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Differentially methylated CpG sites associated with the high-risk group of prostate cancer

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Abstract: Prostate cancer (PC) is one of the most common and socially significant oncological diseases among men. Bioinformatic analysis of omics data allows identifying molecular genetic changes associated with the disease development, as well as markers of prognosis and response to therapy. Alterations in DNA methylation and histone modification profiles widely occur in malignant tumors. In this study, we analyzed changes in DNA methylation in three groups of PC patients based on data from The Cancer Genome Atlas project (TCGA, https://portal.gdc.cancer.gov): (1) high- and intermediate-risk of the tumor progression, (2) favorable and unfavorable prognoses within the high-risk group, and (3) TMPRSS2-ERG-positive (tumors with TMPRSS2-ERG fusion transcript) and TMPRSS2-ERG-free cases within the high-risk group. We found eight CpG sites (cg07548607, cg13533340, cg16643088, cg18467168, cg23324953, cg23753247, cg25773620, and cg27148952) hypermethylated in the high-risk group compared with the intermediate-risk group of PC. Seven differentially methylated CpG sites (cg00063748, cg06834698, cg18607127, cg25273707, cg01704198, cg02067712, and cg02157224) were associated with unfavorable prognosis within the high-risk group. Six CpG sites (cg0138171, cg18060519, cg19507244, cg24492886, cg25605277, and cg26228280) were hypomethylated in TMPRSS2-ERG-positive PC compared to TMPRSS2-ERG-negative tumors within the high-risk group. The CpG sites were localized, primarily, in regulatory genome regions belonging to promoters of the following genes: ARHGEF4, C6orf141, C8orf86, CLASP2, CSRNP1, GDA, GSX1, IQSEC1, MYOF, OR10A3, PLCD1, PLEC1, PRDM16, PTAFR, RPII-844P9.2, SCYL3, VPS13D, WT1, and ZSWIM2. For these genes, analysis of differential expression and its correlation with CpG site methylation (β-value level) was also performed. In addition, STK33 and PLCD1 had similar changes in colorectal cancer. As for the CSRNP1, the ARHGEF4, and the WT1 genes, misregulated expression levels were mentioned in lung, liver, pancreatic and androgen-independent prostate cancer. The potential impact of changed methylation on the mRNA level was determined for the CSRNP1, STK33, PLCD1, ARHGEF4, WT1, SCYL3, and VPS13D genes. The above CpG sites could be considered as potential prognostic markers of the high-risk group of PC.

Keywords: high-risk group; methylation; prognosis; prostate cancer; TCGA; TMPRSS2-ERG.

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1 Introduction

Prostate cancer (PC, MeSH · D011471) is a common malignant neoplasm in men worldwide [1]. Currently, to predict the course of PC, patients are stratified into appropriate risk groups based on the following criteria: pathological stage of the tumor (pT), prostate-specific antigen (PSA) level before surgery, and Gleason score [2]. However, these criteria often incorrectly reflect the aggressive tumor phenotype. The solution to this problem can be the study of tumor molecular genetic characteristics using modern approaches. Bioinformatic analysis of omics datasets (genome, transcriptome, and methylome) enables identifying molecular changes that can be associated with the tendency of a tumor to disseminate or can predict the time from radical prostatectomy to disease progression.

Epigenetic changes occur in all types of malignant tumors and include perturbation of both the DNA methylation and the histone modification patterns [3, 4]. These changes can be associated with various clinical and pathological characteristics and, in some cases, allow to conclude about the prognosis [3]. Aberrant CpG methylation was found in various malignant tumors even at the early stages [4]. However, it is necessary to clearly distinguish between the role of aberrant methylation of the promoter regions and global hyper/hypomethylation throughout the genome, including intergenic and intronic regions. Hypermethylation of CpG islands can contribute to genetic instability and enhance cell growth, proliferation, and invasion [4]. For PC, global DNA hypomethylation is almost always associated with the late stages of the disease and is usually found in metastatic tissues [5].

