Differential gene methylation patterns in cancerous and non-cancerous cells

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Abstract. Large-scale projects, such as The Cancer Genome Atlas (TCGA), Human Epigenome Project (HEP) and Human Epigenome Atlas (HEA), provide an insight into DNA methylation and histone modification markers. Changes in the epigenome significantly contribute to the initiation and progression of cancer. The goal of the present study was to characterize the prostate cancer malignant transformation model using the CpG island methylation pattern. The Human Prostate Cancer EpiTect Methyl II Signature PCR Array was used to evaluate the methylation status of 22 genes in prostate cancer cell lines: PC3, PC3M, PC3MPro4 and PC3MLN4, each representing different metastatic potential *in vivo*. Subsequently, it was ascertained whether DNA methylation plays a role in the expression of these genes in prostate cancer cells. Hypermethylation of *APC, DKK3, GPX3, GSTP1, MGMT, PTGS2*, *RASSF1, TIMP2* and *TNFRSF10D* resulted in downregulation of their expression in prostate cancer cell lines as compared to WT fibroblasts. Mining of the TCGA data deposited in the MetHC database found increases in the methylation status of these 9 genes in prostate cancer patients, further supporting the role of methylation in altering the expression of these genes in prostate cancer. Future studies are warranted to investigate the role of these proteins in prostate cancer development.

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Introduction

Prostate cancer (PCa) is a heterogeneous, multifaceted and biologically complex disease. Changes in the cancer genome and epigenome have been extensively studied in recent years using innovative high throughput methods. Projects aimed at accelerating the expansion of knowledge concerning the genetic landscape of cancer, including PCa, were launched with various platforms based on next-generation sequencing (NGS) and microarrays to discover molecular aberrations at the DNA, RNA, protein and epigenetic levels. The main and first large-scale cancer genomic project was The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/). A pilot study of TCGA started approximately 10 years ago to discover major genetic alterations in large cohorts of selected tumors, i.e. brain, lung, ovarian cancer. Phase II of the project was expanded to over 30 human tumors including prostate adenocarcinoma (1). The comprehensive characterization of 333 primary prostate cancers in the TCGA Network revealed novel molecular features (1). Methylation of CpG islands in gene promoters is the main epigenetic mechanism for gene expression silencing. Aberrant methylation pattern, e.g., increased methylation frequency of tumor-suppressor genes, is a common molecular feature of the majority of human cancers including prostate cancer (2). Genes that protect cells from neoplastic transformation are not only known as tumor suppressors, but often their products function as tumor cell invasion factors and are involved in cell metabolism and DNA repair. Failure of these functions leads to carcinogenesis (3).

The necessity for precise prostate cancer diagnostics and disease prognosis encourages the search for novel biomarkers and basic scientific research. Microarray-based gene signatures are used for cancer diagnostics, tumor classification and prognosis, and prediction of response to therapies (4,5). There are several types of signatures that have been evaluated for cancer diagnostics: Based on gene expression-in the breast (5), colon (6) and lung cancers (7); based on methylation analysis-in colon cancer (8); and based on miRNA expression data (9). Profiling in prostate cancer is still at the exploration stage.

The aim of the present study was to evaluate the gene methylation profile using a prostate cancer cell line model and The Human Prostate Cancer EpiTect Methyl II Signature PCR Array designed to evaluate the following genes: *APC, CAV1, CDH1, CDKN2A, DKK3, DLC1, EDNRB, GPX3, GSTP1, MGMT, MSX1, PDLIM4, PTGS2, RARB, RASSF1, SFRP1, SLC5A8, TIMP2, TNFRSF10D, ZNF185*. For our study, we chose prostate cancer cell lines PC3, PC3M, PC3MLN4 and PC3MPro4 which shared the same origin but each of them demonstrated different levels of metastatic capabilities in a mouse model of human prostate cancer. They provided a system to associate the level of expression with malignancy.

Oncogenesis is associated with abnormal regulation of those genes, which are responsible for various functions in cells, such as cell signaling, cytoskeletal architecture, cell-cell contacts, cell motility, reorganization of the extracellular matrix (ECM) and many other mechanisms. Changes in these processes are important determinants of tumor invasion and metastasis. Molecular mechanisms underlying these processes have been under evaluation in the last few years: *APC* (10), *CAV1* (11), *CDKN2A* (12), *CDH1* (13), *DLC1* (14), *DKK3* (15), *EDNRB* (16), *GPX3* (17), *GSTP1* (18), *MGMT* (19), *MSX1* (20) *PDLIM4* (21), *PTGS2* (22), *RARB* (23), *RASSF1* (24), *SFRP1* (25), *SLC5A8* (26), *TIMP2* (27), *TNFRSF10D* (28), *ZNF185* (29).

For our study, we chose a prostate cancer metastasis model (30,31) and wild-type normal skin fibroblasts (32,33). After evaluation of methylation using The Human Prostate Cancer EpiTect Methyl II Signature PCR Array, we examined the expression status of the genes to confirm whether methylation regulated them. Although several genes [for example *APC* (34), *GPX3* (35) *PDLIM4* (21)] have been analyzed in the PC3 cell line, this was the first study to use this qPCR method to describe gene expression and methylation in PC3-derived cell lines (PC3M, PC3MLN4 and PC3MPro4). Finally, gene methylation data in prostate cancer patients derived from the TCGA project were evaluated.

Materials and methods

Cell line cultures. Prostate cancer cell lines, PC3, PC3M, PC3MLN4 and PC3MPro4 (36), and reference human WT fibroblast cell lines, VH10 and VH25 (32,33), were kindly provided by Professor S. Huang and Dr A. Bialkowska, respectively. Prostate cancer cell lines were cultured in cultured dishes with a growth area of 100 mm^2 in L-glutamine RPMI-1640 medium (GE Healthcare Life Sciences, Marlborough, MA USA). The fibroblast cell lines (VH10 and VH25) were cultured in High Glucose DMEM medium (GE Healthcare Life Sciences). RPMI and DMEM were supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences) and 1% antibiotic/antimycotic solution (GE Healthcare Life Sciences): 100 U/ml of penicillin, 100 μg/ml of streptomycin and 0.25 μg/ml of amphotericin B. The cells were maintained at 37˚C in a 5% CO_2 atmosphere and a relative humidity of 95%.

