

Original Article

Correlation between methylation of E-cadherin gene and malignancy of prostate cancer

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Received November 30, 2015; Accepted January 31, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Prostate cancer is one common malignant tumor in males with unclear pathogenesis mechanism. As one epigenetic regulation mechanism, DNA methylation of the whole genome and specific gene(s) play critical roles in pathogenesis, progression, diagnosis and treatment of prostate cancer. E-cadherin gene is involved in cell metabolism and has been suggested to be related with malignancy of multiple tumors. This study thus investigated the correlation between E-cadherin methylation and malignancy of prostate cancer. Gradient concentrations of 5-Aza-CdR (5 μ M, 10 μ M and 20 μ M) were used to treat prostate cancer cell line LNCaP, whose mRNA level of E-cadherin was detected by RT-PCR. A total of 82 prostate cancer patients were recruited to detect the methylation status of promoter region of E-cadherin gene by pyrophosphate sequencing. Real-time fluorescent quantitative PCR (qRT-PCR) was employed to determine mRNA level of E-Cadherin. Methylation and mRNA levels of E-cadherin were analyzed by SPSS. With elevated concentrations of 5-Aza-CdR, mRNA level of E-cadherin was gradually increased. DNA methylation level of tumor tissues was significantly elevated with increased Gleason score ($P < 0.05$) and higher TNM stage ($P < 0.05$) but was not related with age, smoking or drinking habit ($P > 0.05$). DNA methylation level was negatively correlated with mRNA expression of E-Cadherin gene. Methylation in tumor tissues was significantly higher than that in tumor adjacent tissues ($P < 0.05$). DNA methylation level of E-Cadherin gene can work as one important predictive index for malignancy of prostate cancer.

Keywords: DNA methylation, prostate cancer, E-cadherin gene

Introduction

Prostate cancer is one malignant tumor that severely affects male health, as it most commonly occur in elder males over 60 years old. American Africans had relatively higher incidence of prostate cancer than that in Asian countries, with an increasing trend of incidence in most countries [1]. Current diagnostic methods for prostate cancer include digital rectal examination (DRE) and serum prostate-specific antigen (PSA). However, parts of prostate hypertrophy patients also manifested as PSA level change, while ~25% prostate cancer patients had no significant change of PSA, causing certain difficulty in diagnosis. Therefore, the development of one early diagnostic method with convenience and reliability is of critical importance.

DNA methylation is one common epigenetic mechanism, and has been found to be closely

related with tumor pathogenesis [2], thus can work as one potential biological marker for tumors. In tumor cells, hyper-methylation of specific tumor-suppressor gene and hypomethylation of the whole genome frequently occur. DNA methylation of promoter region is one important process for suppressing or silencing target genes. The methylation of tumor suppressor gene usually occurs at the early stage of cancer [3]. Therefore, alternation of DNA methylation may become one potential biological marker reflecting early tumor pathogenesis and malignancy grades.

E-cadherin, or CDH1 is one critical protein for cell-to-cell and cell-to-matrix adhesion. Its abnormal expression can break the connection between cytoskeletons, thus leading to tumor proliferation, infiltration and metastasis. Various studies have been performed regarding the methylation level of E-cadherin in prostate

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cancer cells but obtaining inconsistent results from 0% to 77% [4-6]. Such differences might be caused by different promoter regions or tissues selected, in addition to test methods. 5-Aza-CdR is one methyltransferase inhibitor, and can restore gene expression by de-methylation [7]. Previous study found the restoration of E-Cadherin expression in prostate cell line after treating with 5-Aza-CdR [4], 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 precisely quantitative determination of methylation level of specific gene region within short time periods [8]. Therefore, this study selected prostate cancer tissues with different malignant stages, and analyzed their DNA methylation level of E-Cadherin gene by pyrophosphate sequencing, in order to provide evidences for early diagnosis and staging of prostate cancer.

Materials and methods

Reagents and equipment

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

Clinical information

A total of 82 prostate cancer patients who received surgery in the department of urology in our hospital from January 2014 to July 2015 were recruited in this study. Gleason score was employed to classify cancer tissues. Adjacent normal tissues (>2 cm from the edge of tumor) were also collected during the surgery. Tissue samples were kept in -80°C fridge for further use. The assay of PSA was performed on an automatic chemiluminescence immunoassay.

Cell culture and 5-Aza-CdR treatment

LNCaP prostate cancer cells were cultured in RPMI1640 medium containing 10% fetal bo-

vine serum (FBS) 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 μM, 10 μM and 20 μM concentrations, with paralleled DMSO treated control group. Culture medium was changed every 24 hours. After 72 hours, cells were collected by trypsin digestion. 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 was determined for purity and concentration under spectrometer. The integrity of RNA was identified in 1% agarose gel electrophoresis. 1 μg RNA 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-denature for 5 min, followed by 40 cycles each containing 95°C denature for 15 sec, 60°C annealing for 60 sec. Triplicated experiments were performed on ViiA7 fluorescent quantitative PCR cycler using β-actin as the internal reference. Primers were: E-Cadherin-F, 5'-ATTGC TCACA TTTCC CAACT C-3'; E-Cadherin-R, 5'-GTCAC CTTC A GCCAT CCT-3'; β-actin-F, 5'-AAACT GGAAC GGTGA AGGTG-3'; β-actin-R, 5'-AGTGG GGTGG CTTT AGGAT-3'. Relative level of mRNA was determined by 2^{-ΔΔCt} method using CT values in adjacent tissues as the control group.

