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Original Paper

Gene Expression, DNA Methylation and Prognostic Significance of DNA Repair Genes in Human Bladder Cancer

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Key Words
Dna repair genes • Expression • DNA methylation • Human bladder cancer

Abstract
Background/Aims*:** This study investigated the gene expression and DNA methylation of selected DNA repair genes (*MBD4*, *TDG*, *MLH1*, *MLH3*) and *DNMT1* in human bladder cancer in the context of pathophysiological and prognostic significance. ***Methods*:** To determine the relationship between the gene expression pattern, global methylation and promoter methylation status, we performed real-time PCR to quantify the mRNA of selected genes in 50 samples of bladder cancer and adjacent non-cancerous tissue. The methylation status was analyzed by methylation-specific polymerase chain reaction (MSP) or digestion of genomic DNA with a methylation-sensitive restriction enzyme and PCR with gene-specific primers (MSRE-PCR). The global DNA methylation level was measured using the antibody-based 5-mC detection method. ***Results: The relative levels of mRNA for *MBD4*, *MLH3*, and *MLH1* were decreased in 28% (14/50), 34% (17/50) and 36% (18/50) of tumor samples, respectively. The *MBD4* mRNA expression was decreased in 46% of non-muscle invasive tumors (Ta/T1) compared with 11% found in muscle invasive tumors (T2-T4) (P<0.003). Analysis of mRNA expression for *TDG* did not show any significant differences between Ta/T1 and T2-T4 tumors. The frequency of increased *DNMT1* mRNA expression was higher in T2-T4 (52%) comparing to Ta/T1 (16%). The overall methylation rates in tumor tissue were 18% for *MBD4*, 25% for *MLH1* and there was no evidence of *MLH3* promoter methylation. High grade tumors had significantly lower levels of global DNA methylation (P=0.04). There was a significant association between shorter survival and increased expression of *DNMT1* mRNA (P=0.002), decreased expression of *MLH1* mRNA (P=0.032) and the presence of *MLH1* promoter methylation (P=0.006). ***Conclusion:*** This study highlights the importance of DNA repair pathways and provides the first evidence of the role of MBD4 and MLH3 in bladder cancer. In addition, our findings suggest that *DNMT1* mRNA and *MLH1* mRNA expression, as well as the status of *MLH1* promoter methylation, are attractive prognostic markers in this pathology.

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Introduction

Inactivation of DNA repair genes in cancer has been reported for several DNA repair pathways, including base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). DNA damage pathways impact on genome stability by stimulating DNA damage-induced apoptosis and are highly relevant to cancer therapy [1]. Studies have shown that not only the deficiency but also single nucleotide polymorphisms (SNPs) in DNA repair genes are associated with higher risk of cancer development, progression, and shorter survival [2, 3]. Defects in DNA repair genes may increase anti-metabolite radiation sensitization by increasing anti-metabolite incorporation into DNA and also predict response to neoadjuvant cisplatin-based chemotherapy in muscle-invasive bladder cancer [4, 5].

Bladder cancer (BC) is a key public health problem, with an estimated 429 000 new cases and 165 000 deaths in 2012 and is the ninth most commonly diagnosed cancer in the world (in both sexes) [6]. The most common type of BC is a urothelial carcinoma, which constitutes more than 90% of bladder cases [7]. Approximately 75% to 85% of patients present with non-muscle invasive disease (NMIBC) confined to the mucosa (stage Ta, CIS) or submucosa (stage T1), and 25% with muscle-invasive and metastatic cancer (MIBC) T2-T4 [8]. Bladder cancer is strongly associated with the exposure to chemical agents that lead to DNA damage [9]. DNA repair processes are considered as the primary mechanisms that act at the level of DNA to prevent genetic and epigenetic alterations from occurring [10]. In the case of BC, the major well-known prognostic factors are grade and stage. In the post-genomic era with the massive amount of sequence data and bioinformatics, there is an intensive search for markers specific for the disease that would be not only easy to measure, but also inexpensive and sensitive enough for population screening. A biomarker-driven decision making on the best drug candidates and selection of the most successful approaches could improve the outcomes for patients with bladder cancer. Genome-wide transcriptome, copy number variation, protein, and epigenetic analyses have allowed defining molecular subclasses and predict clinical outcome [11]. However, this type of analysis requires further validation of potential cancer-gene associations, with the focus on the prognostic significance of molecular and epigenetic markers in bladder cancer.

As in colorectal and other cancers, tumorigenesis and progression of bladder cancer might involve mismatch repair genes [12]. In contrast to DNA repair genes, such as *MGMT* and *MLH1*, the expression pattern of *MBD4*, *TDG* or *MLH3* is usually not evaluated. In this study, the BER enzymes, MBD4 (methyl-CpG-binding domain protein 4) and thymine DNA glycosylase (TDG) were chosen because of their biochemical similarity, and interaction between MBD4 and MLH1 (human mutL homolog 1), the major mismatch repair protein [13, 14]. Moreover, MBD4 in addition to glycosylase domain contains a methyl-binding domain and binds to methylated CpGs and represses transcription indirectly via recruitment of corepressors that modify chromatin transcription [15]. MBD4 interacts with DNA methyltransferase (DNMT1) what makes DNMT1 an essential element of the transcription repression complex [16]. It has been shown that DNMT1 is not only engaged in maintenance of methylation patterns but also may be responsible for *the novo* methylation of CpG islands [17, 18]. In addition, overexpression of *DNMT1* may indicate the CpG island methylator phenotype (CIMP) of cancer.