Methylation analysis of the cell lines. Methylation analysis was performed using EpiTect Methyl II PCR Array, Signature Panel (cat. no. EAHS-051Z; Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's protocol, as follows. DNA from the PC3, PC3M, PC3MLN4 and VH10 cells was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Inc.) according to the protocol, with additional incubation with RNase A. The absence of RNA contamination was tested using agarose gel electrophoresis. Subsequently, incubation with methylation-sensitive (Ms), methylation-dependent (Md), and double (Msd) restriction endonuclease was performed. After digestion, quantitative PCR (qPCR) was performed using primer mixes pre-dispensed into 96-wells to evaluate the methylation status of the 20 (from 22) following genes: *APC, CAV1, CDH1, CDKN2A, DKK3, DLC1, EDNRB, GPX3, GSTP1, MGMT, MSX1, PDLIM4, PTGS2, RARB, RASSF1, SFRP1, SLC5A8, TIMP2, TNFRSF10D* and *ZNF185*. The methylation status of selected gene promoters was analyzed using an integrated Excel-based template (SA Bioscience, Qiagen). Raw threshold cycle values of both digests along with mock digestion values were normalized, and the percentage of un/methylated DNA was automatically calculated using the MethylScreen™ technology provided under license from Orion Genomics, LLC, St. Louis, MO, USA). A heatmap was created using free on-line software-Morpheus from Broad Institute (https://software.broadinstitute.org/morpheus/).

RNA isolation and cDNA synthesis. RNA isolation was performed in the cell cultures reaching ~80% confluency in cultured dishes with a growth area of 100 mm^2. Cells were trypsinized and centrifuged (300 x g, 5 min), and the cell pellet was suspended in 600 ml PBS 1X (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and transferred into 3 tubes in equal volumes (200 μl). Total RNA was isolated from each cell line in triplicates with the High Pure RNA Isolation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. RNA concentration was evaluated using NanoDrop 1000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Approximately 1 μg of each RNA sample was used to synthesize complementary DNA (cDNA) with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH) according to the manufacturer's protocol with small modifications. All cDNAs were synthesized using half of the recommended volume of the anchored-oligo$(dT)_{18}$ primer and the random hexamer primer. Denaturation the template-primer mixture was carried out by incubating the tube for 10 min at 65˚C on a thermal cycler block, followed by the addition of the remaining components of the reverse transcriptase mix (RT-mix). The reverse transcription reaction was carried out at 50˚C for 30 min and 85˚C for 5 min. The synthesized cDNA was stored at -20˚C until subsequent use.

cDNA purity control. The absence of genomic DNA in the cDNA samples was tested by cDNA amplification with a set of primers localized in an intron sequence of DNA: Forward primer (Int2F, 5'-ACATGTAATTATCATGTGAATTTATTACGA-3') and reverse primer (Int2R, 5'-CTCAGAGCTTCAGTTATG GAGA-3'). The positive control was genomic DNA routinely used in the laboratory in cDNA purity testing. Agarose gel electrophoresis was performed. Lack of contamination with genomic DNA was reflected by the lack of intron amplification products for the cDNA samples.

Gene expression analysis. Quantitative reverse transcriptase real-time PCR (RT-qPCR) was performed using two

technologies: i) TaqMan Probes-hydrolysis probes dual-labeled with a reporter fluorophore and a dark quencher dye (LightCycler® 480 Probes Master, The Universal ProbeLibrary Set, Human; both by Roche Diagnostics GmbH) specific to target gene, and ii) a double-stranded DNA binding dye (LightCycler 480 SYBR-Green; Roche Diagnostics GmbH). Analyses were performed on LightCycler 2.0 and LightCycler 480 instruments (Roche Diagnostics GmbH). The *PBGD* (for the probe-based assay) and *hMRPL19* (for the SYBR-Green assay) genes were used as a reference. Primers specific for the mRNA sequences of the analyzed genes were designed using the Universal ProbeLibrary Assay Design Center software accessible at www.universalprobelibrary.com. The primers were designed to have intron-spanning sequences to avoid false-positive signals from the possible residual genomic DNA. Samples without reverse transcriptase for each cell line and samples without RNA were used as negative controls. An amount of 2 μl of sample cDNA was added to each reaction with the *PBGD* reference gene (Universal ProbeLibrary Human PBGD Gene Assay; Roche Diagnostics GmbH). The Universal ProbeLibrary probe was 5'end-labeled with fluorescein (FAM) and 3'end-labeled with a dark quencher dye. The UPL Reference Gene probe was labeled with LightCycler® Yellow 555 at the 5'end and with a quencher dye near the 3'end. Real-time PCR was performed in dual color. The fluorescence signal was acquired in two detection channels: FAM (530 nm) and LightCycler® Yellow 555 (610 nm). Real-time PCR was conducted under the following conditions: one cycle at 95˚C/10 min; 45 cycles of denaturation (95˚C/10 sec), annealing (60˚C/30 sec) and extension (72˚C/1 sec). The expression of the second reference gene *hMRPL19* was evaluated using SYBR-Green and 1 μl of sample cDNA. PCR conditions consisted of: One cycle at 95˚C/10 min; 45 cycles of denaturation (95˚C/10 sec), annealing (60˚C/20 sec) and extension (72˚C/5 sec); one cycle of melting curve: 95˚C/5 sec, 40˚C/1 min, 97˚C, according to a previous publication (37). Relative gene expression was calculated using the ΔΔCq method (38). Gene expression was randomly tested in triplicates using the Universal ProbeLibrary Human GAPD Gene Assay (Roche Diagnostics GmbH) which accounted for the third control analysis. Any significant difference in the trends of high or low expression of the targeted gene between *PBGD* and *hMRPL19* was observed.

MethHC database. The datasets for the analysis and visualization of the methylation level of *APC, DKK3, GPX3, GSTP1, MGMT, PTGS2, RASSF1, TIMP2, TNFRSF10D* and *PDLIM4* in prostate adenocarcinoma TCGA were obtained from the MethHC web base (http://methhc.mbc.nctu.edu.tw/php/index.php) and analyzed using tools available under the MethHC open access terms (39).

Statistical analysis. RNA extraction and cDNA synthesis were performed in three biological replicates for each cell line. Gene expression was analyzed at least three times for each biological replicate, and means were calculated from nine values. Promoter methylation status was analyzed in three biological replicates for each cell line. Differences in gene expression and methylation between analyzed cell lines were determined using one-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) post hoc tests in the STATISTICA Software (StatSoft, Inc. Tulsa, OK, USA). $P \leq 0.05$ and $P \leq 0.01$ were considered as statistically significant. Statistically significant differences between the VH10 cell line and prostate cancer cell lines were marked on the figures with asterisks. Data are presented as means with standard deviation (SD).

Results

Methylation profile. The initial data to be established were the methylation levels of genes important in the carcinogenesis process in four cell lines, non-cancerous VH10 and three prostate cancer cell lines, PC3, PC3M and PC3MLN4. The Human Prostate Cancer EpiTect Methyl II Signature PCR Array was used to analyze the methylation status of 20 out of 22 gene promoters. The kit is based on DNA treatment with a methylation-sensitive and methylation-dependent restriction enzyme followed by qPCR. It was found that hypermethylation was the major mechanism of regulation of expression of the analyzed genes (Fig. 1).

