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RESEARCH

Methylation of Integrin α4 and E-Cadherin Genes in Human Prostate Cancer

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Abstract Prostate cancer is the second most common malignancy in men worldwide. Abnormal epigenetic alterations such as DNA methylation and histone modification play an important role in tumor initiation, progression and regulation of cancer-related genes such as integrin α4 and E-cadherin. Expression of these genes was determined by semi-quantitative reverse transcriptase-PCR in prostate cancer cell lines, DU145 and PC3, before and after treatment with 5-aza-2-deoxycytidine and trichostatin A. Laser capture microdissection microscopy was used to obtain exclusively affected epithelial cells from prostate gland biopsies of 30 patients with prostate cancer and 40 with benign prostate hyperplasia. DNA bisulfite modifications followed by methylation-specific PCR were used to evaluate the promoter methylation status of E-cadherin and α4 integrin genes in extracted DNA from patients and aforementioned cell lines. The integrin α4 promoter in DU145 was fully methylated, whereas in PC3 cells, partial methylation was detected. E-cadherin was expressed in both cell lines; trichostatin A and 5-aza-2-deoxycytidine treatment had no effect on E-cadherin expression, however the combined treatment of both drugs or 5-aza-2-deoxycytidine alone increased integrin α4 expression. Integrin α4 and E-cadherin were hypermethylated in 66.6 % and 6.6 % of prostate cancer cases, respectively; no hypermethylation was observed in patients with benign prostate hyperplasia. These results together suggest that aberrant DNA methylation is one of the mechanisms involved in integrin α4 expression and may play an important role in human prostate carcinogenesis. In addition, the higher rate of integrin α4 gene methylation in prostate cancer patients elects it as a potential molecular tumor marker.

Keywords Integrin α_4 · E-Cadherin · DU145 · PC3 · Prostate cancer · DNA methylation

Abbreviations

BPH	Benign prostate hyperplasia
MSP	Methylation-specific PCR
TSA	Trichostatin A
5-aza-dC	5-aza-2-deoxycytidine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
RT-PCR	Reverse transcriptase polymerase chain reaction

Introduction

Prostate cancer is the second most common malignancy and the second most frequent cause of cancer related death in men older than 50 years in western countries [1, 2]. This cancer shows a high level of biological diversity and can exist as localized disease within the prostate, or become highly invasive and metastasize to the brain, lymph nodes and bone [3, 4].

In normal tissue, cells are held together and to extracellular matrix proteins by cell adhesion molecules such as cadherins

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and integrins. Cell adhesion molecules play a critical role in cancer progression and metastasis [5, 6]. Cadherins are transmembrane glycoproteins involved in Ca^{2+}-dependent intercellular adhesion. Epithelial (E)-cadherin, a member of this superfamily, is expressed mainly on the surface of epithelial cells, where it preserves the normal tissue architecture, and its expression is often decreased in invasive or metastatic tumors [6].

Integrins are cell-surface receptors that mediate cell-cell and cell-extracellular matrix interactions, and control tumor cell migration by regulating the adhesive ability of tumor cells [7, 8]. Integrin α4 forms complexes with integrin β1 and β7, which are involved in cell adhesion to fibronectin [9]. A study by Qian et al. showed that high expression of integrin α4 is associated with a loss of tumorigenicity [10].

DNA methylation and histone modifications work together as part of an epigenetic program that integrates gene-silencing [5]. Aberrant methylation of the promoter CpG island of human genes is a mechanism that contributes to the development of human tumors by transcriptional repression of several tumor suppressor genes such as Rb and $p16^{INK4a}$, which can be reactivated by treatment with 5-aza-2′-deoxycytidine (5-Aza-dC), a well-established inhibitor of DNA methyltransferase [11–16]. Experimental evidence indicates that E-cadherin disappears either in part or entirely in malignancies [17]. E-cadherin down regulation by CpG island hyper methylation plays an important role in cancer invasion and dissemination [18].