To date, no consensus has been achieved, and conflicting data regarding the prognostic significance of molecular and epigenetic markers in bladder cancer have been published. Because BC exhibits distinctive presentations regarding stage, grade, size and tumor number, the treatment options for this disease should be individualized. Therefore, the main objectives of the present study were (1) to evaluate the gene expression status for four selected DNA repair genes (*MBD4*, *TDG*, *MLH1*, *MLH3*), including *DNMT1* mRNA expression in BC; (2) to evaluate the promoter methylation status in case of genes with the decreased level of mRNA expression; (3) to determine the global methylation status; (4) to determine the relationship between analysed genes; (5) to identify a prognostic gene signature.

Materials and Methods

Patients

BC tissue samples were collected from patients who underwent transurethral resection or cystectomy at the Department of Urology at the Medical University of Gdansk between 2001 and 2014. A total of 50 patients with BC were evaluated, including NMIBC (Ta/T1) and MIBC (T2-T4). Tumor and adjacent histologically normal tissues were used in the study. The patient characteristics are presented in Table 1. The median age of patients was 64 years (ranging from 47 to 85). The specimens were staged according to the fifth edition of the TNM classification, and each tumor was reviewed for histological grading according to WHO (1973) [19]. Investigations were approved by the Medical University of Gdansk Ethics Committee. At the time of collection samples were immediately frozen in liquid nitrogen, and stored at -80°C.

qPCR analysis

Bladder tumor and adjacent noncancerous tissues were homogenized by MagNA Lyser (Hoffmann-La Roche, Germany) and total RNA isolation was carried out using Chomczynski method [20]. The quality of RNA was validated using the Experion platform and Experion RNA StdSens Analysis Kit (Bio-Rad, USA). The RNA samples were electrophoretically separated on a microchip and subsequently evaluated via laser induced fluorescence detection. Only samples with high 28S:18S ratio were included in the study. Next, double-stranded cDNA was synthesized from 1 µg of total RNA with First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and random hexamers (Thermo Scientific, USA) in a total reaction volume of 20 µl, following the manufacturer's instruction. The RNA samples were treated with RNase-free DNase (Thermo Scientific, USA), to remove residual DNA. The resulting cDNA solution was diluted 40-fold with deionized water and then used for the qPCR measurements. Each sample was measured two times.

Real-time PCR analysis was performed using the following primers sequences: *ATP5* F-5'-TCA CCC AGG CTG GTT CAG A-3', R-5'-AGT GGC CAG GGT AGG CTG AT-3'; *PPIA F-5'*-ATC TGC ACT GCC AAG ACT GAG-3', R-5'-GAA GGA ATG ATC TGG TGG TTA AGA-3'; *DNMT1 F-5'*-CCA AGC AGG CAT CTC TGA C-3', R-5'-GCA GGA TGT TGC AGT CCT CT-3'; *TDG F-5'*-TGA AGC TCC TAA TAT GGC AGT TG-3', R-5'-TTC CAC TGG TTG TTT TGG TTC T-3'; *MLH1 F-5'*-CTC TTC ATC AAC CAT CGT CTG G-3', R-5'-GCA AAT AGG CTG CAT ACA CTG TT-3'; *MLH3 F-5'*-GAC GTA TGT TCC CGA TTT TGT CA-3', R-5'-GCT TCA GAG CTG ATA TAG CCA CT-3'; *MBD4 F-5'*-CCG TCA CCT CTA GTG AGC G-3', R-5'-GCA GAA GCG ATG GGT TCT TGT A-3'. Two housekeeping genes, mitochondrial ATP synthase beta-subunit (*ATP5B*) and peptidylprolyl isomerase A (cyclophilin A) (*PPIA*) were used for the normalization of gene expression. RT-PCR data, normalized to the geometric mean of two endogenous references allowed to apply $\Delta\Delta C_q$ method for a relative genes expression determination [21]. HOT FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Estonia) was used for quantitative real-time PCR performed on CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). ΔC_q for each gene (target and reference) was calculated, and $2^{-\Delta\Delta Cq}$ formula for relative quantification of gene expression was used [22]. By convention, when the fold change was less than 1, the negative of its inverse was used. Expression fold change ≥2 or ≤-2 was considered as significant.

Table 1. Patients' clinical and pathological characteristics, WHO – World Health Organization

Variable category	N (%)
Sex	
Female	14 (28)
Male	36 (72)
Age	
≤64	23 (64)
>64	27 (54)
Stage	
Ta/T1 (non-muscle invasive)	25 (50)
T2-T4 (muscle-invasive)	25 (50)
1973 WHO Grade	
G1	21 (42)
G2	18 (36)
G3	11 (22)
Lymph node status	
pNx	24 (48)
pN0	15 (30)
pN+	11 (22)

Global DNA methylation analysis

Genomic DNA from frozen tumor tissues and adjacent noncancerous tissue fragments were extracted according to Chomczynski method. Standard curve and following DNA concentration were determined with DNA Quantification Kit, Fluorescence Assay (Sigma-Aldrich, USA). In brief, 100 ng of DNA was used for incubation with both capture and detection antibodies using MethylFlash Methylated DNA Quantification Kit (Colorimetric) from Epigentek, USA. Subsequently, measurements of the absorbance of the

sample at 450 nm in a microplate spectrophotometer (BioTek Instruments, USA) were performed with the percentage of the whole genome 5-methylcytosine (5-mC) calculation according to manufacturer's instructions. Genomic methylation levels in study samples were expressed as a 5-mC%.