An increase in the methylation status in the promoter regions of 9 genes in the prostate cancer cell lines compared to that in the VH10 cells was observed: *APC, CDKN2A, EDNRB, RASSF1, SFRP1, SLC5A8, GPX3, PTGS2* and *TIMP2* (Fig. 1). The methylation status for the first 5 genes was higher and achieved statistical significance ($P<0.01$) in all analyzed prostate cancer cell lines (PC3, PC3M and PC3MLN4) compared to the VH10 cell line and was close to 100%. *GPX3* showed an increase in the methylation level to 83% in PC3M and to 90% in PC3 and PC3MLN4 cell lines. The methylation status of the *PTGS2* gene increased to 53% in PC3, to 69% in PC3MLN4 and to 99% in PC3M cells. The *TIMP2* methylation gradually increased to 73% in PC3MLN4, to 90% in PC3M and to 99% in PC3 cells (Fig. 1). All these genes were hypomethylated in the VH10 cell line (0-3%).

Moreover, 4 genes: *DKK3, MGMT, TNFRSF10D, RARB*, were hypermethylated in PC3M and PC3MLN4 cells when compared to the PC3 and VH10 cell lines (Fig. 1). The methylation level of these genes in the VH10 and PC3 cells was in the range 0-3%. The methylation of *DKK3* and *TNFRSF10D* in the PC3M and PC3MLN4 cell lines increased to almost 100%. The *MGMT* gene showed a methylation level of 58% in PC3M and 93% in the PC3MLN4 cells. The *RARB* methylation level in PC3M and PC3MLN4 cells was 65 and 74%, respectively. For the *GSTP1* gene, a much smaller but statistically significant increase in the methylation level in PC3M and PC3MLN4 compared to the PC3 and VH10 cell lines ($P \leq 0.01$) was noted. The *GSTP1* methylation level increased from 0% in PC3 and VH10 to 6% in PC3M and 15% in the PC3MLN4 cell line.

No differences were observed in the methylation status of 4 genes: *ZNF185, CAV1, MSX1* and *DLC1*. *ZNF185* and *MSX1* were hypermethylated, while *CAV1* and *DLC1* were hypomethylated in all analyzed cell lines. The methylation level of the *CDH1* gene exceeded 20% in all analyzed cell lines: VH10 (21%), PC3MLN4 (26%), PC3M (77%) and PC3 (90%). Importantly, we found that the *PDLIM4* methylation status was higher and achieved statistical significance ($P \leq 0.01$) in PC3 (99%) compared to the VH10 cells, while in other prostate cell lines, the gene was hypomethylated (1%).

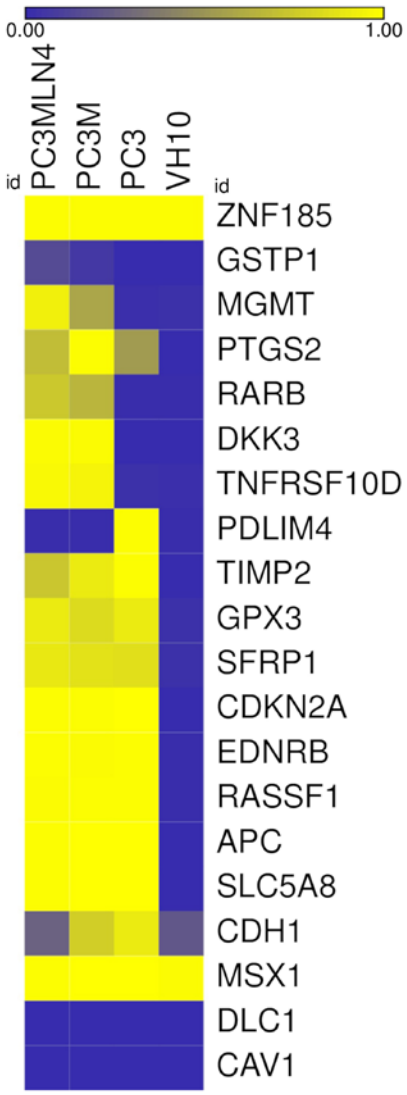

Figure 1. Heatmap of CpG methylation in PC3, PC3M, PC3MLN4 and VH10 cell lines. Each column represents an average of cell line methylation data performed in triplicates; each row represents a described gene. Methylation increases from blue (non-methylated) to yellow (methylated). *ZNF185*, zinc finger protein 185 with LIM domain; *GSTP1*, glutathione S-transferase Pi 1; *MGMT*, O-6-methylguanine-DNA methyltransferase; *PTGS2*, prostaglandin-endoperoxide synthase 2; *RARB*, retinoic acid receptor β; *DKK3*, Dickkopf WNT signaling pathway inhibitor 3; *TNFRSF10D*, TNF receptor superfamily member 10d; *PDLIM4*, PDZ and LIM domain 4; *TIMP2*, TIMP metallopeptidase inhibitor 2; *GPX3*, glutathione peroxidase 3; *SFRP1*, secreted frizzled related protein 1; *CDKN2A*, cyclin dependent kinase inhibitor 2A; *EDNRB*, endothelin receptor type B; *RASSF1*, Ras association domain family member 1; *APC*, APC, WNT signaling pathway regulator; *SLC5A8*, solute carrier family 5 member 8; *CDH1*, cadherin 1; *MSX1*, Msh homeobox 1; *DLC1*, DLC1 Rho GTPase activating protein; *CAV1*, caveolin 1.

Gene expression analysis and its correlation with the methylation pattern. This stage was conducted to ascertain whether methylation of the promoter CpG island regulates gene expression. For this purpose, gene expression alterations were analyzed using the qPCR method. Gene expression analysis included two further cell lines: One prostate cancer cell line PC3MPro4 (with an increased tumorigenic potential but a low incidence of metastasis) and wild-type fibroblasts, VH25. Gene expression in cell lines with an increasing tumorigenic and metastatic potential was analyzed (PC3MLN4 produced a higher incidence of distant metastases).

APC, DKK3, GPX3, GSTP1, MGMT, PTGS2, RASSF1, TIMP2 and TNFRSF10D gene hypermethylation downregulates gene expression. Genes whose expression was downregulated by hypermethylation were characterized. A high methylation level was associated with a decrease in expression in 9 out of the 20 analyzed genes (*APC*, *DKK3*, *GPX3*, *GSTP1*, *MGMT*, *PTGS2*, *RASSF1*, *TIMP2* and *TNFRSF10D*). However, hypermethylation caused a different degree of downregulation of these genes. The expression of all genes in prostate cancer cell lines was compared to that in the VH10 cell line.

