Hypermethylation of p16 and DAPK promoter gene regions in patients with non-invasive urinary bladder cancer

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Submitted: 22 December 2009
Accepted: 24 January 2010

Arch Med Sci 2011; 7, 3: 512-516
DOI: 10.5114/aoms.2011.23421

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Abstract

Introduction: The aim of the study was to examine the frequency of methylation status in promoter regions of *p16* and *DAPK* genes in patients with non-invasive bladder cancer.
Material and methods: Forty-two patients (92.9% men, 73.8% smokers, 71.4% T1G1, 19.1% T1G2, 9.5% T1G3) and 36 healthy controls were studied. Isolation of genomic DNA from blood serum and methylation-specific PCR (MSP) were applied. Methylation status – methylated and unmethylated promoter regions of *p16* and *DAPK* genes were analysed.
Results: Seventeen out of 42 patients (40.5%) had the methylated *p16* gene, while methylation of the *DAPK* gene was seen in 27 of 42 cases (64.3%). In 12 patients (28.6%) both analysed genes were methylated. A statistically significant ($p = 0.046$) higher frequency of *DAPK* gene methylation (71.4%) was observed in patients with lower grade (G1) bladder cancer.
Conclusions: Detection of the aberrant hypermethylation of *DAPK* and *p16* genes in blood DNA from non-invasive bladder cancer patients might offer an effective means for earlier auxiliary diagnosis of the malignancy.

Key words: non-invasive bladder cancer, *DAPK*, *p16*, hypermethylation, methylation-specific PCR.

Introduction

During the last two decades, research concerning the aetiology of cancer disease has been considering not only causes such as carcinogen or mutagen damage of DNA, but also the processes of non-genotoxic cancer aetiology. It is suggested that epigenetic changes which concern inherited changes of gene expression are not connected with changes in the genome sequence.

Epigenetic control of gene transcription includes methylation of the DNA and covalent modifications, such as acetylation and methylation, of chromatin's histone proteins [1, 2]. Both mechanisms are closely linked to each other. Hypomethylation of the genomic DNA, which is connected to the activation of the oncogene, as well as hypermethylation of the promoter regions or exons of tumour suppressor genes, are observed in neoplastic cells.

Excessive methylation of CpG islands was found in anti-oncogene promoter regions, which were associated with the repair of damaged DNA

(*MLH1*, *06-MGMT*, *BRCA1*), metastases and invasiveness of the cancer genesis process (E-*cadherin*, *VHL*, *APC*) metabolism of xenobiotics (*GSTP1*), apoptosis (*DAPK*), cell cycle (*Rb*, *p16*, *p15*, *p14*, *p73*) and other cell processes (*ER*, *RAR-β*) of patients with varied tumour locations [3].

It is suggested that changes in the degree of methylation of DNA in cancer disease may determine the process of cancer genesis, mainly due to 'silencing' of the transcription process.

The product of the *DAPK* gene is considered to be a positive mediator of apoptosis, and moreover it is connected with the suppression of neoplastic processes [4, 5]. The $p16^{INK4a}$ protein belongs to a family of regulators of the cell cycle, called cyclin-dependent kinase inhibitors (CDKI), which bind themselves to cyclin-CDK complexes. The formation of such complexes causes, as a result, the arrest of the cell cycle in the G1 phase. This is also the way through which the $p16^{INK4a}$ protein can stop the proliferation of neoplastic cells [6].

The aim was to examine the frequency of hypermethylation in promoter regions of *p16* and *DAPK* genes in patients with non-invasive bladder cancer.

Material and methods

Forty-two patients, citizens of central Poland, with non-invasive urinary bladder cancer, of different grading (G) were examined. Methylation of promoter regions of the *p16* anti-oncogene, a gene involved in the regulation of the cell cycle, and the *DAPK* gene (death-associated protein kinase), which is involved in processes of programmed cell death, was analysed. The histopathological classification of urinary bladder cancer was confirmed by two independent histopathologists.

The reference group, chosen on the basis of age and gender, consisted of 36 healthy control volunteers. Before blood samples were taken, participants of the study were interviewed with a questionnaire. The questionnaire included questions concerning demographic data, place of residence, history of cigarette addiction and of employment.

The majority of patients in the control group (91.7%) and the study group (92.9%) were men. In the group with urinary bladder cancer 73.8% people smoked cigarettes and in the reference group the smokers constituted 69.4% of the group. In the group of patients with non-invasive urinary bladder cancer, most cases (71.4%) were characterized by a low degree of neoplasm and clinical progression (T1G1). The characteristics of both groups as well as data concerning the clinical progression and the degree of neoplasm in patients with urinary bladder cancer are presented in Table I.