Bisulfite Modification of DNA and Methylation-Specific PCR

DNA methylation of the 5 'region of *MLH1* was determined by chemical conversion of unmethylated cytosines to uracil using methylation-specific PCR (MSP) [23]. Bisulfite treatment was carried out following the procedure of Frommer et al. [24]. Briefly, 2 µg of genomic DNA was passed through a narrow gauge needle several times and denatured with 0.3 M NaOH for 15 min at 37°C. A freshly prepared solution of sodium bisulfite (3.6 M, pH 5.0) and hydroquinone (10 mM) was added to the denatured DNA, and the mixture was incubated at 55°C for 16 h. After purification with Clean-up (A&A Biotechnology, Poland), DNA was desulfonated with 0.3 M NaOH for 15 min at 37°C, neutralized with 5 M ammonium acetate (pH 7.0), ethanol precipitated, and resuspended in Tris-EDTA (pH 7.5). PCR amplification of bisulfite-treated DNA was performed with the set of primers and conditions described previously [25]. The reaction mixture (25 µl) contained bisulfite-treated DNA as a template, primers (3 µM), dNTPs (1.25 mM), *Taq* DNA polymerase (Sigma-Aldrich, USA) in 1 X reaction buffer (16.6 mM ammonium sulfite, 67 mM Tris, pH 8.8; 6.7 mM $MgCl_2$, and 10 mM 2-mercaptoethanol). DNA from leukocytes was used as a negative control for methylated alleles. Placental DNA treated *in vitro* with SssI methyltransferase, and subsequently treated with sodium bisulfite was used as a positive control for methylated alleles (New England BioLabs, USA). Placental DNA (1 µg) (Sigma-Aldrich, USA) was methylated with SssI (4 U) in the presence of S-adenosylmethionine (160 µM) for 2 hours at 37 °C. The DNA was then purified using Clean-up kit (A&A Biotechnology, Poland). All PCR reactions were performed on PTC-200 thermocycler (BioRad, USA). MSP products were separated on 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Methylation-sensitive restriction enzyme digestion PCR (MSRE-PCR)

DNA methylation analysis of *MLH3* and *MBD4* promoter regions was performed based on digestion of genomic DNA with a methylation-sensitive restriction enzyme and PCR with gene-specific primers. According to Thermo Scientific EpiJET Methylation Analysis Kit (MspI/HpaII) instruction, DNA methylation status at a specific locus was evaluated. Methylation-sensitive restriction enzyme cleavage by Epi MspI and Epi HpaII (isoschizomers with different sensitivity to methylation) allowed for 1 µg DNA digestion for the night and afterward samples amplification by PCR with gene-specific primers designed to amplify genomic fragments located within CpG islands. Primer design was performed for genes *MLH3* and *MBD4* using Primer3 software, and primer sequences were as follows: *MLH3*-M F-5'-TAAAAGCTGTGGTGGCACTG-3', R-5'- CCACCACACTCGGCTAATTT-3'; *MBD4*-M F-5'-CGTCTTCCTCGAGAATGGAT-3', R-5'-GGGCGGAGTAAGATGTGAAA-3'. Percentage of methylation was calculated according to the manufacturer's instruction. A cutoff of 20% was used to name a sample methylated.

Statistics

Statistical analysis was performed using the STATISTICA software version 10 and Matlab software version R2011a. Genes expression status in patients' samples was investigated with the t-test or nonparametric Mann-Whitney U-test, as appropriate. The Spearman's rank correlation was used to examine the relationship between two continuous variables. The Kruskal-Wallis ANOVA by Ranks test allowed for the assessment of the difference between total methylation status in tumor tissue versus grade. The associations between methylation of the 5' region of the *MLH1* and *MBD4* in the tumor specimen versus sex, age, stage, and grade were examined using a two-sided Fisher's exact test. In bivariate association clinical variables were dichotomized as follows: age at diagnosis ($\leq$64 versus >64 years); tumor stage Ta/T1 versus T2-T4; tumor grade G1-G2 versus G3.

Survival was defined as the time from primary therapy to death, patients who were alive at the last follow-up were censored at the time of the last follow-up. The total length of the study at the time of analysis was 13 years, and median follow-up was three years. Survival probability curves were prepared using the Kaplan-Meier method and assessed by the log-rank test. Significance was set at $P<0.05$.

Results

mRNA expression

The mRNA expression of the genes: *MBD4*, *TDG*, *MLH1*, *MLH3* and *DNMT1* was determined by qPCR in both bladder cancer and adjacent noncancerous tissue. Genes selected for this study code for the proteins that are at least partially connected, as a group. The presence of functional associations between these proteins was evaluated and confirmed by the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database [26].

