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Prognostic significance of *DAPK* and *RASSF1A* promoter hypermethylation in Non-Small Cell Lung Cancer (NSCLC)

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Abstract. The epigenetic inactivation of tumor suppressor genes may play an important role in the development and progression of many cancer types, including lung cancer. Therefore, we investigated the association between the aberrant promoter methylation of 2 genes: the *Death-Associated Protein Kinase* (*DAPK*) and the *Ras Association Domain Family 1A* (*RASSF1A*) by using methylation-specific PCR, and the clinicopathological features and prognosis in 70 radically resected non-small cell lung cancers (NSCLCs). Hypermethylation of the *DAPK* and *RASSF1A* promoters was found in 24 (34%), and in 18 (26%) tumor DNA samples, respectively. Regarding different clinicopathological features of NSCLCs, the *DAPK* promoter methylation was more frequently observed in squamous cell carcinoma (46%) than in adenocarcinoma (25%) and large cell carcinoma (22%), but there were no significant statistical differences (p=0.3). On the other hand, a statistically significant trend was observed between the *RASSF1A* methylation and a histological type of tumor (p=0.06). 45% of adenocarcinoma tumors showed *RASSF1A* promoter methylation in comparison to 17% of squamous cell carcinomas and 22% of large cell carcinomas. When both markers were analyzed according to the tumor-node-metastasis (TNM) staging system, no statistically significant differences were observed between stage I, II and IIIa, and the *DAPK* (p=0.2) and *RASSF1A* methylation (p=0.1). In comparison, when stage I and II were grouped together and considered *vs.* stage IIIa, a significant association between *RASSF1A* methylation and the TNM was found (p=0.03). The group of patients with tumors showing *DAPK* promoter methylation had significantly poorer overall survival rates (p=0.02) than the patients with tumors that did not show *DAPK* promoter methylation. However, the association between the *RASSF1A* promoter methylation status and the overall survival rates was not statistically significant (p=0.48). In conclusion, this paper supports the importance of epigenetic gene regulation in lung cancer progression and prognosis.

Keywords: lung cancer, *DAPK*, *RASSF1A*, methylation, prognosis

Introduction

Lung cancer is one of the most common malignancies in the world. Despite major advances in the lung cancer treatment over the past two decades, the prognosis of patients with lung cancer has improved only minimally. The poor outcome of the disease may be attributed to its late diagnosis, low cure rate for advanced stage tumors, and the poor understanding of biology of the lung tumors. Additionally, there is a need for improved clinical stratification methods that can identify lung cancer patients with high risk of recurrence and poor prognosis after curative surgical resection.

Until today the TNM staging system has remained the most powerful tool for medical decision making in NSCLC patients.

Whereas the 5-year survival rate for patients with stage I disease is about 70%, it decreases to 30% in stage IIIa [1].

On the other hand, however, TNM staging system makes it difficult to accurately predict the prognosis for each patient. For example, even patients with stage I disease, have only a 65-80% survival rate at 5 years, after they undergo a curative surgery. It is therefore important to evaluate more accurate tools, independent from TNM staging system to predict prognosis in these patients.

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Table 1. The sequence of primes, conditions of the reaction, and sizes of PCR products in MS-PCR

Gene	Forward primer (5'-3')	Reverse primer (5' – 3')	Product size (bp)
DAPK	M: ggatagtcggatcgagttaacgtc U: ggaggatagttggattgagttaatgtt	M: ccctcccaaacgccga U: caaatccctcccaaacaccaa	98 106
RASSF1A	M: gtgttaacgcgttgcgtatc U: tttggttggagtgtgttaatgtg	M: aaccccgcgaactaaaaacga U: caaaccccacaaactaaaaacaa	93 105

In recent years, it has become apparent that lung cancer represents not only the result of multiple mutations, but also the loss of gene function by aberrant methylation of CpG islands in promoter regions. Since the aberrant methylation of normally unmethylated CpG-rich areas of the promoter region has been shown to lead to the silencing of mRNA expression, this epigenetic alteration is considered to be a significant mechanism for inactivation of these tumor suppressor genes in lung cancer [2].

Aberrant promoter methylation has been described for several genes in lung cancer, including the newly identified tumor suppressor genes *DAPK* and *RASSF1A*.

The *DAPK* gene is located on chromosome 9q34. 1. It encodes a proapoptotic protein involved in the apoptosis initiated by THN-α, IFN-γ, Fas and TRAIL. Aggressiveness of malignant tumors has been associated with the methylation of the promoter region of the *DAPK* gene and the loss of *DAPK* expression [3]. Analysis for DAPK mRNA and protein in neoplastic B-cell lines, bladder carcinoma cells, and renal cell carcinoma cells found a lack of expression. The expression was partially restored by treatment with 5'-aza-2'-deoxycitidine (a demethylation agent) indicating a role for methylation in down regulation of *DAPK* [4].

The *RASSF1A* gene is located within a 120-kb region of chromosome 3p21, a region that is epigenetically inactivated at high frequency in NSCLC. RASSF1A has been shown to bind to the Ras-GTP binding protein Nore1, consistent with its role as a negative effector of Ras oncoprotein [5].

It has also been suggested that RASSF1A is an important tumor suppressor gene acting at the level of G1/S phase cell cycle progression [6].

It has been shown that the *RASSF1A* promoter is hypermethylated in lung cancer cells and that the exogenous expression of *RASSF1A* expresses tumorigenesis in nude mice [7]. Additionally, it has been reported that the *RASSF1A* gene is frequently inactivated in primary lung cancers by the *de novo* methylation of CpG islands in the promoter region [8,9].