In the case of 5 of the previously mentioned genes hypermethylated in prostate cancer cell lines versus the fibroblasts, a high methylation level was associated with downregulation of their expression (Fig. 2). The majority of gene expression results were separately normalized in respect to *PBGD* and *hMRPL19*. In reactions normalized in respect to *PBGD*, the most significant decrease in expression in prostate cancer cell lines was observed for the *PTGS2* gene (10-fold in PC3MLN4 and PC3MPro4, and 6-fold in PC3 and PC3M cells; $P \leq 0.05$). *TIMP2* expression decreased by gradually increasing factors: 5-fold in PC3MPro4, 8-fold in PC3MLN4, 10-fold in PC3M, and 80-fold in PC3 cells. *APC* was downregulated 2-fold in prostate cancer cell lines. The most significant decrease in *RASSF1* expression was noted in PC3M (9-fold), and the lowest in PC3MPro4 (~3-fold). The *GPX3* expression decreased from 12-fold in PC3MLN4 to 100-fold in PC3M when compared to the VH10 cells. However, this observation was based on the *GPX3* expression analysis using *hMRPL19* as a single reference gene. The level of the *GPX3* expression analyzed with *PBGD* as the reference gene and the TaqMan probe was undetectable (data not shown).

In contrast, *DKK3*, *GSTP1*, *MGMT* and *TNFRSF10D* were hypermethylated in the PC3M and PC3MLN4 cells, while the methylation level of these genes in the PC3 cell line was similar to that in VH10, close to 0%. A decrease in the expression of these genes was observed corresponding to the hypermethylation of their promoters (Fig. 3.)

The *DKK3* gene showed 10-fold lower expression in the PC3 cell line. Other prostate cancer cell lines showed a much greater decrease in the expression of the *DKK3* gene (100-fold). The *MGMT* gene was expressed at the lowest level in PC3MLN4 cells (8-fold lower compared to VH10), while in PC3M and PC3MPro4, the decrease was 2- and 4-fold, respectively. *TNFRSF10D* was downregulated 2-fold in prostate cancer cell lines. Interestingly, with *hMRPL19* as the reference gene, a 3-fold decrease was noted only in PC3, while in other prostate cancer cell lines, *TNFRSF10D* was expressed at a 4,000-fold lower level compared to VH10. Finally, no statistically significant differences in the expression level of *GSTP1* normalized in respect to *PBGD* were found, thus *GAPDH* was tested instead. The expression of *GSTP1* was downregulated 300-fold in PC3M and PC3MLN4, while in other cell lines it was comparable to the controls. With *hMRPL19* as the reference gene, a similar decrease was noted in all analyzed prostate cancer cell lines (3- to 4-fold). The expression of *APC, DKK3, PTGS2, RASSF1, TIMP2* after normalization in respect to h*MRPL19* was also found to be decreased, similarly as when normalized in respect to *PBGD*.

APC, DKK3, GPX3, GSTP1, MGMT, PTGS2, RASSF1, TIMP2, TNFRSF10D methylation pattern in prostate cancer

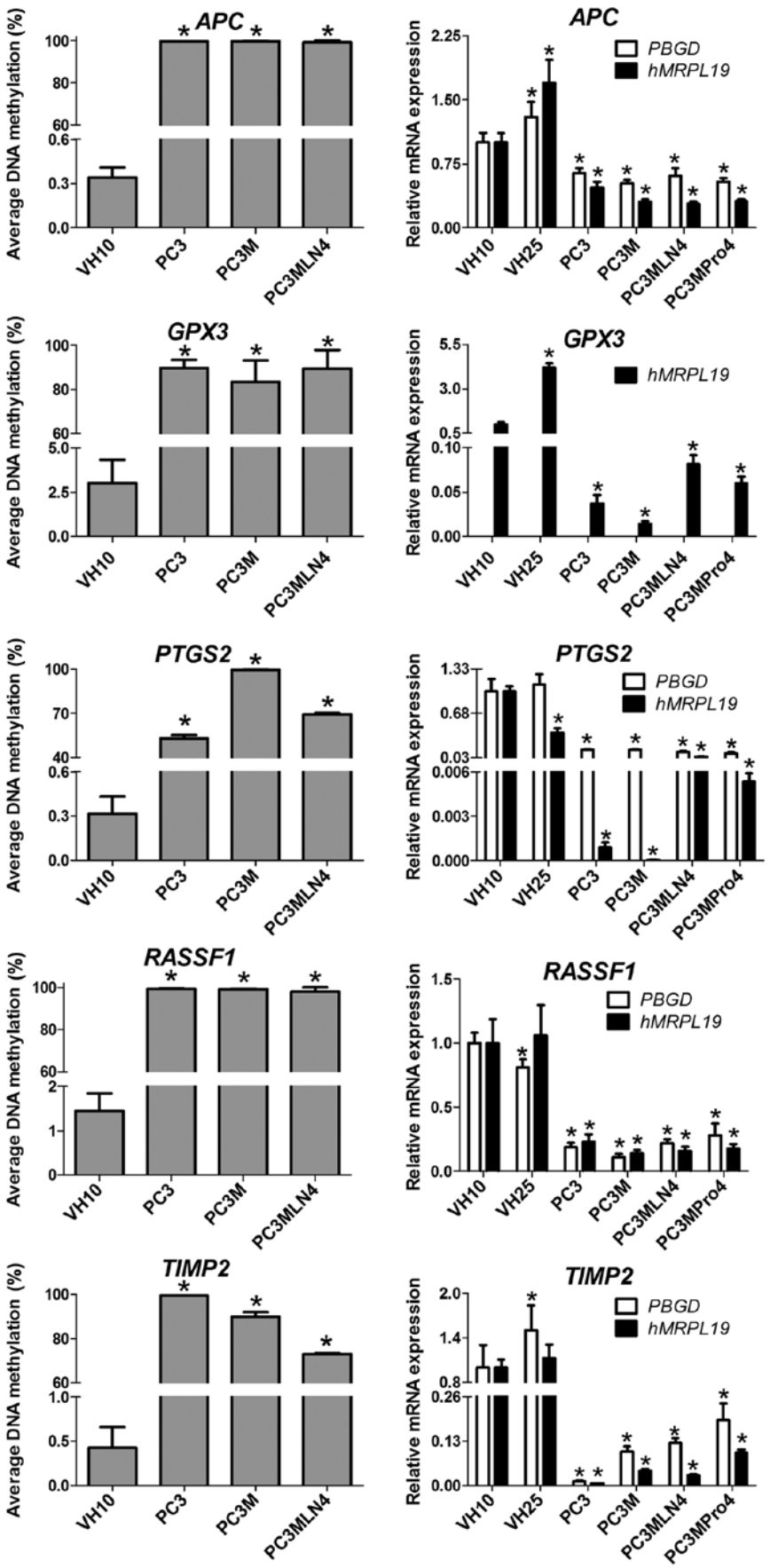

Figure 2. Gene hypermethylation is associated with downregulation of gene expression in prostate cancer cell lines. Left panels show the level of methylation of gene promoters. Gray columns represent an experiment performed in triplicates. Right panels show changes in gene expression. White and black columns represent an experiment performed at least three times in triplicates with *PBGD* and *hMRPL19* used as reference genes, respectively. The *GPX3* expression results were obtained using *hMRPL19* as a single reference gene. *P<0.01, statistically significant difference in comparison with human VH10 fibroblasts. Error bars, SD.