The relative level of *MBD4*, *MLH3*, and *MLH1* mRNA was decreased in 28% (14/50), 34% (17/50) and 36% (18/50) of tumor samples respectively, while the remaining samples showed higher or no difference in expression. In patients with NMIBC (Ta/T1), *MBD4* gene expression was decreased in 46% of tumors compared with 11% found in invasive (T2-T4) tumors, and the difference between these two groups was statistically significant (*P*<0.003, Mann-Whitney U-test) (Fig. 1). Box and whisker plots were constructed for better visualization of the genes that were up-expressed (≥2-fold), down-expressed (≤-2-fold) or equally expressed (2-fold difference threshold). Decreased expression of *MLH1* mRNA was found in 16% of Ta/T1, and 32% of T2-T4 tumors. Despite the higher frequency of *MLH1* mRNA down-expression in T2-T4 tumors, there was no significant difference in the expression level between Ta/T1 and T2-T4 tumors (Fig. 2 A).

The level of *MLH3* mRNA was decreased in 59% of Ta/T1, and only in 12% T2-T4 tumors (*P*=0.008, Mann-Whitney U-test). The median change in *MLH3* mRNA expression in Ta/T1 was 2.4-fold (-11 to 4.7) compared with 1.7-fold (-3.2 to 8.3) in T2-T4 tumors. As shown in Fig. 2 A, the mean fold change of *MLH3* mRNA expression was significantly lower compared with the mean fold change of *MLH1* mRNA expression in Ta/T1 tumors (*P*=0.03, t-test).

The analysis of mRNA expression for *TDG* did not show any significant differences between Ta/T1 and T2-T4 tumors (*P*>0.05; Mann-Whitney U-test), as shown in Fig. 1. Moreover, the *TDG* mRNA expression was not associated with any clinical parameter tested in our study.

Regarding *DNMT1* mRNA, the increased expression was found in 34% (17/50) of tumor samples. The frequency of increased *DNMT1* mRNA expression was higher in T2-T4 (52%) compared with Ta/T1 (16%). The median change in *DNMT1* mRNA expression in Ta/T1 was 1.4-fold (-10 to 3.6) comparing to 2.3-fold (-3.5 to 10.6) in T2-T4 tumors (*P*<0.001, Mann-Whitney U-test). Comparison of *MLH1* mRNA expression with the expression of *DNMT1* mRNA with stratification according to the cancer stage showed that T2-T4 tumors with a

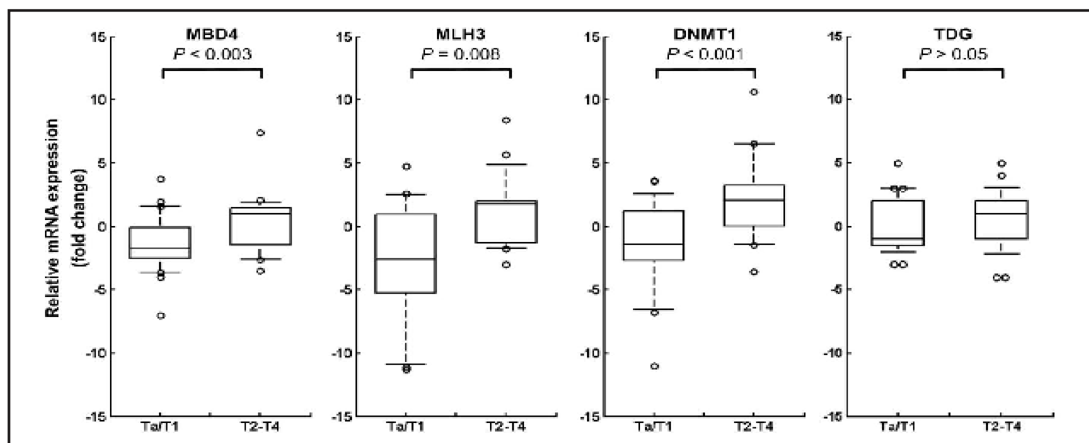

Fig. 1. Relative mRNA expression of MBD4, MLH3, DNMT1, and TDG in bladder cancer according to tumor stage. The expression is normalized to the geometric mean of PPIA and ATP5B. Box-and-whisker plots show the fold change of gene expression. Median and interquartile (25% and 75%) and interdecile (10% and 90%) confidence intervals with individual outsider values are shown.

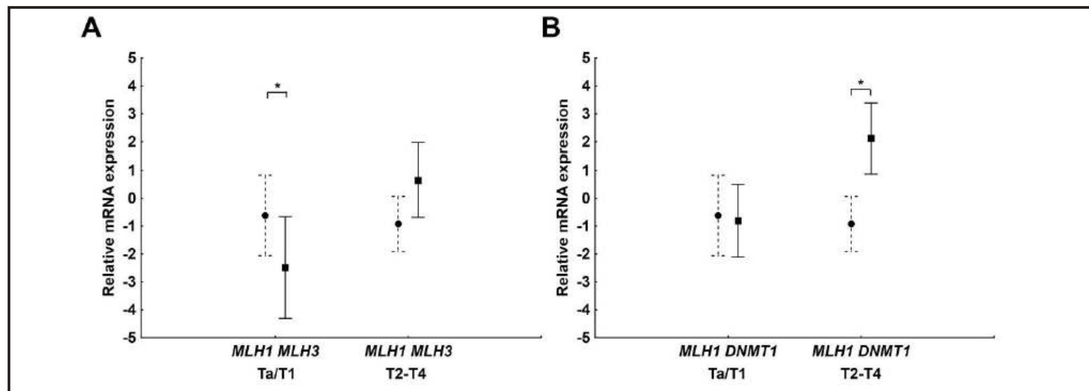

Fig. 2. MLH1, MLH3, and DNMT1 mean mRNA expression levels in bladder cancer. (A) Mean difference between the MLH3 and MLH1 mRNA expression in Ta/T1 and T2-T4 tumors. (B) Mean difference between the DNMT1 and MLH1 mRNA expression in Ta/T1 and T2-T4 tumors. An asterisk (*) indicates a significant difference between the means (*: P<0.05).