Histone deacetylation is another mechanism involved in transcriptional repression of genes [19, 20]. This modification at the E-cadherin promoter mediates repression by recruiting heterochromatin-inducing factors, which can be reversed by trichostatin A (TSA) treatment [21]. The linkage between DNA methylation and histone deacetylation in silencing genes has been addressed before, which bolsters the importance of using 5-aza-dC and TSA together to re-express silenced genes [22].

A preliminary study in our lab had shown no integrin α4 expression in the prostate cancer cell line, DU145. Therefore, in this study we examined whether promoter methylation or histone deacetylation inactivates integrin α4 and E-cadherin gene expression in prostate cancer cell lines, DU145 and PC3. The methylation status of integrin α4 and E-cadherin genes in prostate cancer and BPH patients were also invetigaterd. Overall the results of this study indicated that loss of α4 integrin in human prostate cancer may be due to aberrent DNA methylation which was detected in 66.6 % of prostate cancer patients.

Methods

Cell Culture

DU145 and PC3 human prostate cancer cell lines were obtained from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). Both cell lines were cultured in RPMI 1640 (Biosera, London, UK) containing 10 % fetal bovine serum (Gibco, London, UK), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 C in 5 % CO_2 humidified atmosphere. Cell lines were grown in T-25 flasks and after 24 h, cells were exposed to either 5-aza-dC, (Sigma) at 5 μM for 72 h or TSA (Sigma) at 50 nM for 24 h. Media with 5-aza-dC and/or TSA were changed every 24 h.

Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted from DU145 and PC3 cells with Tripure isolation reagent (Roche, Germany). Total RNA (1 μg) from each sample was used for first-strand cDNA synthesis (cDNA synthesis kit, Fermentase, Lithuania). The prepared cDNA (2.5 μL) was used to study integrin α4, E-cadherin and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression as previously instructed [6, 20, 23]. GAPDH was used as an internal control. Details of the primers used are shown in Table 1. The PCR products (10 μL) were visualized in a 1.5 % ethidium bromide-stained agarose gels.

Study Population and Samples

Appropriate paraffin blocks of formalin-fixed prostate tissues from cancer patients (30) that had radical prostatectomy (from 2004 to 2009) and from BPH patients (40) whom underwent transurethral resection between 2005 and 2007, were retrieved from the department of pathology, Shiraz University of Medical Sciences. H&E-stained section from each sample was evaluated and the suitability of inclusion for the study was determined. All samples of prostate cancer were microscopically dissected to determine the Gleason score, which was<5 in 6 cases, between 5 and 7 (3+4) in 5 cases, and ≥ 7 (4+3) in 19 cases.

Additionally, 5 μm thick sections were cut from biopsies and mounted on Laser Capture Microdissection Microscopy (LCM) slides, deparaffinized with xylene and stained with hematoxylin (without eosin), dissected with the MMI Cell cut (Molecular Machines & Industries, Switzerland), and captured with MMI isolation caps.

Methylation-Specific PCR

DNA was extracted from cell lines using QIAamp DNA mini kit and from laser microdissected sections using a QIAamp DNA FFPE tissue Kit (Qiagen). The concentration and purity of DNA were assessed spectrophotometrically.

Bisulfate conversion of extracted DNA (200 ng) and the positive control for methylated alleles (CPGenome Universal Methylated DNA, UMD, Chemicon) were done using imprint

Table 1 Primer sequence pairs used in the study

Gene	Primer sequence	Temp (°C)	Product size (bp)
E-cadherin	F-GGAAGTCAGTTCAGACTCCAGCC	62	325
	R-AGGCCTTTTGACTGTAATCACACC		
Integrin α4	F-CTCGCCAACGCTTCAGTGATC	60	291
	R-TCGTAAATCAGGGGGCACTCC		
GAPDH	F-ATCTTCCAGGAGGGAGATCC	61	509
	R-ACCACTGACACGTTGGCAGT		
E-cadherin methylated	F-TTAGGTTAGAGGGTTATCGCGT	60	116
	R-TAACTAAAAATTCACCTACCGAC		
E-cadherin unmethylated	F-TAATTTTAGGTTAGAGGGTTATTGT	58	97
	R-CACAACCAATCAACAACAACACA		
Integrin α4 methylated	F-GAGTTATTTCGCGTTTTG	58	186
	R-GCGCTACTTCTCCGAATA		
Integrin α4 unmethylated	F-TAGAGTTATTTTGTGTTTTG	54	193
	R-ACACTACTTCTCCAAATACA		

