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ORIGINAL ARTICLE

Investigation of Methylation Status of Homeobox D3 (HOXD3) and Protocadherin 17 (PCDH17) in Patients with Prostate Cancer

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OBJECTIVE

Prostate cancer is one of the most common cancers in male gender. Despite recent advances in the diagnosis and the treatment methods, more reliable molecular biomarkers have been needed for the diagnosis and evaluation of response to treatments such as chemotherapy, anti-androgen therapy, and radiotherapy. The aim of this study is to investigate promoter methylation status of HOX3D and PCDH17 genes in prostate cancer in Turkish population.

METHODS

A total of 46 patients with prostate cancer were included in this study. Tissue samples obtained from 36 patients with benign prostate hyperplasia were used as controls. Methylation status of HOXD3 and PCDH17 genes was determined by quantitative Methylation-Specific PCR with commercially available primer sets.

RESULTS

Both HOXD3 and PCDH17 promoter methylation was determined significantly higher in patients with compare to controls (p=0.0198 and p=0.0386, respectively). A significant but weak correlation was found between methylation status and pre-operative PSA level for HOXD3 (Spearman's rho=0.259, p=0.02) and PCDH17 gene (Spearman's rho=0.324, p=0.006).

CONCLUSION

Our results indicated that HOXD3 and PCDH17 promoter methylation levels are higher in patients with prostate cancer. Further studies with large sample cohorts and clinicopathological data will enlighten presumptive role of HOXD3 and PCDH17 methylation status.

Keywords: HOXD3; PCDH17; methylation; prostate cancer.

Introduction

Prostate cancer ranks second after lung cancer in cancer-related deaths in men.[1] Prostate cancer exhibits geographic variation in incidence and mortality.[2] In America and European countries, these rates are quite high compared to Asian and African countries.[3] The most commonly used methods for diagnosis are measurement of serum PSA levels and pathological examination of biopsy samples. The most encountered

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problem in determining the disease with biopsy is "blind biopsies." As a result of the biopsies taken without using the imaging method, the cancerous area may be completely overlooked and false negative results can be obtained.[4] Measurement of PSA levels is another method used in the diagnosis of the disease, predicting the response to treatment and determining the risk of recurrence.[5] However, its clinical use is controversial due to insufficient sensitivity, specificity, and predictive values of PSA levels. It has the potential to lead to misdiagnosis and unnecessary treatment. Another drawback of using PSA levels in diagnosis and prognosis is that the PSA is the androgen receptor target gene. Therefore, in patients receiving anti-androgen therapy, androgen receptor signal will be down-regulated and PSA expression will be decreased. In this regard, it is unclear whether the decrease in PSA level is due to the decrease in the number of cancerous cells or the decrease in PSA expression as a result of the treatment.[6] In addition, it has been determined that a large number of compounds that do not have antitumor activity can modulate PSA expression.[7] For all these reasons, much more reliable molecular biomarkers have been needed for diagnosis and evaluation of response to treatments such as chemotherapy, anti-androgen therapy, radiotherapy, or combinational multimodal therapy depending on the disease stage.[8] Serial monitoring is applied for localized prostate cancer, whereas in addition to surgery, radiation therapy is used for patients with locally advanced prostate cancer.[4] Abiraterone and enzalutamide are recent chemotherapeutics that can improve prognosis in metastatic prostate cancer patients, especially in those are resistant to traditional hormonal therapy.[7]