low mRNA expression of *MLH1* displayed a higher mRNA expression of *DNMT1*. The difference between the means was significant (*P*=0.02, t-test) (Fig. 2 B). The expression analysis revealed a lack of tight transcriptional regulation between the genes. A weak correlation was observed between *DNMT1* mRNA expression and *MBD4* mRNA expression (r=0.34, *P*=0.03), and between *MLH1* mRNA expression and *DNMT1* mRNA expression (r=0.31, *P*=0.04).

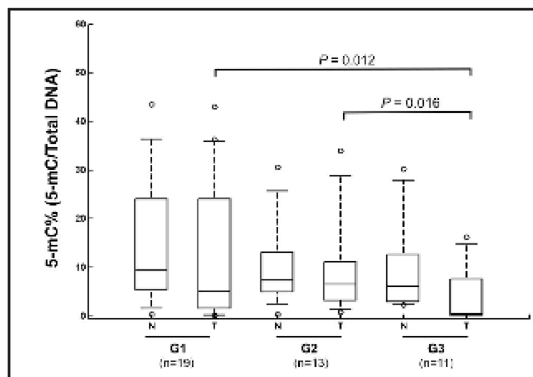

Fig. 3. 5-mC content levels in bladder cancer tissue and the paired adjacent non-cancerous tissue stratified by grade of the resected tumors and measured by methylFlash™ technology. N - non-cancerous tissue; T - tumor; n - number of patients.

Analysis of methylation

MSP or MSRE-PCR was used to examine methylation of 5’ region of *MBD4*, *MLH1*, and *MLH3* in tumor tissue. The overall methylation frequencies in tumor tissue were 18% for *MBD4*, 25% for *MLH1* and there was no evidence of *MLH3* promoter methylation based on MSRE-PCR. High stage tumors displayed aberrant methylation of the *MLH1* promoter of bladder cancer. Moreover, *MLH1* methylation was found only in 36% of cases with decreased expression of *MLH1* mRNA. *MBD4* promoter methylation was found in 66% of cases with decreased *MBD4* mRNA expression. A moderate inverse relationship was found between *MBD4* mRNA expression and *MBD4* promoter methylation (r=-0.53, *P*<0.05). No associations were found between *MBD4* promoter methylation and any clinical parameters.

A global decrease in methylated DNA content is observed in many types of cancer which can account for genomic instability [27]. Therefore, the presence of 5-mC was quantified as a useful addition to hypermethylation analyses of CpG islands. It has been shown that leukocyte DNA hypomethylation is associated with increased risk of developing bladder cancer [28]. We aimed to test the hypothesis that DNA hypomethylation is associated with more aggressive muscle invasive bladder cancer and MethylFlash analysis was used to identify 5-mC in genomic DNA. Global DNA methylation analysis was performed on 43 DNA samples from the tumor and corresponding adjacent noncancerous tissues.

In our study, transitional epithelium from normal adjacent tissue consistently showed a low level of methylation, comparable to the level of methylation in cancer tissue. The median of global DNA methylation (5-mC%) was only slightly lower among tumor tissues