DNA modification kit (Sigma). The methylation status of the promoter region of E-cadherin and integrin α4 was examined by methylation-specific PCR (MSP) as mentioned before [12, 24]. Primers and PCR used are shown in Table 1. The PCR products were evaluated by an ethidium bromide contained agarose gel electrophoresis.

Statistical Analysis

Statistical analyses were performed using SPSS version 16 software package (SPSS Inc, Chicago, IL). Associations between methylation of loci, clinical and biological features were evaluated with chi-square and Fisher's exact test.

Results

RT-PCR and MSP Analysis Before Treatment With 5-aza-dC and TSA

The mRNA expression and methylation status of integrin α4 and E-cadherin was studied in prostate cancer cell lines, DU145 and PC3. As shown in Fig. 1, both cell lines expressed the E-cadherin transcripts. Integrin α4 mRNA expression wasn't observed in DU145, whereas it was detected in PC3 (Figs. 2a, 3a).

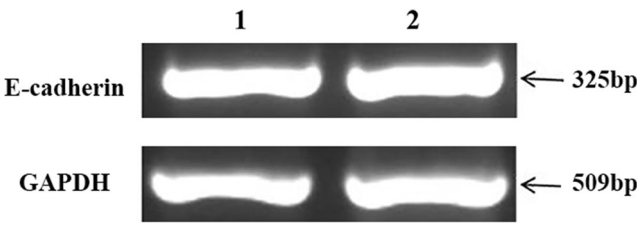

Fig. 1 RT-PCR analysis of E-cadherin gene in DU145 and PC3 cell lines before treatment with 5-Aza-dC and TSA. The expected size for E-cadherin and GAPDH PCR product were 325 bp and 509 bp, resectively. GAPDH were considered as internal control

Biallelic methylation of the integrin α4 promoter was found in DU145 (Fig. 2b), whereas in PC3, partial methylation was observed (Fig. 3b).

RT-PCR and MSP Analysis After Treatment With 5-aza-dC and TSA

To evaluate whether the aforementioned epigenetic mechanisms are involved in modulating transcriptional expression of integrin α4 and E-cadherin genes, DU145 and PC3 cell lines were treated with 5-aza-dC and TSA. Treatment with 5-aza-dC restored integrin α4 expression in DU145 (Fig. 2a); however, the restoration of integrin α4 expression was not significant after treatment with 5-aza-dC in PC3 cells (Fig. 3a). As discussed before [25], the electrophoretic bands were quantified using Syngene software. Remarkably, the combined treatment of 5-aza-dC and TSA increased integrin α4 expression 2.4-fold in DU145 (Fig. 2a) and 1.52-fold in PC3 (Fig. 3a) compared to the single treatment of 5-aza-dC.

In accordance with RT-PCR study, the single treatment of TSA has no effect on the methylation status of this gene while treating DU145 cells with 5-aza-dC resulted in augmentation of the product of unmethylated PCR primer pair. Furthermore, the dual treatment of 5-aza-dC and TSA, made the methylation pattern of integrin α4 promoter nearly disappear (Fig. 2b). However, treatment of 5-aza-dC led to a 1.5-fold increase in the unmethylated status of integrin α4 promoter in PC3, and the combined treatment synergistically led to almost a full restoration of unmethylated status of the gene (3.66-fold increase in unmethylated PCR assay's product) (Fig. 3b). No significant changes in the E-cadherin mRNA expression in either cell lines were observed after the treatments (Fig. 4a, b).

