Prediction efficiency of *PITX2* DNA methylation for prostate cancer survival

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ABSTRACT. This study determined the level of *PITX2* methylation in prostate cancer and benign tissues and its relationship with the postoperative survival rate. Forty-four patients with prostate cancer who underwent radical prostatectomy and 43 patients with benign prostatic hyperplasia were selected. DNA was extracted from the tissues and *PITX2* methylation status was quantitatively analyzed by using the EpiTect MethyLight method. The median follow-up time of the patients was 63 months and was used to analyze the relationship between *PITX2* methylation status with tumor stage and survival rates. Median *PITX2* gene expression in benign tissues was 1.46, which was higher than that of tumor tissues with a median of 0.01 ($P < 0.001$). The median methylation in the controls was less than 0.001%, while the median methylation in the test group was 23.3% ($P = 0.000$). The number of patients with low methylation level in T2 stage was 15, which was more than that in T3 and T4 stages (8 patients); while the number of patients with high methylation levels in T2 stage was 6, which was less than that in T3 and T4 stages (15 patients) ($P = 0.035$). The *PITX2* gene expression level in prostate cancer tissues was lower than that in benign

tissues. A higher degree of *PITX2* DNA methylation was associated with higher tumor stage and lower survival rates. *PITX2* DNA methylation presents a good predictive value for prostate cancer survival.

Key words: DNA methylation; Prostate cancer; *PITX2*; Survival

INTRODUCTION

Prostate cancer is a common malignant tumor in the male urogenital system with increasing incidence (Daniũnaitė et al., 2011; Tang et al., 2013). Radical prostatectomy and radiation therapy are the first choice for treatment, while the prognosis is different according to the clinical stage, pathological grading, and individual differences. PSA, a specific prostate marker, has been widely used in clinical laboratory tests for the diagnosis of prostate cancer. However, PSA detection presents a high false-positive rate and fails to accurately assess the malignancy grade of the tumor. Thus, it does not fully satisfy the clinician's need to determine the treatment modality for patients with prostate cancer (Goessl et al., 2001). Novel specific molecular markers for prostate cancer diagnosis and treatment are needed. DNA methylation changes in the early process of prostate cancer with high detection sensitivity and diversity test methods and is an ideal biomarker (Hoque et al., 2004; Bickers and Aukim-Hastie, 2009). As the most characterized type of epigenetic modification in prostate cancer, DNA methylation has become a hotspot in prostate cancer epigenetic studies and provides a new method for prostate cancer early diagnosis, prognosis assessment, and drug treatment. Many genes, closely related to the occurrence, development, and metastasis of prostate cancer, are methylated. Different tumors present specific methylated genes that could be chosen as biomarkers for the diagnosis and prognosis evaluation. Paired-like homeodomain transcription factor 2 (PITX2) is a downstream transcription factor of the classic Wnt signaling pathway and its mutation can cause a variety of diseases (Tian et al., 2012). This study explored *PITX2* methylation in prostate cancer and benign tissues and its relationship with postoperative survival rate through assessing *PITX2* expression and methylation levels in tissues from patients with prostate cancer.

MATERIAL AND METHODS

General information

Forty-four patients with prostate cancer who underwent radical prostatectomy and 43 patients with benign prostate hyperplasia were selected between May 2004 and November 2007. The mean ages in the two groups were 57.48 ± 4.71 and 56.81 ± 3.52 years old, respectively. The patient's age, weight, and other general clinical data showed no statistical difference ($P > 0.05$). The inclusion criteria were as follows: 1) patients received radical prostatectomy; 2) prostate cancer and benign hyperplasia were confirmed by pathology; 3) patients with no other malignant tumors that affect the test result.

Patients were staged according to the tumor staging standards promulgated by the International Union Against Cancer (Hermanek, 1992). T1 stage is clinical recessive tumor; T2 stage is confined to the prostate; T3 stage invades out of the prostate capsule; T4 stage is fixed tumor or invaded the adjacent tissues outside of the seminal vesicles. Twenty-one

patients presented T2 stage and 23 patients were in stages T3 and T4. Twenty-four patients exhibited a Gleason score of 7, 14 patients presented Gleason scores of less than 7, and 6 patients presented Gleason scores higher than 7. No bone metastasis was found prior surgery in all patients.

Patients with prostate cancer were postoperatively followed up for 36 to 70 months with a median follow-up time of 63 months. This study was approved by the hospital Ethics Committee and the patient or dependents signed the informed consent.