The most commonly described change of the methylation pattern in PC concerns the promoter of the GSTP1 gene [6], which is involved in DNA repair [7]. Its hypermethylation was detected in 90% of PC samples and 50% of hyperplasia prone to malignancy [8]. The GSTP1 [9], APC [10], RASSFIA [11], RARB [3], CCND2 [12], EphA5 [13], and PTGS2 [14] genes were detected to be hypermethylated in PC compared with adjacent normal prostate tissues. Promoter DNA methylation of GSTP1 [15], RARB [16], RASSFIA [17], and APC [18] was widely studied as a non-invasive marker for PC early diagnosis. Hypermethylated GSTP1 promoter detecting in blood or urine are associated with the presence of PC [17]. Tumors carrying a mutation in the IDHI gene, which amount 1% of all PC cases, also have an increased level of DNA methylation [19].

In some cases, subgroups of malignant tumors are featured with the so-called CpG island methylator phenotype (CIMP) that is characterized by intense hypermethylation of the gene promoter regions and is associated with an unfavorable prognosis in colorectal cancer [20, 21]. The existence of the CIMP was firstly demonstrated for colorectal cancer and then was shown for bladder, breast, endometrial, gastric, hepatocellular, and lung cancer, as well as gliomas [21]. The presence of the TMPRSS2-ERG fusion transcript indicates one of the most common molecular subtypes of PC. The presence of this fusion transcript has been considered as a marker of unfavorable prognosis in PC [19]. CIMP has not been found in PC, however, higher overall genome methylation level was shown in the TMPRSS2-ERG-negative cases of PC [22]. It was reported that among TMPRSS2-ERG-positive samples methylation clusters were found; moreover one-third of TMPRSS2-ERG-positive samples of PC has been seen to be characterized by hypermethylated cluster [19]. However, the association of aberrant DNA methylation with the PC prognosis currently remains unclear [23].

The study aims to identify differentially methylated CpG sites associated with the high-risk group of PC, including unfavorable prognosis within the group and TMPRSS2-ERG molecular subtype, based on The Cancer Genome Atlas (TCGA) project data.

2 Materials and methods

2.1 Dataset

The present study includes PC methylation profiling data (Illumina 450k methylation arrays) and RNA-seq data from TCGA project (TCGA-PRAD) [24]. The cohort included PC patients belonging to the Caucasian population. The patients were not receiving
neoadjuvant therapy. The cohort (n = 358) was divided into two PC groups, high (n = 251) and intermediate (n = 107) risk, according to the classification of D’Amico (Table 1) [2]. High-risk group (n = 251) was divided into favorable (n = 83) and unfavorable (n = 21) prognoses groups based on biochemical recurrence (postoperative PSA ≥ 0.2 ng/ml), and TMPRSS2-ERG-positive (n = 75) and TMPRSS2-ERG-negative (n = 79) groups.

2.2 Methods

The analysis of differential CpG methylation was carried out in the R statistical environment (v. 3.5.2) [25]. For comparison of β-value between groups, BiSeq (v.1.22.0) [26] package was used. The Mann–Whitney test, β-regression, and logistic regression modeling were applied. We considered CpG sites (Illumina CpG IDs – cg#) with p-value <0.05 in all three tests as differentially methylated. To retrieve CpG sites mostly differentiating two patient groups, fold-change (Log2FC) and Δβ-value between comparison groups were calculated. Spearman’s rank correlation (standart “cor.test” function) analysis of detected CpG sites with the high-risk group was fulfilled. CpG site annotation (genomic position, gene name, promoter or enhancer) was accomplished by Ensembl [27] and GeneHancer [28] databases, UCSC browser [29], and annotatr (v.1.8.0) [30]. When selecting top-ranked CpG sites the preference was given to ones located in regulatory genomic regions (promoters or enhancers).

Differential expression analysis was carried out on the same samples using edgeR package (v.3.24.3) [31]. The trimmed mean of M-values (TMM) normalization method of count matrix was used; Quasi-likelihood (QLF), Exact Fisher’s (ET), and Mann–Whitney tests were applied for detecting differences between comparison groups. In addition, changes in gene expression level between the comparison groups (Log2FC) and overall gene expression level in the cohort (Log2CPM) were calculated. Spearman’s rank correlation (standart “cor.test” function) analysis of identified CpG sites with their gene expression level was fulfilled. Differentially expressed genes were annotated by biomaRt package (v.2.38.0) [32, 33].