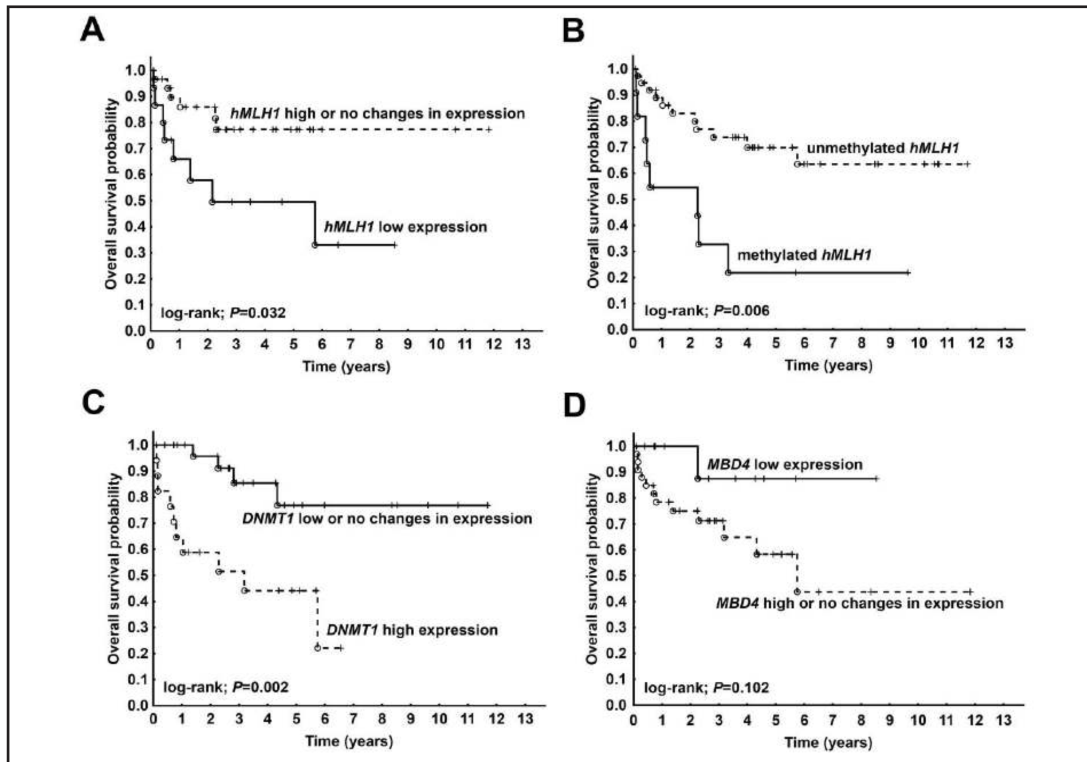

Fig. 4. Kaplan-Meier analysis of disease-specific survival of patients with bladder cancer stratified by (A) mRNA MLH1 expression levels (high, low and no changes), (B) MLH1 promoter methylation (unmethylated, methylated), (C) mRNA DNMT1 expression levels (high, low and no changes), (D) mRNA MBD4 expression levels (low, high and no changes).

than corresponding normal adjacent tissues 6% (range 0.2% – 43%) and 9% (range 2% – 43.5%), respectively. There was no relationship between the mRNA level of *DNMT1* and % of 5-mC in tumor tissue (r=-0.2, *P*=0.4). The association between the % of 5-mC and tumor stage was not statistically significant. However, methylation in tumor tissue was significantly associated with a higher grade (*P*=0.04, Kruskal-Wallis) (Fig. 3).

Association of gene expression and promoter methylation with patient outcome

The Kaplan-Meier method and log-rank test were used to evaluate the effect of mRNA gene expression of four selected genes (*MBD4, MLH, MLH3, DNMT1*) and promoter methylation of the *MLH1*gene on overall survival of 50 patients with bladder cancer. There was a significant association between shorter survival and decreased expression of *MLH1* mRNA (*P*=0.032, log-rank test), and the presence of *MLH1* promoter methylation (*P*=0.006, log-rank test) (Fig. 4). A statistically significant difference in survival rate was observed between patients with high *DNMT1* mRNA and patients with low and no changes in *DNMT1* mRNA expression (*P*=0.002, log-rank test). Interestingly, patients with low *MBD4* mRNA expression showed a better overall survival pattern. However, the difference was not statistically significant. An association between later stage and higher grade and shorter survival was also observed (*P*<0.05, log-rank test).

Discussion

Prognostic models for BC exist, but there is a need to establish a new set of molecular markers that would allow to stratify patients according to survival and select the patients with decreased risk of death [29]. DNA repair pathways are of high importance, as the

defects in DNA repair mechanisms may predispose to cancer. On the other hand, they may have a profound effect on tumor chemoresistance and radioresistance. Therefore, specific DNA repair inhibitors like YM155 (inhibitor of survivin) [30], NU7441 (inhibitor of DNA-PK) [31] or some miRNAs [32, 33] hold great promise for improvement of the effectiveness of chemical and radiation therapy.

In this study, selected genes from DNA repair systems and *DNMT1* were analyzed regarding mRNA expression and promoter methylation. The analysis of global methylation status was also performed. Gene expression analysis showed that *MLH3*, *MBD4*, and *DNMT1* genes were differentially expressed in Ta/T1 versus T2-T4 tumors. Decreased expression of *MLH3* mRNA and *MBD4* mRNA was associated with Ta/T1 tumors while the expression of *DNMT1* mRNA was significantly higher in T2-T4 tumors. The decreased expression of *MLH1* mRNA was observed in both early and advanced stages of bladder cancer.

MLH1 and MLH3 belong to MMR system associated with DNA replication, which function is to correct for deficiencies in DNA polymerase proofreading activity and to signal the presence of DNA damage to the apoptotic machinery [34]. Mutation in *MLH1* or other MMR genes leads to microsatellite instability that is highly associated with hereditary non-polyposis colon cancer (HNPCC) [35]. Inactivation of *MLH1* has also been observed in sporadic cancers like gastric cancer [36], ovarian cancer [37], non-small cell lung carcinoma (NSCLC) [38], bladder cancer [39] and many other types of cancer. In MMR system, MLH1 forms a heterodimer with PMS2 (known as MutLα) or with MLH3 (known as MutLγ) which can be observed in meiotic recombination events [40]. In addition, studies in the mouse model have shown that MLH1/MLH3 heterodimer may be involved in the repair of base-base mismatches and small insertion/deletion loops [41]. Based on its interaction with MLH1, it was postulated that MLH3 might play a role in tumorigenesis. However the functional role for MLH3 remains uncertain, and its prognostic value is unknown. Mutations in *MLH3* gene are rare with the frequency of about 25% in sporadic colorectal cancer with MSI [42]. Taylor et al. [43] identified somatic *MLH3* mutations only in 3 of 57 endometrial cancers. No studies to date have reported on *MLH3* expression in bladder cancer. Our research findings for the first time show the differential *MLH3* expression in BC. *MLH3* expression was decreased in 34% of tumors in our investigations. The lower level of *MLH3* expression was more frequently found in Ta/T1 tumors (59%) compared with muscle-invasive tumors (12%). Interestingly, in Ta/T1 tumors the average fold change in *MLH3* mRNA expression was lower compared with the average fold change of *MLH1* mRNA expression. It may indicate that *MLH1* can functionally interact with another MutL homolog which allows compensating for the *MLH3* deficiency in low stage BC. As the *MLH3* promoter contains CpG islands that could be silenced by hypermethylation, we performed the DNA methylation analysis using the MSRE-PCR. Surprisingly, we did not find any methylation in this region. Also, we did not find any significant correlation between the low expression of *MLH3* mRNA and overall survival. Based on these observations, we conclude that *MLH3* expression is not likely to play a role of importance in bladder cancer, however, how low expression of *MLH3* mRNA in NMIBC may affect the sensitivity to anti-tumor therapy requires further investigation.