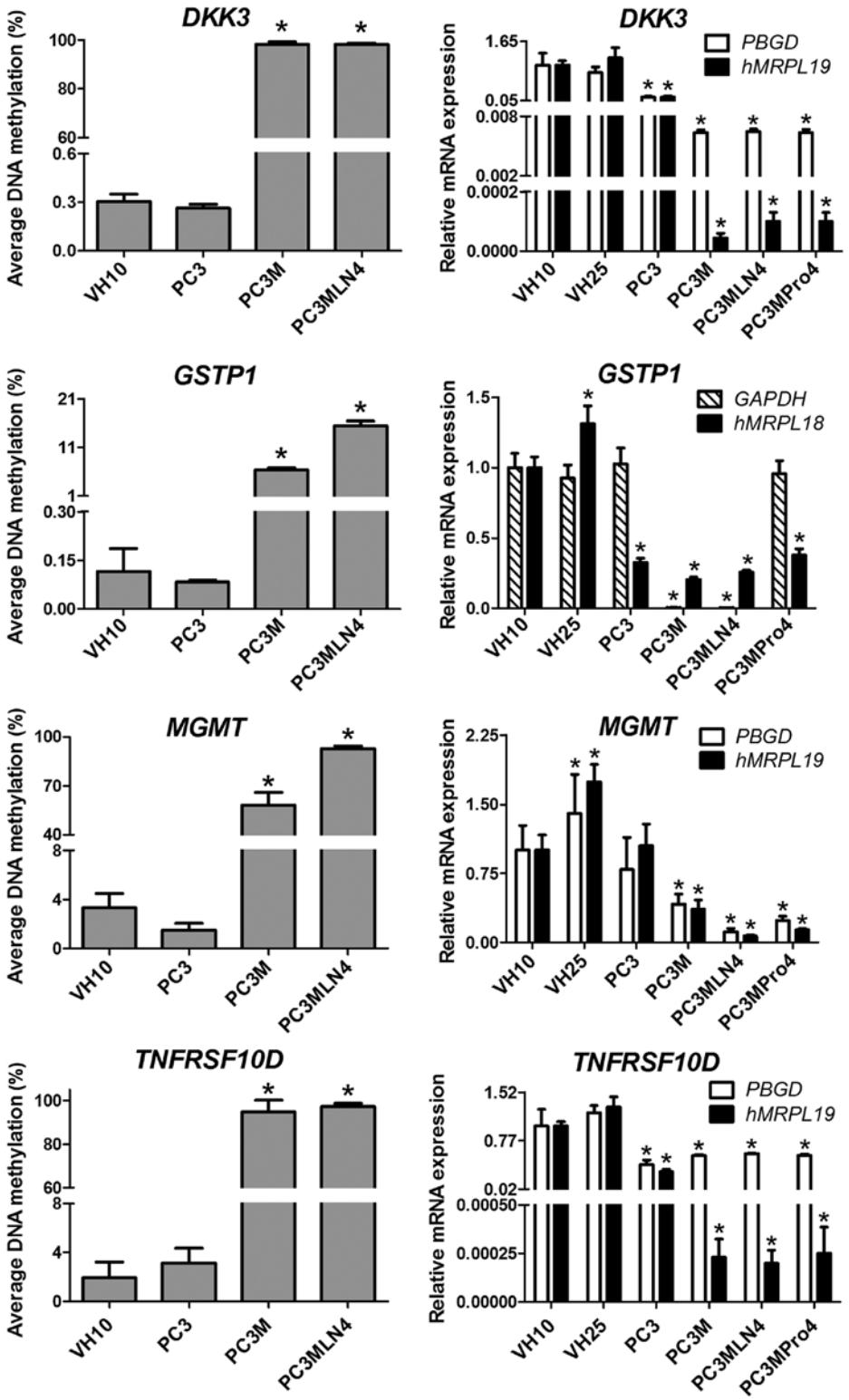

Figure 3. Gene hypermethylation is associated with downregulation of gene expression in PC3M and PC3MLN4 cells compared to PC3 and VH10 cells. Left panels show the level of methylation of gene promoters. Gray columns represent an experiment performed in triplicates. Right panels show changes in gene expression. White, black and striped columns represent an experiment performed at least three times in triplicates with *PBGD*, *hMRPL19* and *GAPDH* used as reference genes, respectively. *P<0.01, statistically significant difference in comparison with the human VH10 fibroblasts. Error bars, SD.

tissue-in silico analysis. This stage aimed to establish whether the obtained methylation results were consistent with the methylation pattern in prostate cancer tissue. As TCGA is a project which uses high-throughput technologies, it was decided to use integrated human data from TCGA deposited in the MethHC web base (39,40). The *APC*, DKK3, *GPX3, GSTP1, MGMT, PTGS2, RASSF1, TIMP2* and *TNFRSF10D* genes were hypermethylated in prostate adenocarcinoma samples compared to normal samples (which represented a normal tissue from the same group of prostate adenocarcinoma patients) with statistical

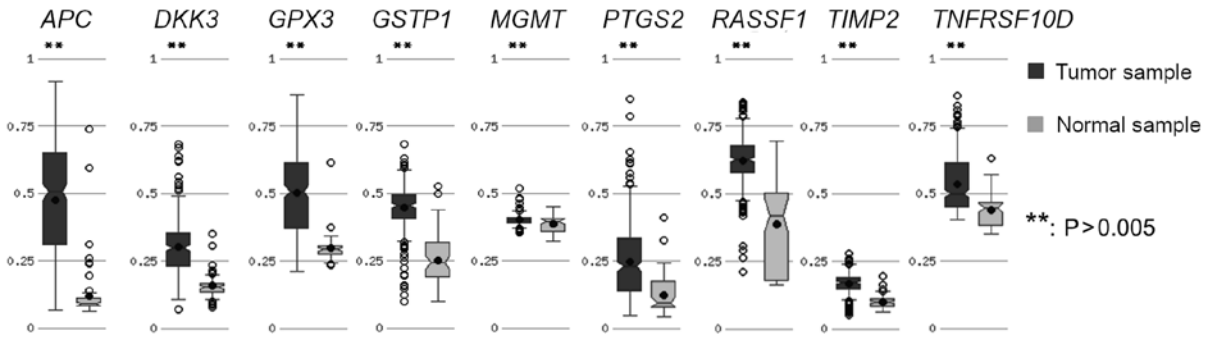

Figure 4. Methylation status of CpG islands in the promoter regions of human prostate cancer from the MethHC database (39). Analysis showed a significant difference in methylation between all presented genes in normal prostate tissue and prostate adenocarcinoma (**P≤0.005). Computational analysis was performed using a dataset (prostate adenocarcinoma TCGA) deposited and tools available under the MethHC open access terms.