Epigenetics are the alterations in gene expression without changing the primary DNA sequence. One of the epigenetic modifications that cause this change is DNA methylation. It occurs by adding methyl groups to the CpG dinucleotides in the genome by the DNA methyl transferase enzyme. Hypermethylation of CpG dinucleotides located in the promoter regions of genes is an epigenetic modification that can cause genes to be silenced. While one allele of the gene is inactivated by genetic mechanisms such as point mutation or deletion, methylation of CpG islands in promoters can inactivate the other allele (Knudson's two-hit hypothesis).[9] Mostly tumor suppressor genes are silenced as a result of these mechanisms. Inactivation by hypermethylation has been detected in genes encoding proteins involved in prostate cancer, cell cycle checkpoints, DNA repair, invasion, and metastasis, as in many types of cancer.[10] It is believed that DNA hypermethylation occurs early in tumor formation and plays a major role in tumor initiation and prognosis.[11] In the literature, it has been shown that hundreds of genes are silenced by promoter DNA methylation in a coordinated manner in methylation studies conducted especially with solid tumors and covering the whole genome. In addition, it is stated that there are tumor-specific methylation models.[12] Similar to genetic changes, epigenetic changes are also inherited and stable. Therefore, they have the potential to be used as a molecular biomarker in cancer patients for early diagnosis, prognosis, and prediction of response to treatment.[13] In studies conducted for this purpose, genes that can be used as biomarkers in prostate cancer have been investigated. Genes with high sensitivity and specificity in prostate cancer have been investigated in single gene studies and gene panels studies and it has been reported that many genes have the potential to become biomarkers through promoter DNA hypermethylation.[10]

In this study, we aimed to investigate promoter methylation status of HOX3D and PCDH17 tumor suppressor genes in prostate cancer in Turkish population. HOXD3 gene is a member of homeobox gene family of transcription factors. Altered expression of many homeobox genes have been found different tumors including prostate cancer.[14] PCDH17 gene belongs to protocadherin gene family and its methylation level is suggested to be important in tumor progression for several cancers.[15] Depending on the treatment received by the patients, these two genes could be important in the diagnosis and evaluation of response to treatment of prostate cancer. Our study will contribute to intensive studies on the use of tumor suppressor genes as molecular biomarkers in the diagnosis and prognosis of prostate cancer.

Materials and Methods

Sample Collection

This study was approved by Istanbul Medeniyet University Goztepe Training and Research Hospital Ethical committee with decision number 2017/0257. A total of 46 prostate tumor samples and 36 non-tumoral (benign) prostate tissue samples were used for this study. Prostate tumor samples were obtained from prostate cancer patients who had operation of radical prostatectomy. Non-tumoral prostate tissues were obtained from patients with benign prostatic hyperplasia. Tumor and non-tumor samples were confirmed with pathology results.

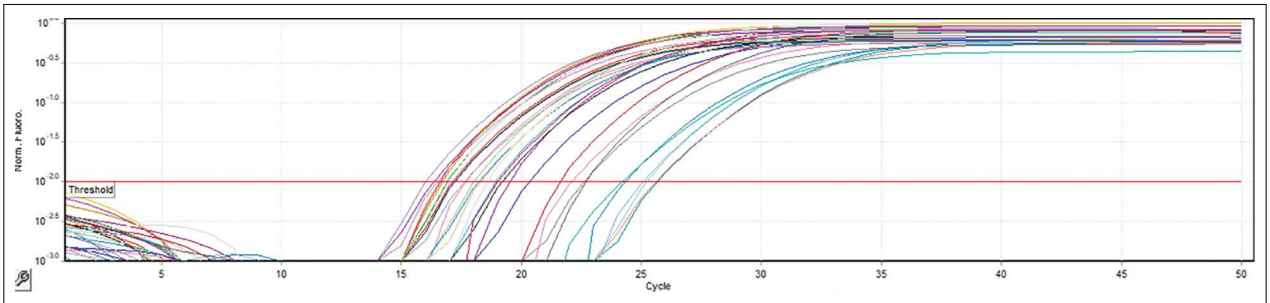
DNA Isolation

Genomic DNA isolation was performed using Invitrogen PureLink Genomic DNA kits using 25 mg of tissue for each sample in accordance with the manufacturer's instructions. For each sample, 25 mg of prostate tissue was incubated mixed with 180 ul PureLink genomic digestion buffer and 20 ul proteinase K in 55°C for 4 h to overnight. The resulting lysate was centrifuged at full speed for 3 min and the sediments were removed. 20 ul RNAase A was added and incubated at room temperature for 2 min. 200 ul genomic binding/lysis buffer was added and homogenized with vortex, then 200 ul 96-100% ethanol was added and vortexed again. The lysate with a final volume of approximately 640 ul was added to the spin column and centrifuged for 1 min at 10000 g. The fluid that went under the membrane was discarded. 500 ul wash buffer 1 was added to the spin column, centrifuged at 10000 g for 1 min and the liquid underneath was discarded, 500 ul wash buffer 2 was added to the column, 3 min after centrifuge at maximum speed, the liquid that passed below was discarded. The spin column was placed in a 1.5 ml tube, elution with 40 ul PureLink genomic elution buffer was performed with the help of centrifuge. DNA concentration and purity parameters were evaluated by nanodrop.