In the present study, the level of *MLH1* was decreased in 36% of tumors and was observed in both Ta/T1 and T2-T4 stages. Despite the fact that *MLH1* mRNA was more frequently decreased in muscle invasive cancer, 32% versus 16% in Ta/T1, there was no statistically significant difference in the level of expression between Ta/T1 and T2-T4 tumors. One of the inactivation mechanisms of the MMR system is promoter methylation of *MLH1* observed in a significant percentage of sporadic colon cancers [44]. The frequency of *MLH1* promoter methylation has been found in 42% of patients with cervical cancer and 13% of non-muscle invasive bladder cancers [12, 37]. In our study, we found *MLH1* methylation in 25% of tumors with the frequency of 32% in MIBC. This is the much higher frequency of *MLH1* methylation than 1% reported by Catto et al. [45] in bladder cancer and similar to 29.6% observed in NMIBC [46]. The *MLH1* gene can be silenced by the methylation of its promoter region, but this is most likely not the only mechanism that results in the lack of gene expression, as only 36% of the tumor samples with decreased expression of *MLH1* mRNA displayed the

transcriptional inactivation of their promoters. This result is not surprising, as recently it was shown that microRNA might cause the downregulation of the mismatch repair proteins such as MLH1, MSH2, and MSH6 [47]. The low frequency of *MLH1* promoter methylation in our study suggests that it is not a common alteration in bladder cancer; however, if present it is strongly associated with shorter overall survival.

The methyl-CpG binding domain protein 4 (MBD4; also known as MED1) can bind methylated DNA and may function not only as a thymine and uracil glycosylase involved in DNA repair and demethylation but also as a methylation-dependent transcription repressor [16, 48]. It has been shown that MBD4 interacts with MLH1 and displays biochemical activities similar to the thymine DNA glycosylase (TDG) [14]. Mutations in the *MBD4* gene have been observed in 20% – 43% of human pancreatic, endometrial and colon carcinomas displaying microsatellite instability [49, 50]. Reduced expression of *MBD4* mRNA was observed in 61% of hepatocarcinoma (HCC) and was significantly correlated with poorly differentiated HCCs [51]. In contrast to reduced expression in HCC, *MBD4* mRNA was overexpressed in glioblastoma [52]. In sporadic colon cancer, promoter methylation of *MBD4* and decreased *MBD4* mRNA expression was observed in an early stage of disease with a trend towards the higher stage and lower *MBD4* expression [53]. In our study, *MBD4* expression was decreased in 28% of tumors. Furthermore, *MBD4* gene expression was decreased in 46% of Ta/T1 tumors compared with 11% found in muscle-invasive tumors, and the difference between these two groups was statistically significant, although the fold change in mRNA expression was rather small. Methylation of the *MBD4* promoter was found only in 18% of tumors. However, a moderate inverse relationship with the relative *MBD4* mRNA level was observed. This may suggest that methylation contributes to gene silencing, but it is not the only mechanism. Low frequency of *MBD4* methylation found in our study indicates the minor importance of this alteration in bladder cancer development. It is consistent with the research on colon cancer where the average frequency of 24% was observed [53]. In contrast, we did not find any association with decreased *MBD4* mRNA expression and more advanced tumor stage, and the frequency of decreased *MBD4* mRNA was higher in low stage tumors. Thus, decreased *MBD4* mRNA expression seems to be associated with NMIBC. Because MBD4 and TDG have functional overlap and the *TDG* mRNA level did not show any differences between tumor and adjacent normal tissue in our study, this may suggest that slight down-regulation of MBD4 function can be overcome by TDG activity. Recently, in human primary breast tumors, the low MBD4 protein expression was found in well-differentiated carcinoma while the MBD4 protein was highly expressed in poorly differentiated tumors samples [54]. In addition, they showed that MBD4-dependent DNA methylation reprogramming is induced by RON/MSP pathway, and is required for breast cancer metastasis [54].

The association of low *MBD4* mRNA expression with a better clinical outcome in our study suggests that a low level of *MBD4* mRNA may be a favorable prognostic biomarker. Nevertheless, because of the relatively low frequency of decreased *MBD4* mRNA expression in NMIBC and a small number of analyzed tumors further investigations are required with a larger number of patients.