Permission to conduct the research was granted by the Local Ethics Commission of Scientific Research (Resolution no. 25/2003 dated 2.06.2003). After being acquainted with the aim and the methods used in the study, as well as the possibility to quit the study at any desired moment, each of the patients included in the study or reference group signed a written informed consent form.

Before any treatment, peripheral blood samples were taken from both groups of patients.

In order to detect the methylation status of the two chosen genes, specifically the *p16* and the *DAPK* gene, in peripheral blood, the MSP method (methylation-specific PCR) was used.

Blood samples collected from each participant were stored at –70°C before DNA isolation. DNA samples were extracted from 200 µl of blood serum using the procedures of QIAamp DNA Blood Mini Kit (Syngen Biotech, Poland). Sodium bisulfite conversion of 1 µg of genomic DNA was performed with CpGenome Modification Kit (Millipore, Biokom, Poland). After bisulfite conversion, the methylation analysis was conducted by the MSP assay. Primers for determination of methylated or unmethylated *p16* and *DAPK* alleles have been described elsewhere [7-9]. A nested, two-stage PCR approach was used for *p16* methylation status analysis described by Palmisano *et al*. After 1st stage PCR with primers specific to methylated or unmethylated template, 280-bp products were 50-fold diluted and 2 µl of diluted amplicons were used in the 2nd stage PCR. Primer sequences, product sizes and annealing temperatures used for MSP analysis are presented in Table II. The methylation status of the *p16* was determined with AmpliTaqGold polymerase (Applied Biosystems, Poland)

Table I. Characteristics of studied groups

		Bladder cancer [pts]	Control group
N		42	36
Sex[2]:	Males	39 (92.9%)	33 (91.7%)
	Females	3 (7.1%)	3 (8.3%)
Age[1]		66.5 ±10.4 (51-92)	57.0 ±17.2 (37-83)
BMI[1]		26.1 ±3.9 (17.7-36.3)	25.7 ±4.0 (20.8-34.0)
Smoking habit[2]:	Yes	31 (73.8%)	25 (69.4%)
	No	11 (26.2%)	11 (30.6%)
Histopathology	T1	42 (100%)	–
	G1	30 (71.4%)	–
	G2	8 (19.1%)	–
	G3	4 (9.5%)	–

[1]Student's t-test and Mann-Whitney U test, NS – not significant, [2]χ^2 test, NS – not significant

Table II. Primer sequence, product size and annealing temperature used for MSP

Gene	Forward primer (F) (5' → 3')	Reverse primer (R) (5' → 3')	Annealing temperature [°C]	Product size [bp]	Ref.
p16	N[1]F GAAGAAAGAGGAGGGGTTGG	NR CTACAAACCCTCTAC	60	280	Palmisano *et al.*, 2000
	M[2]F TTATTAGAGGGTGGGGCGGATCGC	MR GACCCCGAACCGCGACCGTAA	65	150	Herman *et al.*, 1996
	U[3]F TTATTAGAGGGTGGGGTGGATTGT	UR CAACCCCAAACCACACAACCATAA	60	151	
DAPK	MF GGATAGTCGGATCGAGTTAACGTC	MR CCCTCCCAAACGCCGA	56	98	Esteller *et al.*, 1999
	UF GGAGGATAGTTGGATTGAGTTAATGTT	UR CAAATCCCTCCCAAACACCAA	61	106	

N – nested PCR primer, M – methylated-specific primer, U – unmethylated-specific primer

and of the *DAPK* gene with HotStarTaq polymerase (Qiagen, Syngen Biotech, Poland) in a 20 µl volume. CpGenome universal methylated DNA (Millipore, Biokom, Poland) served as a positive control of methylated alleles.

After amplification, PCR products were electrophoresed on 1% agarose with ethidium bromide along with DNA ladder and then visualized and analysed. Samples with ambiguous results were re-tested and 10% of all samples were repeated.

To analyse the material, the χ^2 test, Student's *t*-test and non-parametric Mann-Whitney-*U* test were used. Statistical significance was defined as a value $p < 0.05$.

Results

Analysis of the profile of methylation in patients with urinary bladder cancer shows that 17 out of 42 patients (40.5%) presented hypermethylation of the *p16* gene, while methylation of the *DAPK* gene was seen in 27 of 42 cases (64.3%). In the case of 12 patients (28.6%) both analysed genes were methylated, and in the case of 10 patients (23.8%), promoter regions of *p16* and *DAPK* genes were unmethylated. A statistically significant ($p = 0.046$) higher frequency of hypermethylation of the *DAPK* gene (71.4%) was observed in patients with low grade (G1) urinary bladder cancer in comparison to patients with the G2 and G3 form (55%). Such a dependency was not observed in the case of *p16* gene methylation. A statistically significant difference in the frequency of hypermethylation of the *p16* and *DAPK* genes was not seen in association with age and cigarette smoking.