Methylation Analysis

Methylation analysis was performed in accordance with manufacturer's instructions using Qiagen Epitect Methyl II PCR assay kits. For each sample, 125 ng genomic DNA was mixed with 13 ul restriction digestion buffer up to 60 ul final volume with varying amounts of water, and vortexed. This prepared mix was taken 14 ul at a time and distributed in 4 separate tubes. Enzyme reaction tubes of four different contents were created by placing 1 ul water in the first of these four tubes, 0.5 ul methylation sensitive enzyme A and 0.5 ul water in the second, 0.5 ul methylation dependent enzyme B and 0.5 ul water in the third, and 0.5 ul of each enzyme A and enzyme B in the fourth tube. Thus, the first tube was an enzyme-free mock tube, the second tube contained only enzyme A Ms (methylation sensitive), the third tube contained only enzyme B Md (methylation dependent), and the fourth tube was Msd (sensitive and dependent) creating 4 reaction tubes with a total volume of 15 ul each. Each tube was incubated at 37°C for 6 h and digestion reaction was performed, then the tubes were incubated at 65°C at 20 min to stop the enzyme activity and then stored at -20 degrees for later use in the real time PCR stage.

The qPCR reaction was established with a total volume of 10 ul and with 36 tubes plate in the Qiagen rotor gene q device. The reaction was established with 5 ul Qiagen SYBR green, 0.4 ul Qiagen primary mix (HOXD3 and PCDH17), 2 ul digestion reaction material prepared in the previous stage and 2.6 ul water. For each primer, 4 tubes were created with the final volume of 10 ul for each gene and each sample ; Mo, Ms, Md, Msd, accordingly Mock, enzyme A, enzyme B, and enzyme A+B. qPCR protocol was set to be 95°C 10 min 1 cycle, 99°C 30 s and 72°C 1 min 3 cycle, 97°C 15 s and 72°C 1 min (SYBR reading) 40 cycles in accordance with manufacturer's guidelines, and a melt curve analysis protocol was added. To get reliable results, qPCR reactions were performed in duplicates for each tube, for total of two different gene regions and for each sample examined for methylation. Mo, Ms, Md, and Msd qPCR Ct results were exported to Microsoft Excel from Qiagen rotor gene q 2.1.0.9 software with the threshold value of 0.01 for each reading (Supplemental Fig. 1). CT data for each 4 tubes of each sample and each primer were arranged and appropriately applied into the Epitect Methyl II qPCR primary data analysis Microsoft Excel worksheet, which per-



Suppl. Fig. 1. A representative image of Real-Time PCR results before analysis. Ct values were calculated as described in materials and methods.
PCR: Polymerase Chain Reaction; Ct: Cycle threshold.

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forms methylation analysis for the specific gene region by comparing Mo, Ms, Md, and Msd CT values through specific equations and was specially prepared by Qiagen for the kit, then the results of methylation rates were obtained. Provided by Qiagen, this worksheet was a tool that automatically performs the calculations that the manufacturer had already provided in the kit manual. After the methylation percentages were determined for each sample and each gene region, the necessary statistical analysis was performed.

Statistical Analysis

Differences in gene promoter methylation levels between prostate cancer patients and controls were analyzed and graphs were obtained in GraphPad Prism V7 program (San Diego, CA, USA) using Student's t test. For epidemiological data analysis, Statistical Package for Social Sciences (SPSS) for Windows 22.0 (SPSS Inc., Chicago, Ill., USA) was used. The study data were shown as mean and standard deviation as data fit the normal distribution by Kolmogorov–Smirnov test. Spearman's correlation test was applied for correlation analysis. A $p<0.05$ was accepted as significant.

Results

Study Population

Patients demographics and clinical data are given in Tables 1 and 2. Gleason Scores and ISUP Grades are given in Tables 3 and 4. Total 46 prostate tumor and 36 control DNA samples were used for methylation analysis due to failure in qPCR reaction as a result of low DNA quality or amount of samples.