There have been a few studies on the role of TDG in tumorigenesis. The role of TDG is still being judged because it is considered as a glycosylase and DNA demethylase [55]. In addition, TDG interacts with several transcription factors, suggesting its role in the regulation of gene expression [56]. Interestingly, TDG, on the one hand, is involved in epigenetic regulation of gene expression by preventing hypermethylation of CpG islands, but on the other hand, it may promote mutagenic conversion of both CpA and TpG dinucleotides to CpG ones [13, 57]. TDG down-regulation was observed in the multiple myeloma cell lines and was associated with the less efficient DNA repair activity in response to hydrogen peroxide-induced DNA damage [58]. DNA hypermethylation of *TDG* was observed in ovarian and sporadic colorectal cancer [53]. As these findings suggest that the loss of TDG function may contribute to tumorigenesis, we performed the analysis of *TDG* mRNA expression in bladder cancer. In our study, *TDG* mRNA was universally expressed without significant differences between cancer and the adjacent noncancerous tissue. Also, there was no difference between NMIBC and

MIBC. Our data imply that *TDG* mRNA analysis does not have any diagnostic or prognostic value in bladder cancer.

DNA methylation is typically mediated by DNMT1 (maintenance methyltransferase) as well as DNMT3A and DNMT3B (*de novo* methyltransferases) [59, 60]. In addition, DNMT1, but not the DNMT3, seems to play a role in the MMR pathway [61]. DNA methyltransferase 1 protein overexpression resulting in regional DNA hypermethylation may be associated with the distinct pathway leading to the development of nodular invasive carcinomas with aggressive clinical courses via widely spreading carcinomas *in situ* [62]. As in other cancers, mRNA and protein analysis have shown that bladder cancer specimens expressed a substantially higher level of DNMT1. Namely, 55% of T2-T4 bladder cancer tissues had positive staining for DNMT1. On the contrary, 24% of early stage tumors (CIS, Ta, T1) and 15% of nonmalignant bladder tissues expressed DNMT1 [60]. Immunohistochemical staining demonstrated a positive correlation between tumor invasion depth and DNMT1 expression [60]. In our study, the *DNMT1* mRNA level was significantly higher (52%) in T2-T4 compared with Ta/T1 (16%) tumors. These findings are complementary and similar to the results reported by Wu et al. [60] and confirm high expression of *DNMT1* in invasive bladder cancer. As DNMT1 can participate in multiple complex networks involved in epigenetic signaling and genome stability including interaction with *MLH1* and *MBD4* [63], in our study, we compared the mRNA expression of these genes with each other. Weak correlations between *DNMT1* mRNA expression and both *MLH1* mRNA and *MBD4* mRNA expression were found. However, it should be noticed that T2-T4 tumors with a decreased mRNA expression of *MLH1* displayed a higher mRNA expression of *DNMT1*. This is consistent with the study in the SW48 cell line where depletion of DNMT1 has been reported to markedly potentiate the ability of 5-aza-2'-deoxycytidine to reactivate *MLH1* expression [64]. Thus, suggesting that DNMT1 is required to maintain CpG methylation and aberrant gene silencing, including *MLH1*, in human cancer cells. In our study, the highly significant association was also found between increased *DNMT1* mRNA level and shorter overall survival of patients with bladder cancer. This makes the *DNMT1* mRNA expression an attractive prognostic biomarker.

In addition to epigenetic silencing, the global DNA hypomethylation in cancer genomes has been observed. This phenomenon leads to abnormal expression of genes, especially those which promote cell survival and proliferation. Global DNA hypomethylation has been associated with the progression of lung cancer and has also been suggested as a potential complementary marker in differential diagnosis of thyroid neoplasia [65, 66]. In our study, transitional epithelium from adjacent non-cancerous tissue consistently showed a low level of methylation, comparable to the level of methylation in cancer tissue. We did not observe a strong correlation between the mRNA level of *DNMT1* and % of 5-mC in tumor tissue, and the 5-mC DNA content in tumor tissue was significantly lower in the high grade tumors. The presence of hypomethylation in adjacent noncancerous tissue can be explained by the fact that some epigenetic alterations in the surrounding urothelium are the same like in the tumor tissue. This epigenetic and sometimes genetic similarity may be the result of a field cancerization or a consequence of aging. The presence of hypomethylation in BC in morphologically normal urothelial has been reported in an exfoliative urine cytology of patients with suspected bladder cancer [67]. Our data support the hypothesis of early global demethylation in bladder cancer proposed in the study by Seifert et al. [67]. Despite the fact that the reduced level of 5-mC is associated with high grade tumors, the presence of hypomethylation in adjacent noncancerous makes this type of analysis less attractive for clinical applications in the case of bladder cancer patients.

Conclusion

In conclusion, this study is one of very few in the literature discussing the prognostic value *MBD4* and *MLH3* in the bladder cancer. In addition, these data confirm the importance of DNA repair pathways in bladder cancer and suggest that *DNMT1* mRNA and *MLH1* mRNA

expression, as well as the status of *MLH1* promoter methylation, are attractive prognostic markers of shorter survival of bladder cancer patients.

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Disclosure Statement

The authors declare that there is no Disclosure Statements regarding the publication of this article.

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