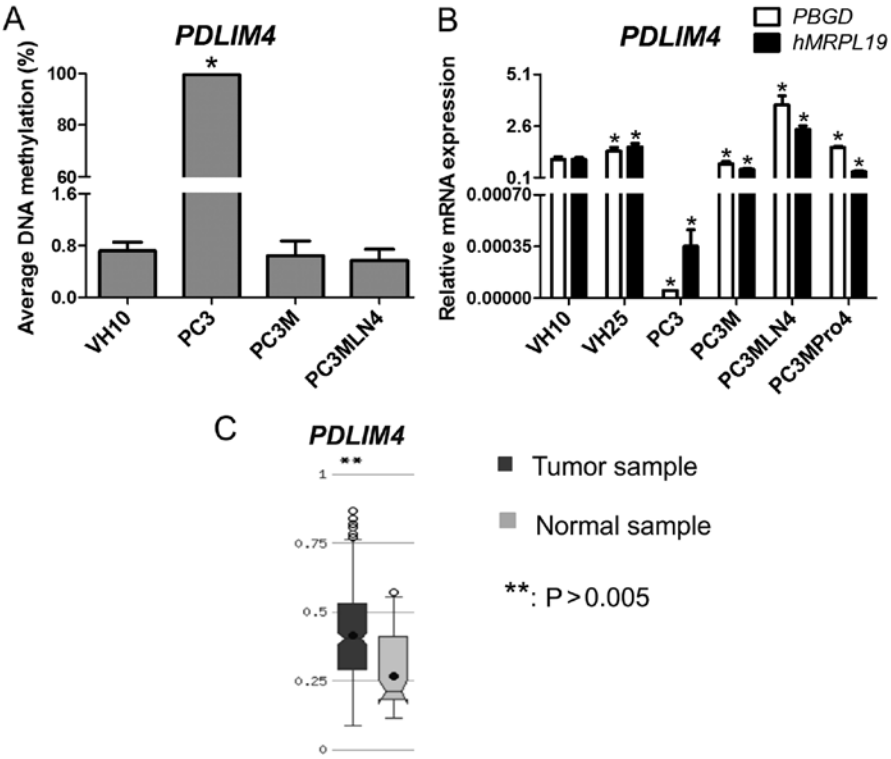

Figure 5. Methylation status of *PDLIM4* and its association with expression pattern. (A) *PDLIM4* gene hypomethylation is associated with upregulation of gene expression in prostate cancer cell lines and fibroblasts. A shows the level of methylation of gene promoters. Gray columns represent an experiment performed in triplicates. (B) Changes in gene expression are shown. White and black columns represent an experiment performed at least three times in triplicates with *PBGD* and *hMRPL19* used as reference genes, respectively. *P<0.01, statistically significant difference in comparison with the human VH10 fibroblasts. Error bars, SD. (C) Methylation status of *PDLIM4* CpG islands in the promoter regions of human prostate cancer tissue from the MethHC database (39). Analysis showed a significant difference between the *PDLIM4* methylation in normal prostate tissue and prostate adenocarcinoma (**P≤0.005). Computational analysis was performed using a deposited dataset (prostate adenocarcinoma TCGA) and tools available under MethHC open access terms.

significance at P≤0.005 (Fig. 4). The greatest difference in the level of methylation between tumor and normal sample was detected in *APC*, the lowest in *MGMT* (Fig. 4).

PDLIM4 hypomethylation upregulates gene expression. Genes whose hypomethylation was found to be associated with a significantly higher expression were analyzed. One gene classified to this group, *PDLIM4*, was hypomethylated in most tested cell lines.

PDLIM4 expression was statistically significantly higher in the PC3M, PC3MLN4 and PC3MPro4 cell lines as well as in fibroblasts compared to that in PC3 cells. *PDLIM4* was hypermethylated in PC3. In other cell lines, *PDLIM4* was hypomethylated which was associated with a various degree of gradual increasing expression: PC3M (0.77±0.13), PC3MPro4 (1.56±0.08) and PC3MLN4 (3.63±0.44). In the VH10 cell line, *PDLIM4* expression was 1.0±0.14. According to the TCGA data deposited in MethHC, the *PDLIM4* gene was hypermethylated in prostate adenocarcinoma compared to normal samples with statistical significance at P≤0.005 (Fig. 5).

Methylation pattern of the DLC1, CAV1, MSX1, ZNF185, CDKN2A, CDH1, RARB genes is not associated with gene expression. Seven genes (*DLC1, CAV1, MSX1, ZNF185,*