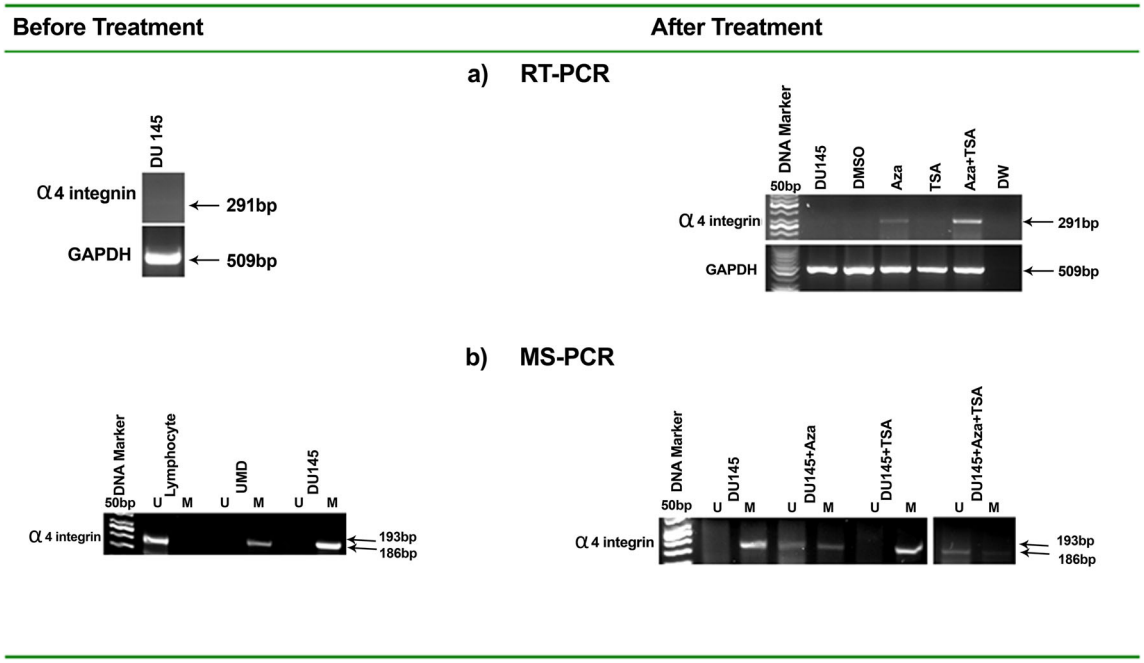

Fig. 2 Integrin α4 gene methylation and expression status before and after treatment with 5-aza-dC and TSA in DU145 cells. Integrin α4 gene selected for verification of expression by RT-PCR (**a**) and promoter methylation status by MSP (**b**). Gene descriptions are indicated on the left and gene names are shown next to the PCR results. Water (RT-PCR and MSP), DMSO, universal methylated DNA (UMD for methylation-specific PCR), lymphocyte DNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH for RT-PCR) were used as controls for Integrin α4 gene

Methylation-Specific PCR Analysis of Integrin α4 and E-Cadherin Genes in Prostate Cancer and Benign Prostate Hyperplasia

Methylation-specific PCR analysis was also done on extracted DNA from 40 BPH patients and 30 prostate cancer samples; tumor lesions were exclusively dissected by laser capture microdissection microscopy. In 3 out of 30 (10 %) cancer samples, complete integrin α4 methylation was observed, whereas in 17 out of 30 (56.6 %) partial methylation was detected. Only 2 (6.6 %) prostate cancer samples showed partial methylation for E-cadherin; the remaining samples had no evidence