DNA extraction and whole-genome bisulfite modification

Whole genome extraction kit was used to collect DNA from both cancer and adjacent tissues following manual instruction. UV spectrometer was employed to detect contents and purity of DNA, as those with A260/A280 ratio between 1.7 and 1.9 were adequate for further experiments. 1 μg DNA was used for bisulfite modification using test kit. Human genomic DNA with Sss I methylation and bisulfite sodium treatment was used as the positive control. DNA after modification and purification was stored at -20°C for further experiments.

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Table 1. E-Cadherin gene methylation and clinical indexes

Clinical index	N	E-Cadherin methylation level	P value
Age			
<60 years	37	62.13±12.24	0.30
≥60 years	45	59.53±10.29	
Smoking			
Yes	61	63.21±8.09	0.14
No	21	60.01±9.21	
Drinking			
Yes	53	61.43±9.87	0.34
No	29	59.32±8.54	
Gleason score			
4-7	49	54.32±7.34	P<0.001
8-10	33	68.34±6.54	
PSA concentration (ng/ml)			
≤20	22	56.32±5.43	P<0.001
>20	60	67.21±6.89	

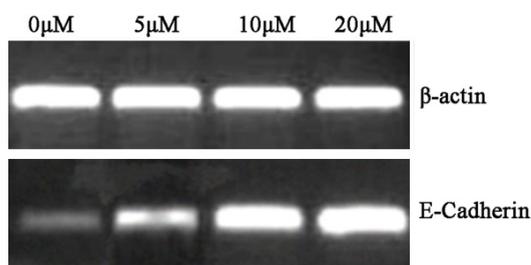


Figure 1. mRNA level of E-Cadherin after 5-Aza-CdR treatment.

Pyrophosphate sequencing for gene methylation level

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

Statistical methods

SPSS13.0 software was used to process all collected data, of which student t-test was used to

compare two independent samples, while enumeration data were compared by chi-square test. The significant level α was defined as 0.05.

Results

Clinical data

Based on patients' information collected, no significant difference has been found in methylation level of E-Cadherin gene regarding age, smoking, and drinking habit ($P>0.05$, **Table 1**). With higher Gleason score, however, E-Cadherin gene methylation level was significantly elevated ($P<0.05$, **Table 1**).

E-Cadherin mRNA expression was regulated by 5-Aza-CdR

In those cells treated with 5-Aza-CdR, RT-PCR and agarose gel electrophoresis revealed significantly elevated expression level of E-Cadherin gene with higher concentration of 5-Aza-CdR (**Figure 1**).

Methylation level and mRNA expression of E-Cadherin gene

Pyrophosphate sequencing and qRT-PCR results showed significantly lowered E-Cadherin mRNA level in tumor tissues compared to adjacent tissue ($P<0.05$, **Figure 2**). The methylation level in tumor tissue was also higher than 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 expression level of E-Cadherin gene in tumor tissues and revealed significantly negative relationship ($r=-0.56$, $P<0.01$). Therefore, expression level of E-Cadherin was decreased with elevated DNA methylation level, suggesting the possible effect of DNA methylation level on protein expression of E-Cadherin gene.

E-Cadherin gene methylation level across different degrees of malignancy

The analysis of E-Cadherin DNA methylation revealed significant difference across different

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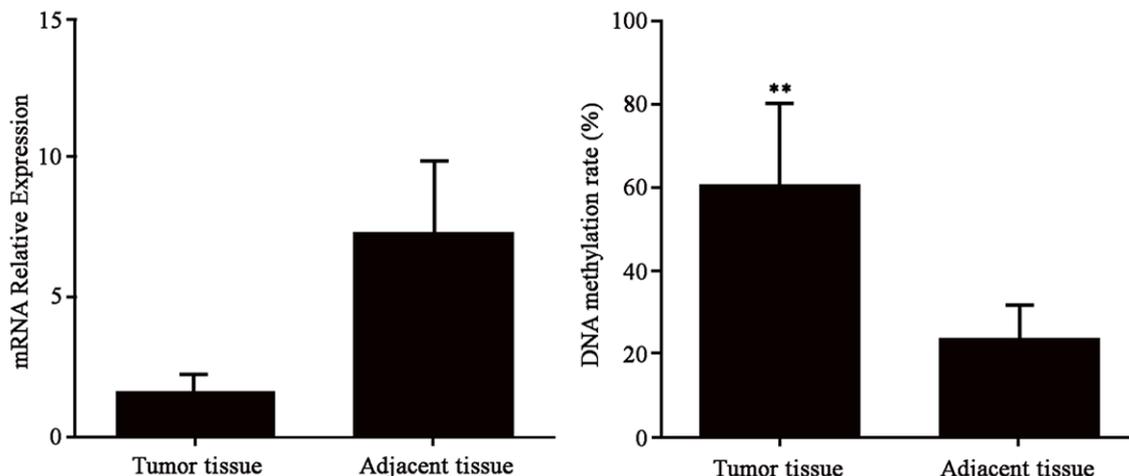


Figure 2. DNA methylation rate and mRNA expression of E-Cadherin gene. **, P<0.05 compared to adjacent tissues.