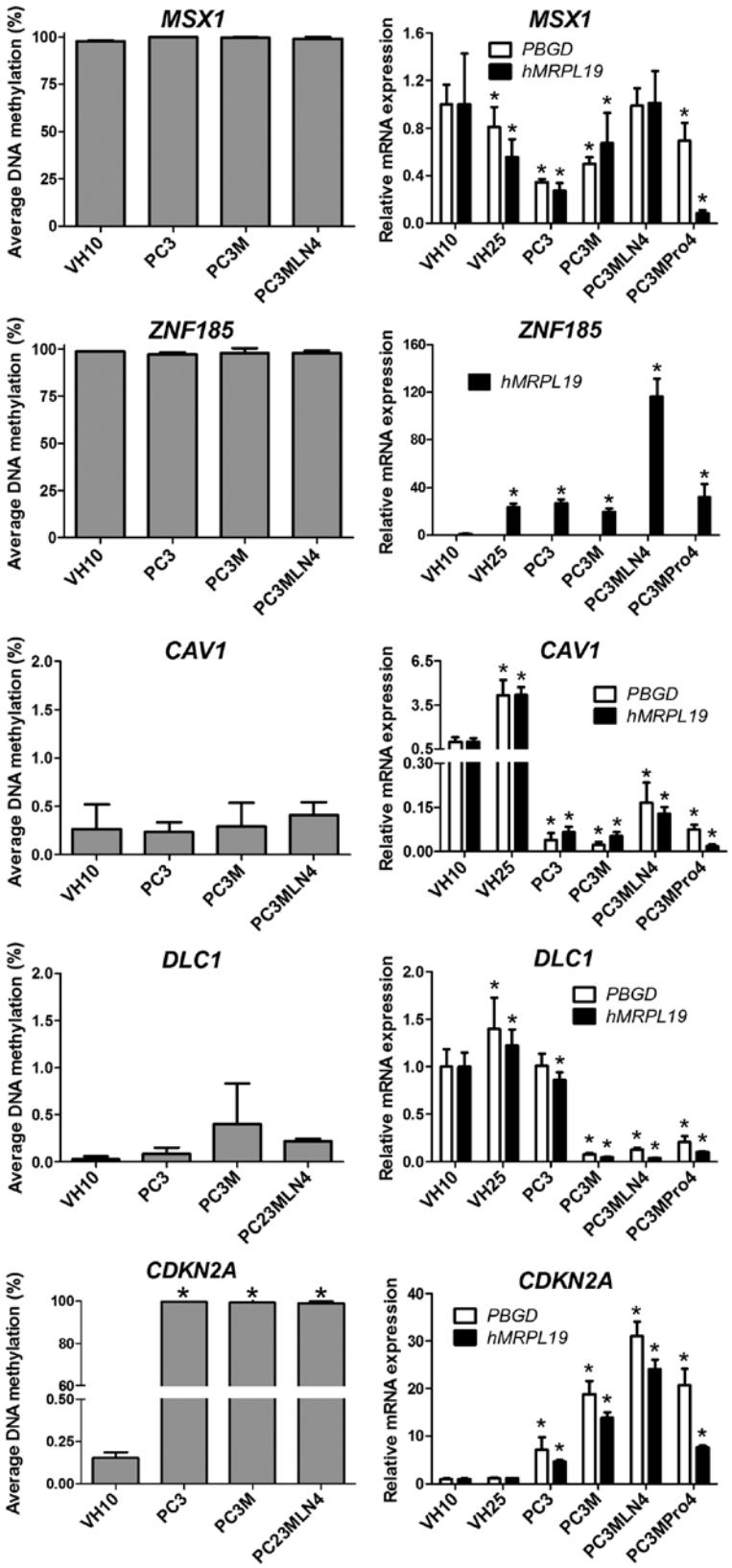

Figure 6. Methylation pattern of *DLC1, CAV1, MSX1, ZNF185, CDKN2A* genes is not associated with gene expression in prostate cancer cell lines. Left panels show the level of methylation of gene promoters. Gray columns represent an experiment performed in triplicates. Right panels show alterations in gene expression. White and black columns represent an experiment performed at least three times in triplicates with *PBGD* and *hMRPL19* used as reference genes, respectively. *P<0.01, statistically significant difference in comparison with the human VH10 fibroblasts. Error bars, SD.

CDKN2A, CDH1 and *RARB*) whose methylation pattern was not associated with gene expression were distinguished (Fig. 6).

Two genes, *DLC1* and *CAV1,* were hypomethylated in fibroblasts as well as in prostate cancer cell lines. Nevertheless,

differences in the expression levels of these genes were shown. In respect to *PBGD* as the reference gene, *DLC1* expression was at similar level in the PC3 and VH10 cell lines. In the other prostate cancer cell lines, a decrease in expression was found: 5-fold in PC3MPro4, 7.5-fold in PC3MLN4 and 12-fold in PC3M compared to that in the VH10 cell line (1.0±0.2). *CAV1* expression was downregulated in all analyzed prostate cancer cell lines: From 40-fold in PC3M to 6-fold in PC3MLN4.

Two other genes, *ZNF185* and *MSX1,* were hypermethylated in all analyzed cell lines. An interesting observation concerned *MSX1*: When only prostate cancer cell lines were considered, a gradual increase in the relative expression level was observed for PC3 (0.34±0.03), PC3M (0.50±0.05), PC3MPro4 (0.69±0.15), PC3MLN4 (0.99±0.15). However, *MSX1* expression in prostate cancer cell lines was lower than in VH10. The *ZNF185* gene was shown to be upregulated in prostate cancer cell lines: 27-fold in PC3, 19-fold in PC3M, 28-fold in PC3MPro4, and 116-fold in PC3MLN4 compared to VH10 cells. Interestingly, the values of relative expression of *ZNF185* obtained in PC3, PC3M and PC3MPro4 were comparable with the expression level in the second WT control, VH25. Therefore, they can be considered as falling within the normal range. The *ZNF185* expression results were presented with *hMRPL19* as the reference gene. In respect to *PBGD* as the reference, the gene expression pattern was consistent; however, the difference in the expression level between VH10 and PC3MLN4 was much higher (data not shown).

In the case of the *CDKN2A* gene, its methylation pattern as well as expression level differed between prostate cell lines and fibroblasts. Although the *CDKN2A* gene was hypermethylated in prostate cancer cell lines, it was upregulated in these cells compared to VH10 and VH25. The increase in expression was gradual: From 7-fold in PC3, 19-fold in PC3M, 21-fold in PC3MPro4, to 31-fold in the PC3MLN4 cell line.

Expression of *DLC1, CAV1, MSX1* and *CDKN2A* after normalization in respect to h*MRPL19* indicated a pattern similar to that obtained with *PBGD* as the reference gene. Despite the varied methylation pattern of the *CDH1* and *RARB* genes in prostate cancer cell lines and fibroblasts, no statistically significant differences in expression level were noted (data not shown).

Discussion

Abnormal expression of genes in cancer cells can arise from epigenetic changes, but also changes in the number of copies and/or the presence of sequence mutations. Changes in the epigenome are often crucial for the functioning of cells and thus are often associated with carcinogenesis, metastasis and response to chemotherapy. Therefore, unique expression and methylation patterns have been introduced in diagnostics as prognostic and predictive biomarkers. Since the best-known expression profiling performed 15 years ago in breast cancer (41), determination of the unique signature of gene expression (6,42) or promoter methylation is used more frequently, for example *MGMT* methylation in tumor tissue as a biomarker in glioma (43) or *SEPT9* methylation in plasma as a biomarker in colorectal adenocarcinoma (44).

Over the last few years, a broad spectrum of different technologies has been introduced for the quantitative and qualitative measurement of DNA methylation status. The most commonly used method is sodium bisulfite conversion of genomic DNA to differentiate and detect unmethylated and methylated cytosines using methylation-specific PCR, MassARRAY EpiTYPER, hybridization-based promoter and CpG island microarrays. In this study, a less-known methodology based on input DNA treatment with a methylation-sensitive and methylation-dependent restriction enzyme followed by qPCR was used. Commercial EpiTect Methyl II PCR Array, Signature Panel: EAHS-051Z (Qiagen) kit was selected for methylation analysis in prostate cancer to examine the methylation profile of CpG islands in 22 cancer-related genes in prostate cancer cell lines with increasing tumorigenic and metastatic potential (30). According to the EpiTect Methyl II PCR Array System manufacturer, every target region is selected within one CpG island or CpG-dense area predicted from both the UCSC database and published data with functional annotation (45). Further analyses included determination of gene expression and comparison of data from prostate cancer cell lines with the methylation status of CpG islands in the promoter regions of human prostate cancer from the MethHC database (39).

The expression and methylation status of *GSTP1, APC, RASSF1A, MGMT* and *PTGS2* have previously been well characterized in prostate cancer (46), and gene expression has frequently been evaluated using microarrays. High-throughput methods, such as microarrays, are an excellent screening tool, although the results require validation using quantitative real-time RT-PCR (qPCR) assays. Likewise, the results of methylation profiling have often been obtained using less advanced technologies, such as methylation-specific PCR (MSP) (46). Our study for the first time analyzed these genes in PC3-derived cell lines (PC3M, PC3MLN4 and PC3MPro4) in reference to fibroblasts (VH10 and VH25). qPCR used in this study allowed a precise determination of relative gene expression, normalized in respect to the expression of different reference genes.