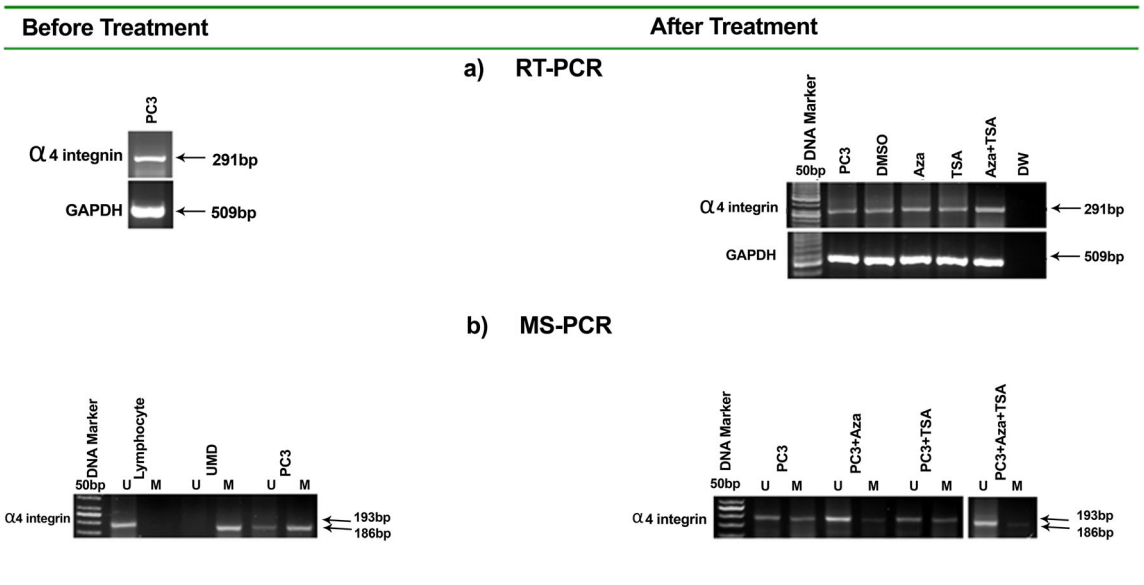

Fig. 3 Integrin α4 gene methylation and expression status before and after treatment with 5-aza-dC and TSA in PC3 cells. Integrin α4 gene selected for verification of expression by RT-PCR (**a**) and promoter methylation status by MSP (**b**). Gene descriptions are indicated on the left and gene names are shown next to the PCR results. Water (RT-PCR and MSP), DMSO, Universal methylated DNA (UMD for MSP), lymphocyte DNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH for RT-PCR) were used as controls for Integrin α4 gene

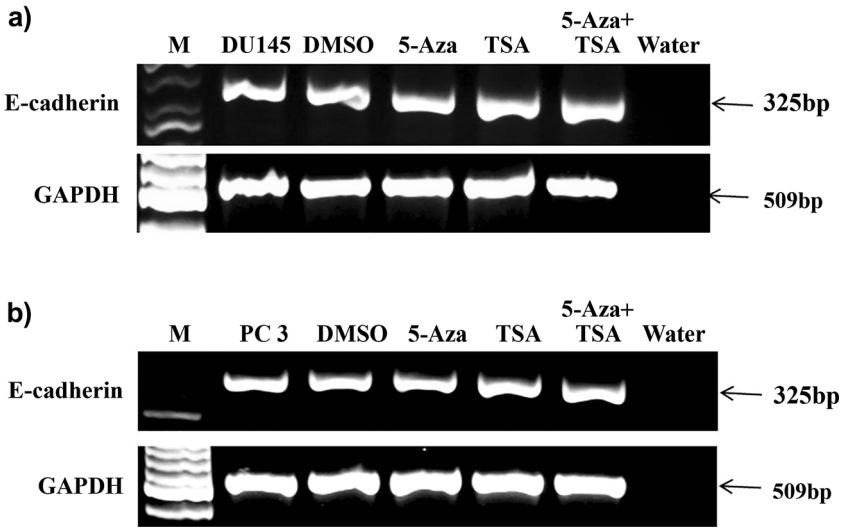

Fig. 4 E-Cadherin gene expression status before and after treatment with 5-aza-dC and TSA in (**a**) DU145 cells and (**b**) PC3 cells. E-cadherin gene selected for verification of expression by RT-PCR. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as internal control. Water and DMSO were also used as controls

of methylation (Fig. 5). The clinical characteristics of prostate cancer patients and methylation status of integrin α4 and E-cadherin promoters have been summarized in Table 2.