Table 1: Clinicopathologic characteristics of the cohort.

| Criteria                          | Parameter       | High risk, n | Intermediate risk, n |
|----------------------------------|-----------------|--------------|---------------------|
| Gleason score                    |                 |              |                     |
| 6                                |                 | 8            | 13                  |
| 7                                |                 | 82           | 94                  |
| 8                                |                 | 51           | –                   |
| 9                                |                 | 108          | –                   |
| 10                               |                 | 2            | –                   |
| Mean preoperative PSA (ng/ml)    |                 | –            | 13.2                |
| Biochemical recurrence (postoperative) | Yes           | 45           | 2                   |
| PSA ≥ 0.2 ng/ml                  |                 | No           | 183                 |
| Mean age (yr)                    |                 | –            | 62                  |
| Pathologic tumor stage (pT)      |                 |              |                     |
| pT2a                             |                 | –            | 5                   |
| pT2b                             |                 | 4            | 2                   |
| pT2c                             |                 | 19           | 96                  |
| pT3a                             |                 | 118          | –                   |
| pT3b                             |                 | 102          | –                   |
| pT4                              |                 | 7            | –                   |
| Pathologic lymph nodes (pN)      |                 |              |                     |
| pN0                              |                 | 172          | 81                  |
| pN1                              |                 | 63           | –                   |
| Clinical distant metastases (cM) |                 |              |                     |
| cM0                              |                 | 251          | 107                 |
| cM1                              |                 | –            | –                   |
| Molecular subtype                |                 |              |                     |
| 1-ERG                            |                 | 80           | 34                  |
| 2-ETV1                           |                 | 15           | 6                   |
| 3-ETV4                           |                 | 9            | 3                   |
| 4-FLI1                           |                 | 1            | 1                   |
| 5-SPOP                           |                 | 13           | 7                   |
| 6-FOXA1                          |                 | 4            | 1                   |
| 7-IDH1                           |                 | 2            | –                   |
| 8-other                          |                 | 35           | 22                  |
| Total                            |                 | –            | 251                 |
|                                  |                 |              | 107                 |
3 Results

3.1 Differentially methylated CpG sites associated with the high-risk group of PC

We identified eight hypermethylated CpG sites (p-value ≤0.05; FC >1; Δβ-value >0) under comparing high and intermediate-risk groups: cg07548607, cg13533340, cg16643088, cg18467168, cg23324953, cg23753247, cg25773620, and cg27148952 (Figure 1a). These CpG sites were located in the promoters of the following genes [27–30]: ZSWIM2, GDA, CSRNPI, IQSEC1, PLEC1, STK33, PLCD1, and C6orf141, respectively (Table 2).

The differential expression analysis showed that just CSRNPI, STK33, and PLCD1 genes were significantly downregulated (p-value ≤0.05) in the high-risk group (Table 3). Moreover, expression levels of the CSRNPI and STK33 genes negatively correlated with β-values of their CpG sites; Spearman’s rank correlation coefficients were −0.19 and −0.13 respectively (Table 3).

According to literature, cancer-associated hypermethylation was previously shown for the STK33 [34–36], IQSEC1 [37], and PLCD1 [38–43] genes, however, a decrease in the expression was observed only for IQSEC1 [37].
Table 2: Differentially methylated CpG sites associated with the high-risk group of PC.