DNA extraction

Prostate tissue was homogenized with cracking liquid and combined with an equal volume of chloroform, mixed, and centrifuged at 2500 rpm for 10 min. The upper water phase was mixed with an equal volume of isopropyl alcohol and centrifuged at 2500 rpm for 10 min. NaCl (5 M) was added to the water phase to a final concentration of 0.3 M. Another 2.5 time volume of ice anhydrous ethanol was added to collect the white precipitate. The white precipitate was diluted by 1 mL TE. The DNA concentration was calculated as follows: (μg/mL) = A_{260} nm x 50 x 1/optical path x dilution ratio. The DNA was then further purified by agarose gel electrophoresis.

Real-time PCR

SuperScript II transcription enzyme was used for reverse transcription and the real-time PCR was performed by using commercially specific primers and the corresponding mRNA TaqMan probe on ABI 7900 (Table 1). The TaqMan probes and PCR primers allowed us to distinguish methylated and unmethylated DNA. The DNA fragments were first treated with bisulfite and used for real-time PCR. cDNA dilution series was used to standardize each test and the experimental deviation of each sample was lower than 10%. The *TBP* gene was used as the reference.

Table 1. Primer sequences.

Gene	Primer (5'→3')
PITX2	ataagcttgccaccatggagaccaactgccgcaaactgg
GFP-SF1	ccatcttcttcaaggacgacgac

Methylation analysis

After treatment with sodium bisulfite, DNA was amplified with EZ DNA methylation kit. The EpiTect MethyLight quantitative method was used to analyze *PITX2* gene methylation level. A probe was designed complementary to the loci to be detected with a fluorescent dye on its 5'-end and a quenching fluorescence probe on its 3'-end for real-time PCR.

Statistical analysis

All statistical analyses were performed using the SPSS11.5 software (Chicago, IL, USA). Measurement data are reported as means ± standard deviation and enumeration data

are reported as a percentage. Differences between groups were analyzed using the chi-square test, with $P < 0.05$ considered statistically significant.

RESULTS

PITX2 expression in prostate cancer

Real-time PCR results showed that the median value for *PITX2* gene expression was 1.46 in benign tissues and 0.01 in carcinoma tissues. *PITX2* expression in prostate cancer tissues was obviously lower than that in benign tissues ($P < 0.001$). *PITX2* expression and Gleason score did not significantly correlate with the tumor stage.

PITX2 methylation level in prostate cancer and benign tissues

PITX2 methylation level was detected in the two groups. *PITX2* gene expression was significantly related to DNA methylation in the 44 tumor specimens. As shown in Figure 1, the median value of the methylation level in tumor (23.3%) was markedly higher than that in benign tissues (<0.001%; $P = 0.000$).

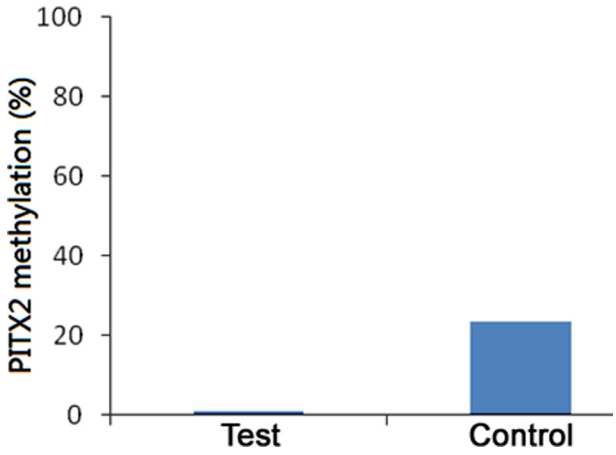

Figure 1. *PITX2* methylation percentage in test and control groups ($P = 0.000$).

Association of *PITX2* methylation with tumor staging

The median value of *PITX2* DNA methylation in prostate cancer was 23.3%. We divided the patients with a methylation degree lower than the median value (23.3%) as the low methylation subgroup and the patients with a methylation degree higher than the median value as the high methylation subgroup. The number of patients with low methylation in T2 stage was higher than that in T3 and T4 stages, while the number of patients with high methylation in T2 stage was less than that in T3 and T4 stages ($P = 0.035$; Table 2). No significant correlation between tumor stage and *PITX2* expression and Gleason score was observed.

Table 2. Association of PITX2 methylation with tumor staging.

PITX2 methylation level	Low methylation subgroup (N = 23)	High methylation subgroup (N = 21)
T2 stage	15 (65%)	6 (29%)
T3 and T4 stages	8 (35%)	15 (71%)
χ^2	5.909	
P	0.035	

Association of *PITX2* methylation with survival rate

In the subgroup of patients with low methylation, a higher number of patients exhibited a survival rate longer than 5 years, while, in the subgroup of patients with high methylation, a higher number of patients showed a survival rate shorter than 5 years (P = 0.034; Table 3).