HOXD3 Promoter Methylation

HOXD3 promoter methylation level was determined using real-time prob-based PCR analysis (qMSP). HOXD3 promoter was found 57.8 % methylated (42.11% unmethylated) in patients while the methylation level was 43.06% (56.93% unmethylated) in the control group. As compared to the controls, HOXD3 promoter methylation was detected significantly higher in prostate cancer patients (p=0.0198) (Fig. 1 and Table 5).

PCDH17 Promoter Methylation

PCDH17 promoter methylation level was also detected using the same qMSP protocol. Mean methylation level was calculated as 37.22% (62.77% unmethylated) in patients with prostate cancer. In the control group, methylation level was determined as 23.04% (76.95% unmethylated). PCDH17 methylation was detected

Table 1 Characteristics of the study population

Characteristic	Mean±SD
Age	67.17±7.57
Height	170.86±5.33
Weight	78.46±10.60
BMI	26.83±3.15
Age at diagnosis	67.00±7.39
PSA	14.31±29.40
Smoking pack/year	26.2 ±17.5

BMI: Body mass index; PSA: Prostate-specific antigen

Table 2 Clinicopathological characteristics of patients (n=46)

Characteristic	n	%
Smoking (n=46)		
None	8	17.39
<20	15	32.60
20<	23	50
Family history (n=46)		
No	40	86.95
Yes	6	13.04
Comorbidity (n=46)		
No	12	26.08
Yes	34	73.91
High Blood Pressure		
Yes	21	45.65
Diabetes Mellitus		
Yes	10	21.73
Chronic obstructive pulmonary disease		
Yes	2	4.34
Hypothyroidism		
Yes	3	6.52
Diagnosis Stage (n=46)		
Localized psa T1-2	38	82.60
Locally advanced psa T3-4	6	13.04
Metastatic pca N+and/or M+	2	4.34
Recent stage (n=46)		
Localized pca	36	78.26
Locally advanced pca	7	15.21
Metastatic pca	3	6.52
Biochemical Recurrence (n=46)		
Yes	10	21.73
No	36	78.26
Progression (n=46)		
Yes	3	6.52
No	43	93.47

psa: Prostate-specific antigen; pca: Prostate cancer

Table 3 Gleason Scores of prostate cancer patients

Gleason Score (n=46)	n	%
3+3	9	19.56
3+4	15	32.60
4+3	9	19.56
4+4	6	13.04
4+5	6	13.04
NA	1	2.17

NA: Not available

Table 4 ISUP Grades of prostate cancer patients

ISUP Grade (n=46)	n	%
1	9	19.56
2	15	32.60
3	9	19.56
4	6	13.04
5	6	13.04
NA	1	2.17

ISUP: International Society of Urological Pathology; NA: Not available

higher in the patients as compared to the control group and this difference was statistically significant (p=0.0386) (Fig. 2 and Table 6).

Promoter Methylation and PSA Level

To analyze prognostic significance of HOXD3 and PCDH17 gene promoter methylation levels in prostate cancer, correlation analysis was applied to seek for association of methylation status and PSA level. A week but positive correlation was found for HOXD3 (Spearman's rho=0.259, p=0.02) and PCDH17 gene methylation (Spearman's rho=0.324, p=0.006) and PSA levels of patients. It appears that increase in methylation status of genes associated with elevated PSA level (Fig. 3).

Discussion

Prostate cancer is one of the most common cancer especially in elderly men.[3] Most of the prostate tumors can be detected at the local stage and cured by radical prostatectomy. However, a large number of patients have biochemical recurrence or develop metastatic cancer.[7] Prostate cancer is a heterogenous disease as other cancer types and scientist have applied multiple approaches to develop reliable, novel detection methods.[16] Aberrant DNA methylation have been shown to play important roles in cancer development and progression.[9] Determination of DNA methylation has been suggested to involve in the development of diagnostic biomarkers as well as identification of therapeutic targets.[10] In addition to PSA detection, methylation status of several genes either individually or in combination has been studied in prostate cancer to discover reliable biomarkers in the diagnosis of the disease.[17]