| CpG site ID (Illumina 450k) | Position (hg19) | Gene (region) | Linear regression, p-value | Logistic regression, p-value | Mann-Whitney, p-value | Spearman's correlation coefficient, $r_s$ | p-value | Δβ-value | FC |
|-----------------------------|----------------|--------------|---------------------------|------------------------------|----------------------|--------------------------------------|--------|----------|----|
| cg07548607                  | chr2: 187713964| ZSWIM2 (promoter) | 3.78E-02*                 | 2.36E-02*                     | 1.76E-07*             | 0.24                                | 4.28E-06* | 0.12     | 2.14 |
| cg13533340                  | chr9: 74764495 | GDA (promoter)    | 2.77E-02*                 | 7.36E-03*                     | 1.80E-04*             | 0.24                                | 3.65E-06* | 0.10     | 2.02 |
| cg16643088                  | chr3: 39188743 | CSRNPI (promoter) | 1.02E-02*                 | 6.14E-03*                     | 2.01E-07*             | 0.28                                | 8.89E-08* | 0.11     | 1.67 |
| cg18467168                  | chr3: 13114803 | IQSEC1 (promoter) | 4.65E-03*                 | 2.26E-03*                     | 5.34E-04*             | 0.18                                | 5.86E-04* | 0.07     | 1.70 |
| cg23324953                  | chr8: 145013728| PLEC1 (promoter)  | 3.33E-04*                 | 8.77E-03*                     | 7.37E-03*             | 0.17                                | 1.37E-03* | 0.07     | 1.85 |
| cg23753247                  | chr11: 8615842 | STK33 (promoter flank) | 1.97E-02*                 | 8.05E-05*                     | 1.03E-02*             | 0.20                                | 1.56E-04* | 0.06     | 1.81 |
| cg25773620                  | chr3: 38071309 | PLCD1 (promoter)  | 4.47E-04*                 | 3.78E-03*                     | 5.32E-04*             | 0.19                                | 2.91E-04* | 0.07     | 1.68 |
| cg27148952                  | chr6: 49518347 | C6orf141 (promoter flank) | 5.14E-03*                 | 1.44E-02*                     | 1.56E-02*             | 0.18                                | 7.06E-04* | 0.06     | 1.63 |
| cg00637488                  | chr1: 3352986  | PRDM16 (promoter) | 4.85E-02*                 | 4.15E-02*                     | 1.38E-02*             | -0.22                               | 2.70E-02 | -0.06    | -1.15|
| cg06834698                  | chr11: 7961985 | OR10A3 (promoter) | 1.18E-02*                 | 1.51E-02*                     | 4.05E-02*             | -0.19                               | 4.36E-02 | -0.09    | -1.18|
| cg18607127                  | chr5: 175630310| RPI1-844P9.2 (promoter) | 1.24E-02*                 | 3.31E-02*                     | 4.83E-03*             | -0.24                               | 1.36E-02 | -0.08    | -1.11|
| cg25273070                  | chr7: 76037066 | TF binding site (ENSR00001009205) | 4.72E-02*                 | 4.31E-02*                     | 1.59E-03*             | -0.32                               | 1.04E-03 | -0.09    | -1.18|

*p-value ≤ 0.05.
ARHGEF1

−0.19

2.90E-01

−0.19

3.70E-04

−0.19

2.90E-01

OR10A3

−0.33

6.91E-04

CLASSP2

0.10

3.13E-01

GSX1

−0.03

7.90E-01

C8orf86

−0.58

7.40E-11

ARHGEF4

−0.22

6.98E-03

MYOF

−0.03

6.89E-01

WT1

−0.23

4.42E-03

PTAFR

0.09

2.50E-01

SCYL3

−0.40

2.11E-07

VPS13D

−0.25

1.56E-03

* p-value < 0.05.

and PLCD1 [38–43] (Table 4). CSRNP1 and C6orf141 were found to be downregulated with no studied methylation status.

3.2 Differentially methylated CpG sites associated with the unfavorable prognosis in the high-risk group of PC

We identified seven differentially methylated CpG sites (p-value ≤0.05) in the unfavorable prognosis group of PC compared with the favorable one: cg00063748, cg06834698, cg18607127, cg25273707, cg01704198, cg02067712, and cg02157224. Among them, the cg01704198 and cg02067712 sites were hypermethylated (FC >1; Δβ-value >0), when other CpG sites were characterized by the hypomethylation status (FC <1; Δβ-value <0) (Figure 1b). Six identified CpG sites were localized in the promoter regions of the PRDM16, OR10A3, RP11-844P9.2, CLASP2, GSX1, and C8orf86 genes; the cg25273707 CpG site belonged to the transcription factor (TF)-binding region (Table 2) [27–30].

Differential expression analysis revealed no significant expression changes of the above genes between the unfavorable prognosis group and the favorable one within the high-risk group of PC (Table 3).

However, several studies noticed that the PRDM16 gene was hypermethylated and downregulated in lung cancer (Table 4) [48–50]. The CLASP2 gene showed differential expression levels in lung, gastric, and bladder cancers [51].