Table 3. Association of PITX2 methylation with survival rate.

PITX2 methylation level	Low methylation subgroup (N = 23)	High methylation subgroup (N = 21)
Cases with survival >5 years	18 (78%)	10 (48%)
Cases with survival <5 years	5 (22%)	11 (52%)
χ^2	4.454	
P	0.034	

DISCUSSION

Prostate cancer is difficult to diagnose at the onset of the disease and, once diagnosed, the cancer has progressed. Epigenetic changes are widespread in patients with prostate cancer and take place at the beginning of the prostate tumor formation (Yegnasubramanian et al., 2004; Berger et al., 2009). It can be used for clinical screening and early detection. DNA methylation occurs in the cytosine of CpG dual nucleotides (Vanaja et al., 2009; Desotelle et al., 2013) and combines methyl groups to the 5th carbon atom of CpG dual nucleotide cytosine to form 5-methyl cytosine under the catalysis of the DNA methyltransferase (Yegnasubramanian et al., 2008; Khor et al., 2011). CpG dual nucleotides are usually distributed in the CpG islands in the genome (Kilinc et al., 2012). CpG island methylation is not common in normal cells, while it is widespread in tumor cells (Perry et al., 2006). PITX2, a transcription factor downstream of the canonical Wnt signaling pathway, could regulate the expression of genes involved in cell growth such as *CCDN1* and *CCDN2*. This study analyzed the relationship between *PITX2* methylation and prostate cancer prognosis, to determine whether DNA methylation could be used as a prognosis marker for prostate cancer.

Real-time PCR was performed using extracted RNA from benign prostate and tumor tissues. *PITX2* expression in prostate cancer tissues was significantly lower than that in benign tissues, while the opposite was observed in term of *PITX2* methylation. This suggested that *PITX2* methylation was high in tumor tissues and can be used to identify prostate cancer from benign prostatic hyperplasia. Additionally, *PITX2* methylation could be used as a prognosis tool based on the *PITX2* methylation degree. The CpG islands in the promoter region of tumor cells are methylated (Schulz and Hatina, 2006; Zheng et al., 2013). DNA methylation might suppress gene expression. Our results indicate that *PITX2* was highly methylated and expressed at a low level in cancer tissues. Hypermethylation is the third mechanism in tumor suppressor gene inactivation besides gene silencing and gene mutations, and is the only mechanism in

some cases (Steiner et al., 2010). Several studies confirmed that *PITX2* hypermethylation can cause tumor suppressor gene inactivation and chromosome instability, which leads to abnormal prostatic cell proliferation (Bañez et al., 2010). *PITX2* methylation and expression changes have also been found in breast cancer tissue and cell lines (Mirza et al., 2012).

The median value of *PITX2* DNA methylation in prostate cancer was 23.3%. The number of patients with low methylation in T2 stage was higher than that in T3 and T4 stages, while the number of patients with high methylation in T2 stage was less than that in T3 and T4 stages. This indicated that a higher DNA methylation degree of *PITX2* was associated with higher tumor stage and lower survival rates. CpG island hypermethylation can inhibit tumor suppressor gene expression, leading to the loss of control of tumor growth. *PITX2* can also regulate the androgen receptor and insulin-like growth factor 1 receptor pathways to affect prostate cell growth (Liu et al., 2011). In addition, excessive activation of the Wnt signaling pathway can lead to abnormal cell proliferation and tumor formation. PITX2 might influence tumor development through its participation in the Wnt signaling pathway.

In the subgroup of patients with low methylation a higher number of patients exhibited a survival rate longer than 5 years, while, in the subgroup of patients with high methylation, a higher number of patients exhibited a survival rate shorter than 5 years. This suggested that higher *PITX2* methylation levels are associated with worse 5-year survival rate. *PITX2* hypermethylation, leading to related gene silencing, plays an essential role in prostate cancer formation. It results in the reduction of *PITX2* expression and leads to the worse prognosis (Ummanni et al., 2011). Since patients in the high methylation group exhibited higher tumor stage, higher malignant degree, and more severe disease, they presented the worse prognosis and lower 5-year survival rate. Taken together, our results indicate that *PITX2* methylation degree can be used as a predictor for the survival of patients with prostate cancer.

In summary, *PITX2* methylation was closely associated with the occurrence and development of prostate cancer. *PITX2* expression in prostate tissue was significantly lower than that in benign tissues. The *PITX2* gene expression level in prostate cancer was lower than that in benign tissues. A higher DNA methylation degree of *PITX2* was associated with higher tumor stages and lower survival rate. *PITX2* DNA methylation presents a good predictive value for prostate cancer survival.

Conflicts of interest

The authors declare no conflict of interest.

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