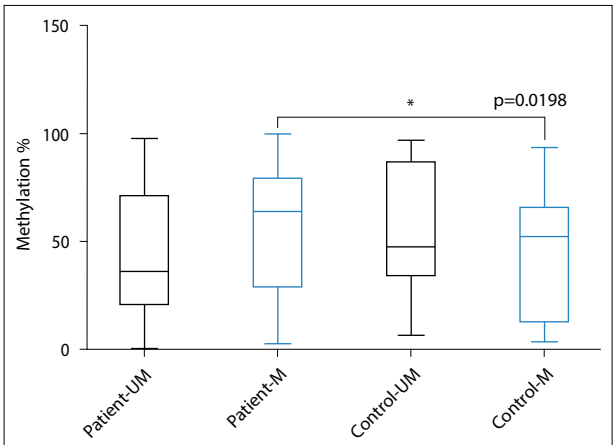

Fig. 1. HOXD3 promoter methylation level in prostate cancer patients and in control group. Data presented as mean±Standart Deviation (SD).
HOXD3: Homeobox D3, UM: Unmetile, M: Metile.

Table 5 HOXD3 promoter methylation levels in prostate cancer patients and controls

	Patients (n=46)		Control (n=36)		
Gene	UM	M	UM	M	p
HOXD3, %	42.11	57.80	56.93	43.06	0.0198

HOXD3: Homeobox D3; UM: Unmethylated; M: Methylated

In this study, we determined the promoter methylation of HOXD3 and PCDH17 genes by quantitative Methylation-Specific PCR (qMSP). HOXD3 is a member of Homeobox genes and they are a family of transcription factors.[18] Methylation of homeobox genes has been determined as a common event in prostate cancer and especially methylation of HOXD3 gene have been shown to promote gene silencing.[19] The relationship between HOXD3 methylation and prognosis of prostate cancer has been previously determined. HOXD3 methylation has been found significantly increased in patients with higher Gleason score, an indicator of disease progression.[20] This data proposed that HOXD3 methylation level might have a

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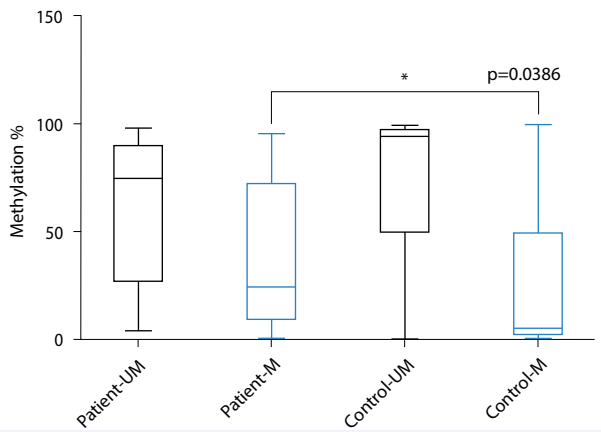

Fig. 2. PCDH17 promoter methylation level in prostate cancer patients and in control group. Data presented as mean±Standart Deviation (SD).
PCDH17: Protocadherin 17, UM: Unmethylated, M: Methylated.

Table 6 Mean promoter methylation levels in prostate cancer patients and controls

	Patients (n=46)		Control (n=36)		
Gene	**UM**	**M**	**UM**	**M**	**p**
PCDH17, %	62.77	37.22	76.95	23.04	0.0386

PCDH17: Protocadherin 17; UM: Unmethylated; M: Methylated

prognostic value. In addition to individual methylation status, HOXD3 was combined with TGF-β, RASSF1A and APC genes to identify a panel of epigenetic markers.[21] It was found that the presence of high methylation levels in two or more genes mentioned above might predict biochemical recurrence.[21] In another study, CRIP3 gene methylation was analyzed together with HOXD3, TGF-β, and APC genes. Similar to our study, qMSP was applied to determine methylation status.[22] It was found that when combined with PSA level at diagnosis, methylation status of this gene panel might be indicator of biochemical recurrence.[22] In our study, we determined that HOXD3 methylation level was significantly higher in patients with prostate cancer as compared to healthy controls.