3.3 Differentially methylated CpG sites associated with the TMPRSS2-ERG molecular subtype in the high-risk group of PC

When studying the molecular subtype of TMPRSS2-ERG in the high-risk group, we identified six hypomethylated CpG sites (p-value ≤0.05; FC >1; Δβ-value >0) (cg01138171, cg14060519, cg19570244, cg24492886,
cg25605277, and cg26228280) that were localized in the intron of ARHGEF4, and promoters of MYOF, WT1, PTAFR, SCYL3, and VPS13D, respectively (Figure 1c, Table 2) [27–30].

Differential expression analysis showed that the ARHGEF4, WT1, SCYL3, and VPS13D genes were significantly upregulated (p-value ≤0.05) in TMPRSS2-ERG-positive tumors (Table 3). Furthermore, expression levels of the above genes negatively correlated with β-values of their CpG sites; Spearman’s rank correlation coefficients were −0.22, −0.23, −0.40, and −0.25 respectively (Table 3).

Presently, there are no data on the methylation status of ARHGEF4, MYOF, WT1, PTAFR, SCYL3, and VPS13D in the literature (Table 4). Nevertheless, ARHGEF4 [52, 53], MYOF [54–56], WT1 [57, 58], PTAFR [59] were upregulated in pancreatic, breast, and prostate cancers.

| Gene     | Pathology                        | Alteration                      | Relation                      | Reference |
|----------|----------------------------------|---------------------------------|-------------------------------|-----------|
| CSRNP1   | Hepatocellular carcinoma         | No methylation data, downregulated | Tumor progression            | [44]      |
|          | Lung squamous cell carcinoma     | No methylation data, downregulated | Tumor progression            | [45]      |
| IQSEC1   | Non-small cell lung cancer       | Hypermethylated, downregulated   | Tumor progression            | [37]      |
| PLEC1    | Pancreatic cancer                | No methylation data, upregulated | –                             | [46]      |
| STK33    | Colorectal cancer                | Hypermethylated, no expression data | Tumor progression            | [34, 35] |
|          | Head and neck cancers            | Hypermethylated, no expression data | Tumor progression            | [36]      |
| PLCD1    | Colorectal cancer                | Hypermethylated, downregulated   | Tumor progression            | [40, 42] |
|          | Breast cancer                    | Hypermethylated, downregulated   | –                             | [38]      |
|          | Gastric cancer                   | Hypermethylated, downregulated   | –                             | [39]      |
|          | Chronic myeloid leukemia         | Hypermethylated, downregulated   | –                             | [41]      |
|          | Endometrial cancer               | Hypermethylated, downregulated   | –                             | [43]      |
| C6orf141 | Oral squamous cell carcinoma     | No methylation data, downregulated | Tumor progression            | [47]      |
| PRDM16   | Lung cancer cell lines (A549 and HTB-182) | Hypermethylated, downregulated | –                             | [48]      |
|          | Non–small cell lung cancer       | Hypermethylated, downregulated   | –                             | [49]      |
|          | Gastric cancer                   | No methylation data, downregulated | Unfavorable prognosis        | [50]      |
| CLASP2   | Muscle-invasive bladder urothelial cancer | No methylation data, upregulated | High-stage tumors, lymph node metastases | [51]      |
| ARHGEF4  | Pancreatic cancer                | No methylation data, upregulated | Unfavorable prognosis        | [52, 53] |
| MYOF     | Pancreatic cancer                | No methylation data, upregulated | Poor survival outcome        | [54, 55] |
|          | Triple-negative breast cancer    | No methylation data, upregulated | Poor survival outcome        | [56]      |
| WT1      | Prostate cancer                  | No methylation data, upregulated | Androgen-independent stage   | [57, 58] |
| PTAFR    | Breast cancer                    | No methylation data, Upregulated | Bone metastases              | [59]      |
4 Discussion

DNA methylation is one of the main mechanisms of gene expression regulation. In adult normal somatic cells, oncogene silencing is maintained by the promoter methylation, when promoter methylation of tumor suppressor genes does not occur [4]. Altered DNA methylation leads to the deregulation of gene expression patterns and disruption of crucial cellular processes, such as DNA repair, cell adhesion, cell cycle control, and apoptosis, contributing to the development of cancer [4, 60]. Cancer-associated genome-wide hypomethylation more often occurs than individual gene hypomethylation [60]. At the same time, hypermethylation can be seen in promoters of individual genes in carcinogenesis a lot [60]. In this study, we found both hypermethylation and hypomethylation of CpG sites of individual genes associated with the high-risk group of PC. Identified genes have not been previously reported as oncogenes or tumor suppressor genes.

