acid receptor-β2 and Ras association domain-containing protein 1 (RASSF1a) also play important roles in the progression of prostate cancer, as their downregulation is related with hypermethylation of the CpG island in promoter regions (Jerónimo et al., 2004; Yaqinuddin et al., 2013).

The expression of E-Cadherin is the result of the interaction between microenvironment and epigenetic factors. The decreased expression is closely correlated with metastasis and prognosis of squamous carcinoma (Diniz-Freitas et al., 2006; Alt-Holland et al., 2008). The abnormal methylation of its promoter has been discovered in multiple tumors (Costa et al., 2010), including prostate cancer, with major effects on tumor infiltration (van Horssen et al., 2012). This study treated prostate cancer cells with 5-Aza-CdR and found that, with higher dosage of the drug, mRNA levels of E-Cadherin were significantly elevated. This suggests the possible role of E-Cadherin downregulation caused by hypermethylation of the CpG island in promoter regions. Meanwhile, E-Cadherin methylation level was closely correlated with its mRNA level, further strengthening such a hypothesis. Based on previous studies on the methylation status of E-Cadherin in prostate cancer, significant differences exist in promoter methylation status of the E-Cadherin gene. Researchers utilized the real-time methylation-sensitive PCR technique to analyze paraffin-embedded prostate cancer tissue samples and found the methylation level as high as 24% (Woodson et al., 2004). Another study utilized the same PCR approach on formaldehyde-fixed paraffin-based tissue sections and identified the methylation rate of E-Cadherin at 61% (Singal et al., 2004). This study performed the pyrophosphate sequencing technique and revealed higher methylation levels in cancer tissues than adjacent tissues (60.21 vs 23.12%). This study and previous ones all suggest higher methylation levels in prostate cancer tissues than non-tumor tissues, but obtained different rates of methylation. This may be due to the artifacts incurred during the processes of tissue fixation and embedding. The relatively short target fragment of pyrophosphate sequencing in this study may also bias results from methylation-sensitive PCR. In addition, different target sequences selected may lead to differential results. This study utilized tumor tissues obtained during surgery as the sample in combination with next-generation sequencing techniques, thus obtaining more reliable results.

Based on different malignancy grades of patients, we found higher methylation levels of E-Cadherin in stage III tumor tissues (73.4%) compared to stage I and stage II tumors (47.32 and 65.21%, respectively). All these values were higher than tumor adjacent tissues (23.12%, P < 0.05). In summary, with increasing malignancy of tumors, the methylation level
of the E-Cadherin promoter region was elevated. The alteration of E-Cadherin methylation also occurs in breast cancer (Fukagawa et al., 2015) and skin squamous carcinoma (Wu et al., 2014) in addition to prostate cancer but not in normal tissues, suggesting the specificity of E-Cadherin gene methylation in tumor diagnosis and staging. Therefore, E-Cadherin gene methylation levels may provide evidence for diagnosis and clinical staging of prostate cancer. In clinical practice, however, tissue samples may not be available during the primary diagnosis. The study of peripheral E-Cadherin methylation level requires further study.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the anonymous reviewers for reviewing this manuscript.

REFERENCES


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DNA methylation in prostate cancer