Methylation of the *p16* and *DAPK* genes was not observed in blood serum of patients from the control group. Table III presents the results of hypermethylation of promoter regions in the *p16* and *DAPK* genes.

Discussion

It has been suggested that changes in the degree of methylation in DNA regulatory regions, observed during the course of neoplastic disease, can influence the process of cancer genesis through 'silencing' of the process of transcription. Some studies have found that the hypermethylation of promoter regions mainly involves genes responsible for control of the cell cycle and apoptosis, DNA damage repairing genes, and genes connected with the metabolism of xenobiotics [7].

In the presented project, the anti-oncogene *p16* and *DAPK* were chosen to analyse the hypermethylation of promoter regions in genomic DNA isolated from blood serum of bladder cancer patients. The *p16* gene is involved in the regulation of the cell cycle while the *DAPK* gene is committed

to a process of programmed cell death. *p16* and *DAPK* genes are closely connected with the process of cancer genesis, by inducing the suppression of cell proliferation. So far, in Poland, research concerning the promoter hypermethylation of genomic DNA from blood has not been conducted. At the same time, in world literature, the role of epigenetic regulation of the *p16* and *DAPK* genes in urinary bladder cancer has not been completely explained.

Past researchers have used neoplastic tissues to estimate the degree of DNA hypermethylation. Many authors claim that peripheral blood, serum and plasma can be good diagnostic material for the detection of changes, such as DNA hypermethylation, in nucleic acids [8-10]. Valenzuela *et al*. observed a statistically significant dependence between the methylation of *p16* in the promoter region in tissue and plasma, while examining 86 people with urinary bladder cancer. Dominguez *et al*. achieved similar results, assessing, among other things, the hypermethylation of promoter *p14* and *p16* regions in 27 urinary bladder cancer patients [11].

Excessive methylation of CpG islands (cytosine, phosphorylated guanine), which occurs in the promoter regions, is observed in various types of tumours. As a consequence, hypermethylation can lead to the decrease of transcription activity of specific genes and can play an important role in the induction of the cancer genesis process. Some authors claim that even up to 65% of cases of neoplastic disease may be linked to these epigenetic changes [9].

Hypermethylation of promoter regions in the *p16* and *DAPK* genes does not occur in healthy people. This confirms our observations.

Many authors have observed hypermethylation, in 7% to 60% of cases, of the *p16* gene, in patients with urinary bladder cancer [12-16]. In our study, patients with urinary bladder cancer, in whom hypermethylation of the *p16* gene was seen, made up 40.5%.

In the case of *DAPK*, hypermethylation was found in 4% to 45.5% of patients with urinary bladder cancer [14-16], but when counting all of the patients examined in our centre, this proportion was 64.3%. The observed variation could be the result of differences in the sensitivity of our method of detection of DNA methylation as well as of the heterogeneity of the sample taken [17]. Some of the authors also paid attention to the correlation between DNA methylation and the time of progression of the neoplastic disease. Higher frequency of hypermethylation in the *DAPK* gene was observed in the beginning stages of neoplastic disease [18-21].

Tada *et al*. claimed that the assessment of the changes in *DAPK* methylation can be a prognostic factor of non-invasive urinary bladder cancer recurrences [22]. They proved that the percentage of recurrences of the disease was 88% among those patients in whom *DAPK* hypermethylation was found, while it was only 28% among those patients in whom gene methylation was not seen. We observed a higher frequency in *DAPK* gene hypermethylation (71.4%) among patients with urinary bladder cancer of lower malignancy (grade G1) in comparison to patients with grades G2 and G3 (55%). A longer follow-up of these patients could help us explain the above observations.

Table III. Percentage of *DAPK* and *p16* gene methylation

Gene methylation	No. of pts	Percentage
DAPK methylation	27/42	64.3
p16 methylation	17/42	40.5
DAPK and p16 methylation	12/42	28.6
No methylation	10/42	23.8

Higher frequency (p = 0.046) of DAPK methylation (71.4%) in patients with lower grading (G1) in comparison to G2 and G3 (55%). No such dependency in methylation of p16. No methylation of p16 and DAPK in healthy control volunteers. p16 and DAPK methylation; smokers vs. non-smokers – no significance

In conclusion, detection of the aberrant methylation of *DAPK* and *p16* genes in blood DNA from non-invasive bladder cancer patients might offer an effective means for the earlier auxiliary diagnosis of the malignancy. The usefulness of the above-mentioned research, in clinical practice, still has to be evaluated.

Acknowledgments

This study was supported by a Ministry of Science and Higher Education grant (No. N N403 188434) and by an internal grant (IMP1.3/2004).

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