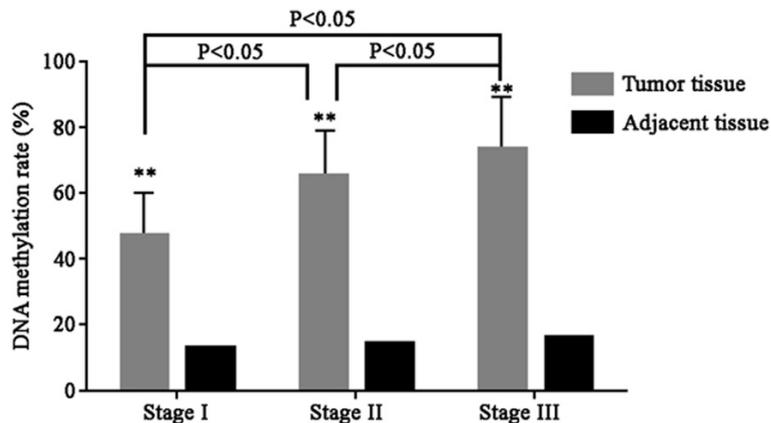


Figure 3. DNA methylation in tumor and adjacent tissues across different TNM stages. **, P<0.05 compared to adjacent tissue.

TNM stages. Stage III tumor tissues had significantly higher methylation rate (73.4%) compare to stage I (47.32%) and stage II (65.21%) tumors (Figure 3). Tumors with higher malignancy had elevated methylation level. Therefore, E-Cadherin gene methylation level was increased with higher malignancy, further affecting gene expression.

Discussion

Epigenetic mechanism includes DNA methylation, histone covalent modification and chromosome remodeling. Abnormal epigenetic control is believed to be closely correlated with tumor pathogenesis. As increasing researches have focused on the effect of abnormal DNA methylation on tumor occurrence, novel biologi-

cal markers for early diagnosis and treatment of tumor may be developed.

Prostate cancer is the result of the interaction between multiple tumor-suppressor genes and oncogenes [9]. APC is one tumor-suppressor gene that modulates Wnt signaling pathway, with significantly higher methylation level in prostate tumor tissues compared to hypertrophy tissues (P<0.001) [10]. ERG gene participates in tumor angiogenesis, infiltration and metastasis, its ab-

normal methylation at promoter region also occurred in prostate cancer patients [11]. In addition, RAR- β 2 and RASSF1a gene also played important roles in the progression of prostate cancer, as their down-regulation is related with hyper-methylation of CpG island in promoter regions [12, 13].

The expression of E-Cadherin is the result of interaction between microenvironment and epigenetic factors. The decreased expression is closely correlated with metastasis and prognosis of squamous cell carcinoma [14, 15]. The abnormal methylation of its promoter has been discovered in multiple tumors [16] including prostate cancer with major effects on tumor infiltration [17]. This study treated 5-Aza-CdR with prostate cancer cells and found that with

higher dosage of drugs, mRNA level of E-Cadherin was significantly elevated, suggesting the possible role of E-Cadherin down-regulation caused by hyper-methylation of CpG island in promoter regions. Meanwhile, E-Cadherin methylation level was closely correlated with its mRNA level, further strengthening such hypothesis. Based on previous studies about methylation status of E-Cadherin in prostate cancer, significant differences existed in promoter methylation status of E-Cadherin gene. Researches utilized real-time methylation-sensitive PCR technique to analyze paraffin-embedded prostate cancer tissue samples and found the methylation level as high as 24% [18]. Other study utilized the same PCR approach on formaldehyde-fixed paraffin-based tissues sections and identified the methylation rate of E-Cadherin at 61% [19]. This study performed pyrophosphate sequencing technique and revealed higher methylation level in cancer tissues than adjacent tissues (60.21% vs. 23.12%). This study and previous ones all suggested higher methylation level in prostate cancer tissues than non-tumor tissues, but obtained different rates of methylation. This may be due to the artifacts during processing 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 combined with next-generation sequencing technique, thus obtaining more reliable results.

Based on different malignancy grades of patients, we found higher methylation level 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 promoter region was also elevated. The alternation of E-Cadherin methylation also occurs in breast cancer [20] and skin squamous cell carcinoma [21] besides prostate cancer but not in normal tissues, suggesting the satisfactory specificity of E-Cadherin gene methylation in tumor diagnosis and staging. Therefore, E-Cadherin gene methylation level may provide evidences for

diagnosis and clinical staging of prostate cancer. In clinical practice, tissue samples, however, may not be available during the primary diagnosis. The study of peripheral E-Cadherin methylation level requires further study.

Disclosure of conflict of interest

None.

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