Discussion

Prostate cancer is the most prevalent malignant tumors in males in western countries which has been shown to be affected by both genetic and epigenetic alterations [26, 27]. The epigenetic alterations play major roles in carcinogensis [28]. It is known that epigenetically repressed adhesion molecules such as integrins and E-cadherin promote tumor progression and metastasis by changing the cell-cell and cell-ECM adhesion strength [8]. The function and expression of integrins are deregulated in several types of cancers, including prostate cancer [7, 8, 10, 15, 25, 29]. The downregulation of integrin

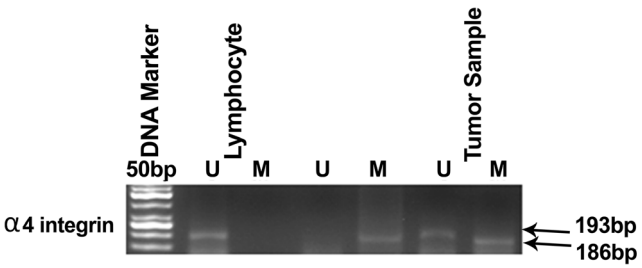

Fig. 5 Methylation-specific PCR analysis of the integrin α4 gene in prostate cancer. Representative example of methylation-specific PCR reactions for promoter methylation analysis of the integrin α4 gene in patients with prostate cancer. Lanes marked U or M indicate unmethylated or methylated genes, respectively. The expected size was 185 bp for the methylated PCR product, and 193 bp for the unmethylated PCR product of integrin α4. Lymphocyte DNA and universal methylated DNA (UMD) were used as negative and positive controls for the reactions

α4 in melanoma and fibrosarcoma cells correlates with tumor development and progression [10, 30]. In regards to prostate cancer, DNA methylation may lead to silencing of tumor suppressor genes that are involved in cell cycle control, DNA repair, apoptosis, androgen response and metastasis [31, 32]. Although the silencing effect of DNA methylation on E-cadherin transcript gene expression has been studied before [33], the underlying molecular mechanism of loss of integrin α4 expression in prostate cancer cells has yet to be surveyed. In this study, the role of DNA methylation and histone deacetylation in regulating the integrin α4 and E-cadherin expression were examined. We also investigated the occurrence of promoter methylation in E-cadherin and integrin α4 genes in prostate cancer and BPH patients. Our results show that the promoter hypermethylation of the integrin α4 gene, which was frequently detected in the prostate cancer biopsies used in this study, is involved in the transcriptional silencing of this gene [34].

Hypermethylation and histone deacetylation of the CpG islands in the promoter region are two important mechanisms that control the expression of candidate cancer driver genes [31, 35]. This study has made known that integrin α4 expression is lost in DU145 cells and mildly expressed in PC3 cells. It was previously shown that loss of integrin α4 expression in gastric cancer cell lines are due to DNA promoter hypermethylation, but not histone deacetylation. This led us to study whether any of these epigenetic alterations are involved in controlling the expression of this integrin subunit in prostate cancer cell lines. The occurrence of DNA methylation in the CpG island of integrin α4 gene was also analyzed by MSP; biallelic DNA methylation in integrin α4 gene was detected in DU145 cell lines, while PC3 only harbors a partial DNA methylation in this locus. In agreement with the study done by Park et al. [12], upon treating DU145 with the DNA methyltransferase inhibitor, 5-aza-dC, the expression of integrin α4

Table 2 Characteristics of prostate cancer patients, integrin α4 and E-cadherin promoter methylation (M) and unmethylation (U) status