Comparison of the high- and intermediate-risk groups of PC revealed eight hypermethylated CpG sites in promoters of different genes. The decreased expression has been found only for three out of eight genes (CSRNP1, STK33, and PLC1). For these genes, we observed a negative correlation of CpG site methylation status (β-value levels) and expression changes. Spearman’s rank correlation coefficients were statistically significant but had low values. Thus, we can conclude that there is a tendency of the impact of these CpG site hypermethylation on the gene expression. The hypermethylation of other identified CpG sites was not associated with expression alterations of corresponding genes. Notably, aberrant methylation of the STK33, and PLC1 genes was observed in other cancers. In particular, often promoter hypermethylation of the PLC1 gene was shown to be associated with its downregulation in breast [38], gastric [39], and colorectal cancers [40], as well as chronic myeloid leukemia [41]. In colorectal cancer, PLC1 promoter hypermethylation and its decreased expression were correlated with tumor progression [42]. The hypermethylation of the STK33 gene promoter was associated with progression of colorectal [34, 35] and head and neck cancers [36]; no data on the altered gene expression were previously reported. For IQSEC1 gene, we did not observe a significant expression change correlated with the CpG methylation status. However, hypermethylation of the IQSEC1 gene promoter and its downregulation was reported in lung cancer [37]. Methylation status of CSRNP1 has not been earlier studied, however, the gene expression was decreased in hepatocellular [44] and lung cancers [45] correlating with tumor progression.

Seven differentially methylated CpG sites were found under comparison of the favorable and unfavorable prognosis within the high-risk group of PC. Additional analysis of differential expression of genes with identified CpG sites revealed no significant expression changes. Therefore, aberrant methylation of identified CpG sites does not influence the gene expression. Two genes (PRDM16 and CLASP2) genes have been previously shown to be involved in cancer. Promotor hypermethylation and downregulated expression of the PRDM16 gene was observed in lung cancer [49]. In gastric cancer, decreased PRDM16 expression was associated with an unfavorable prognosis [50]. Methylation status of the CLASP2 gene has not been studied; however, the gene upregulation was detected in bladder cancer [51].

The analysis of TMPRSS2-ERG-positive tumors within the high-risk group of PC revealed six hypomethylated CpG sites in different genes, among which significant upregulation was observed for ARHGEF4, WT1, SCYL3, and VPS13D. Expression changes in these genes were negatively correlated with the β-value levels of the identified CpG sites. Thus, hypomethylation of cg01138171, cg19570244, cg25605277, cg26228280 CpG sites can potentially upregulate the expression of the corresponding genes. In the literature, there are no data on the methylation status of the identified genes. However, the ARHGEF4 and WT1 genes were characterized by increased expression in pancreatic [52, 53] and prostate cancers [57, 58] that correlated with unfavorable prognosis and poor survival of patients.

Likewise our study, the STK33 and the PLC1 genes had similar both methylation changes and expression signatures in colorectal cancer, indicating their potential effect on the gene expression. With regards to the CSRNP1, the ARHGEF4, and the WT1 genes, shifted expression were noticed in lung, liver, pancreatic and androgen-independent prostate cancer. However, methylation or expression changes in SCYL3 and VPS13D have never been marked in any cancer.
5 Conclusion

Thus, we found differential methylation of several CpG sites associated with the high-risk group of PC. Furthermore, aberrant methylation was related to individual CpG sites located predominantly in the gene promoter regions. CSRP1, STK33, PLCD1, ARHGEF4, WT1, SCYL3, and VPS13D were also characterized by significant changes in the mRNA levels negatively correlated with the methylation status of identified CpG sites. Identified CpG sites could be considered as potential prognostic markers of the high-risk group of PC.

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