Recently, cadherin protein superfamily gained much attraction by scientist in cancer research. Expression of classical cadherins, protocadherins (PCDH) and cadherin-related proteins have been associated with various steps in cancer development and progression.[23] PCDHs are located on chromosome 13q21.2 and their downregulation due to promoter methylation has been determined in various cancer types.[15] PCDH17, a member of PCDHs, have been identified as a tumor suppressor gene and it is often found inactivated by promoter methylation in different tumors, including laryngeal squamous cell carcinoma,[24] urothelial carcinomas,[25] bladder cancer,[26] gastric, and colorectal cancers.[27] In patients with prostate cancer,

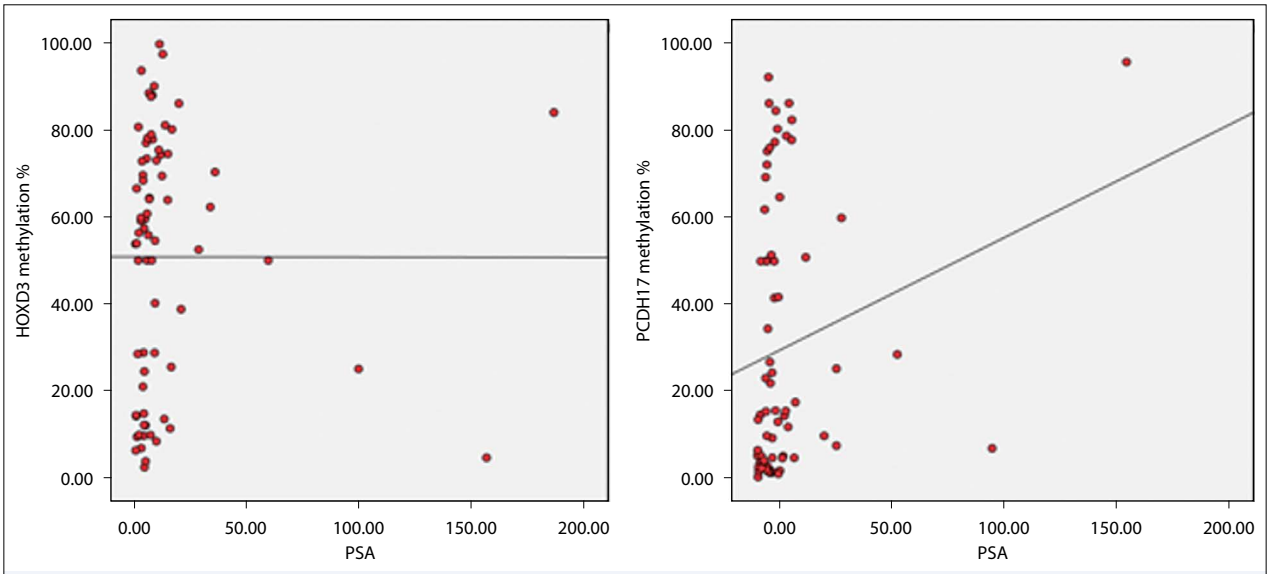

Fig. 3. Correlation of HOXD3 and PCDH17 promoter methylation and PSA level in prostate cancer patients. R^2=5.266E-7 and 0.040, respectively.
HOXD3: Homeobox D3; PCDH17: Protocadherin 17; PSA: Prostate-specific antigen.

regarding to its clinical significance, it was shown that methylation of PCDH17 is significantly associated with higher Gleason score, advance pathological stage and high level of pre-operative PSA level.[28]

In this study, PCDH17 methylation level was also determined significantly higher in patients with prostate cancer compared to controls. Furthermore, both HOXD3 and PCDH17 promoter methylation showed significant but weak correlation with pre-operative PSA level. This weak correlation could be explained by the small number of our study group. This was the limitation of our study. In addition, among our study group, we had clinicopathological parameters for fewer patients, which prevented us to obtain substantial data on prognostic value of HOXD3 and PCDH17 genes.

To conclude, in compliance with the literature, our results showed that HOXD3 and PCDH17 promoter methylation levels are higher in patients with prostate cancer. Further studies with large sample cohorts and clinicopathological data will enlighten presumptive role of HOXD3 and PCDH17 methylation in development of novel diagnostic and prognostic markers in prostate cancer.

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