Variables	N (%)	Integrin α4 (M) n (%)	Integrin α4 (U) n (%)	*P	E-cadherin (M) n (%)	E-cadherin (U) n (%)	*P
Total	30	20 (66.6)	10 (33.4)		2 (6.6)	28 (93.4)	
Age (years)							
<68.5	15 (50)	8 (53.3)	7 (46.7)	0.2	0 (0)	15 (100)	
≥68.5	15 (50)	12 (80)	3 (20)		2 (13.3)	13 (86.7)	1
Differentiation							
Well	10 (33.3)	2 (20)	8 (80)		0 (0)	10 (100)	
Moderate	12 (40)	10 (83.3)	2 (16.7)	0.00	0 (0)	10 (100)	
Poor	8 (26.6)	8 (100)	0 (0)		2 (25)	6 (75)	0.06
Poor	8 (26.6)	8 (100)	0 (0)		2 (25)	6 (75)	0.06
II	18 (60)	10 (55.5)	8 (44.5)	0.2	0 (0)	18 (100)	
III	8 (26.6)	6 (75)	2 (25)		0 (0)	18 (100)	
IV	4 (13.3)	4 (100)	0 (0)		2 (50)	2 (50)	0.5
Gleason score							
<5	10 (33.3)	4 (40)	6 (60)		0 (0)	6 (100)	
5–7	1 (3.3)	0 (0)	1 (100)	0.02	0 (0)	5 (100)	
≥7	19 (63.3	16 (80.2)	3 (15.8)		2 (10.5)	17 (89.5)	0.5

*P-value from Fisher's exact test

was reconstituted. This is in agreement with the data submitted in the GEO database by Kim H (GDS1697), which shows that the silenced α4 integrin mRNA expression in DU145 cell is restored upon treatment with 5-aza-dC [36]. Although the dual treatment of TSA and 5-aza-dC increased this integrin's expression significantly, the single treatment of TSA had no effect. In contrast, single treatment of 5-aza-dC (in accordance with the GDS1697 data) or TSA didn't significantly increase the mRNA expression in PC3 cells. However, 5-aza-dC and TSA synergistically augmented the expression of integrin α4 mRNA prominently. Overall these results indicate that DNA methylation and not histone deacetylation is the main molecular mechanism involved in the transcriptional silencing of integrin α4 gene. There is growing evidence of the linkage between DNA methylation and histone deacetylation machineries, which might explain higher efficacy of the dual treatment compared to the single treatment of 5-aza-dC. In addition, using MSP analysis, we showed that DNA methylation in the promoter region of integrin α4 occurs frequently (66.6 %) in prostate cancer patients. In contrast, no DNA methylation was detected in our BPH samples. The consequence of loss of integrin α4 expression in prostate cancer has yet to be addressed. The moderate adhesion strength mediated by cell adhesion molecules is crucial for cancer cell migration and metastasis which is regulated by different molecular mechanisms: alterations of integrins expression pattern and the secretion of matrix metaloproteinases [37, 38]. It has been shown that the integrin repository of a cancer cells has a significant impact on their migratory phenotype [37, 38]. For instance the overexpression of integrin α4 subunit has shown to suppress metastasis in gastric cancer, melanoma, and lymphoma

Even though it is known that the transcriptional silencing of E-cadherin gene, mediated by DNA methylation is common in prostate cancer, in this study the methylation status of this gene was also studied. Downregulation of E-cadherin expression is associated with de-differentiation, invasion and metastasis in cancer cells [33]. Both DU145 and PC3 cells expressed E-cadherin, a finding that confirmed the results obtained by Graff et al. [39] In agreement with this study and Li et al. [17], the E-cadherin promoter was not methylated in both cell lines.

Although Li et al. [17] reported 70 % E-cadherin methylation in advanced prostate cancer samples, the frequency of DNA methylation of E-cadherin gene in our sample population was 6.6 %. The reason for this discrepancy might be due to differences in the sample size and/or the tumor stage of the samples used in these studies.

Overall the results of this study indicated that loss of α4 integrin in human prostate cancer is due to aberrent DNA methylation which was detected in majority of prostate cancer patients. This candidates integrin α4 as a molecular marker in prostate cancer. The consequence of loss of integrin expression in prostate cancer cells needs to be studied further.

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Conflict of Interest The authors declare that they have no conflict of interest.

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