According to the results obtained earlier in prostate cancer, it was shown that 12 genes out of 20 analyzed were hypermethylated: 8 genes were hypermethylated in prostate cancer cell lines compared to VH10 (*APC, CDKN2A, EDNRB, GPX3, PTGS2, SLC5A8, TIMP2, RASSF1)*, and 5 genes were hypermethylated in PC3M and PC3MLN4 compared to PC3 and VH10 cell lines (*DKK3, MGMT, TNFRSF10D, RARB* and *GSTP1)*. Furthermore, 3 genes (*CAV1, DLC1, PDLIM4)* were found to be hypomethylated in prostate cancer cell lines. Interestingly, methylation was found to regulate the expression of half of the analyzed genes (*APC, DKK3, GPX3, GSTP1, MGMT, PDLIM4, PTGS2, RASSF1, TIMP2* and *TNFRSF10D*) in PC3-derived cell lines and fibroblasts VH10 and VH25.

APC hypermethylation leads to the stabilization of β-catenin in the cytoplasm due to deregulation of β-catenin degradation (10). Previous studies have also demonstrated that *APC* hypermethylation is a common occurrence in the PC3 cell line (34), prostate cancer and its progression (47), as well as in other cancers, such as colon and gastric cancer (48).

DKK3 hypermethylation has been observed in pancreatic cancer lines and in breast cancer tissue (49,50). It has been found that this gene is also associated with β-catenin expression. *DKK3* overexpression in transfected cells resulted in a decrease in β-catenin expression (49). Inactivation of the *DKK3* gene is also common in prostate cancer, in which

the level of the Dkk3 protein is inversely correlated with the Gleason degree, and the lowest level was noted in tumors that are probably metastatic (51,52).

A wide-spectrum analysis of androgen-dependent (LNCaP, and Du145) and androgen-independent (PC3) prostate cancer cell lines allowed characterization of *GPX3* as a novel tumor-suppressor gene, as the level of *GPX3* expression was associated with prostate tumor stage (35). Our findings in cell lines and the MethHC database are consistent with the widespread hypermethylation of *GPX3* in prostate cancer (53-55).

The methylation status of *GSTP1*, as for *APC*, is common in prostate cancer (56,57). However, evidence of a relationship between the level of *GSTP1* methylation and disease progression is contradictory. Some studies have shown that the hypermethylation of this gene is associated with prostate cancer progression (58-60). On the other hand, the prognostic value of *GSTP1* methylation has not been demonstrated (47). In our study, methylation as well as the expression level provided prognostic information, but the methylation level increased only to 15% in PC3MLN4 cells.

MGMT is an important glioblastoma prognostic and predictive biomarker in clinical use (43). Reports regarding prostate cancer are inconclusive (46,56,61). The TCGA results showed that the increase in methylation between tumor and normal tissue was small; however, the difference was statistically significant. On the other hand, *MGMT* hypermethylation in prostate cancer has been previously reported (46,61). Our results revealed no significant difference in the *MGMT* methylation level between the PC3 and VH10 cell lines. The *MGMT* gene was hypermethylated only in the PC3M and PC3MLN4 lines, which was associated with a decrease in expression in these cell lines.

PTGS2 was also hypermethylated in all prostate cancer cell lines compared to fibroblasts. However, the methylation status of *PTGS2* varied between the prostate cancer cell lines and it seems that methylation is the main mechanism of *PTGS2* regulation in prostate cancer cell lines, as well as in prostate cancer patients-TCGA data (39). Recent studies employing qPCR for methylation analysis have shown that *PTGS2* hypermethylation is a potential sensitive and specific prostate cancer biomarker in ctDNA isolated from the blood of PCa patients (62), as well as in prostate cancer tissue (63). Although *PTGS2* hypermethylation has been observed in 68% cases of PCa versus 15% of BPH tissues (62), it seems that the methylation status of *PTGS2* alone is not sufficient. However, it definitely should be included in molecular profiling to improve efficiency.

A previous study demonstrated that *PDLIM4* can function as a tumor suppressor in prostate cancer cells (21). *PDLIM4* mRNA expression was found to be reduced in PC3 prostate cancer cells (21), which is consistent with our results, but notably, in other prostate cancer cell lines derived from PC3, *PDLIM4* expression increased gradually with the increase in PNC. Our results may suggest a novel oncogenic function of *PDLIM4* in prostate cancer cell lines derived from PC3. Putatively, methylation is the main mechanism of *PDLIM4* regulation in prostate cancer cell lines, as in renal cancer and acute myelogenous leukemia (64,65).

RASSF1 hypermethylation, which has been reported in many types of cancers, including prostate cancer, can lead to disorders in the DNA repair pathway and cell cycle control (66). The relationship between the *RASSF1* methylation level and prostate cancer aggressiveness has been noted (66,67). Downregulation of *TIMP2* has also been correlated with cancer progression and metastasis (68). The results obtained in prostate cancer indicate an antitumor effect of the Timp2 protein (69). However, reports of this phenomenon are contradictory (70). Our results showed hypermethylation of the *RASSF1* and *TIMP2* genes in prostate cancer cell lines compared to fibroblasts.

Hypermethylation of *TNFRSF10D* has been noted; for example, in melanoma and prostate cancer (71,72). In prostate cancer cell lines in our study, the *TNFRSF10D* gene was hypermethylated only in the PC3M and PC3MLN4 cells. Nevertheless, a decrease in expression was observed in all tested prostate cancer cell lines. The *TNFRSF10D* expression level was similar in all prostate cancer cell lines in respect to PBGD as the reference gene, while with hMRPL19, greater downregulation in PC3M, PC3MLN4 and PC3MPro4 was observed. This observation confirms how important it is to use at least two reference genes for the analysis of relative gene mRNA expression.

It should be noted that the results of CpG island methylation analysis in the promoter regions obtained in the prostate cancer cell lines for those 10 genes were consistent with clinical data obtained from 336 prostate cancer patients in the TCGA project. Moreover, the methylation signature panel used in this study included genes methylated in prostate cancer cell lines, but no changes in their expression (*EDNRB* and *SLC5A8*) were shown. This also included genes with an altered expression level between prostate cancer cell lines and fibroblasts, but was not consistent with the methylation pattern. Those genes are probably regulated by other mechanisms, such as small RNA molecules, e.g., miRNAs (73), changes in chromatin conformation (74) or histone modifications (75).

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

Conceptualization of the study design was achieved by MAL. The research methodology was conceived by KK, MAL, AB and SH. Software analysis of data was performed by KK and MAL; validation of the data was accomplished by KK; formal analysis was conducted by KK and MAL. Investigation was carried out by KK; resources were the responsibility of JK and MAL. Data curation was performed by MAL. Writing; original draft preparation was carried out by KK and MAL; Writing; review and editing was accomplished by AB, JK and SH; visu-

alization was conducted by KK; supervision was conducted by SH and MAL. Software analysis of data was performed by KK; validation of the data was accomplished by KK; formal analysis was conducted by KK and MAL. Investigation was carried out by KK; resources were the responsibility of JK and MAL; project administration was conducted by JK and MAL; and funding acquisition by MAL. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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