In the present study, we investigated the association between the aberrant promoter methylation of the *DAPK* and *RASSF1A* genes, and the clinicopathological features and prognosis in radically resected NSCLCs.

Material and methods

Patients. The study includes 70 NSCLC patients (33 stage I, 22 stage II and 15 stage IIIa) examined by the Chest Oncology Group and operated in the Thoracic Surgery Unit at the Bialystok Medical University. All of these patients underwent surgical resection.

Pretreatment staging procedures included physical and blood examinations, chest radiographs and tomographs, bronchoscopy, computed tomography (CT) of the thorax and ultrasound scanning of liver. In addition, radioisotopic scans of bones, examination of bone marrow aspirates, and abdominal and brain CT scan were performed when necessary. Selected patients underwent mediastinoscopy. Pathological material has been specially reviewed for this study by the same pathologist. Postoperative, pathological staging (pTNM) was performed by correlating the operative and histological findings.

Methods. Tumor DNA was isolated and purified according to conventional methods. DNA from serum was extracted using a QIAmp Blood Mini Kit (Qiagen, Germany) following the protocol for blood and body fluids, modified as described elsewhere. Purified DNA was chemically modified by sodium bisulfite. The methylation-specific PCR (MS-PCR) was performed according to the method of Herman *et al.* [10].The two sets of primers were used, one specific for DNA methylated at the promoter region of each gene and the other specific for unmethylated DNA. The DNA sequence of primes, conditions of the reaction, and sizes of PCR products in MS-PCR are described in Table 1.

Statistical analysis. The association between the promoter methylation status of *DAPK* and *RASSF1A* and the clinicopathological characteristics was analysed using chi-squared test. The effect of methylation on patient survival was estimated by the Kaplan-Meier method and the differences between two groups were compared using the log-rank test.

The Cox univariate proportional hazards regression model was used to estimate the HR of factors influencing patient's survival.

The analysis was performed using Statistic 8.0 program.

Results

Methylation status of the CpG islands at the promoter regions of *DAPK* and *RASSF1A* genes was analysed by MS-PCR in frozen NSCLC tissue (Fig. 1). Hypermethylation of the *DAPK* and *RASSF1A* promoters was found in 24 (34%), and in 18 (26%) of the tumor DNA samples, respectively.

Regarding different clinicopathological features of NSCLCs, *DAPK* promoter methylation was more frequently observed in squamous cell carcinoma (46%) than in adenocarcinoma (25%) and large cell carcinoma (22%), but there was no statistically significant differ-

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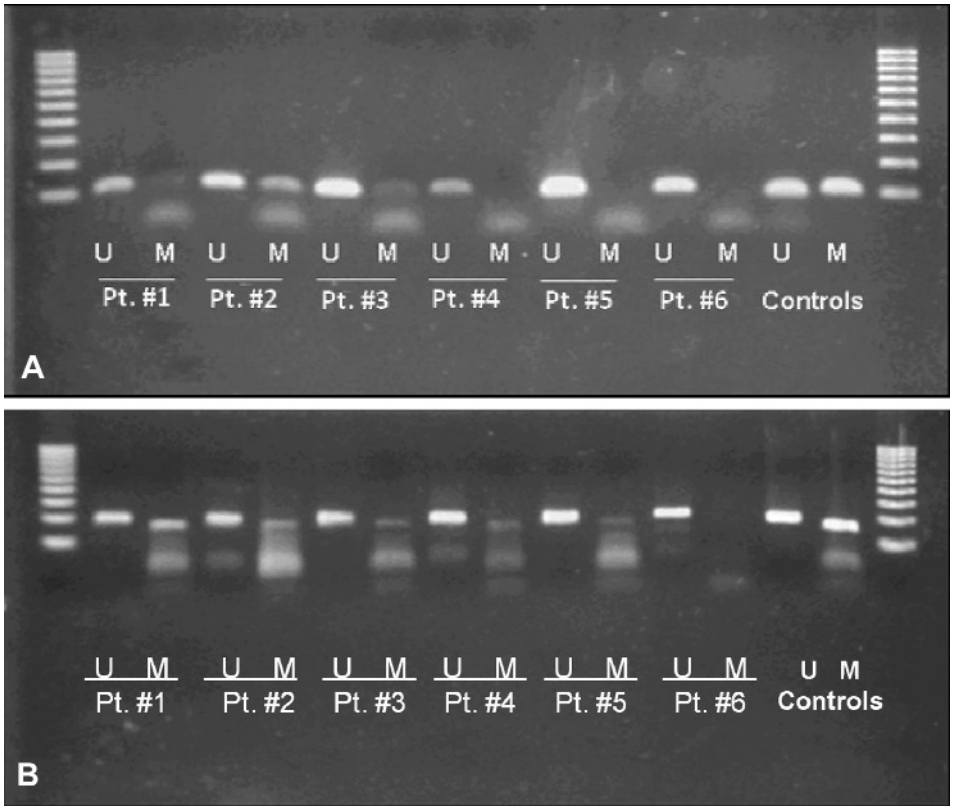

Fig. 1. Methylation analysis of DAPK (**A**) and RASSF1A (**B**) promoters in NSCLC. MSP for DAPK and RASSF1A was performed using unmethylation-specific (U) and methylation-specific (M) primer sets. Molecular weight markers are listed on left side.

ences (p=0.3). On the other hand, a statistically significant trend was observed between *RASSF1A* methylation and histological type of tumor (p=0.06). 45% of adenocarcinoma tumors showed *RASSF1A* promoter methylation in comparison to 17% of squamous cell carcinomas and 22% of large cell carcinomas (Table 2).

When the both markers were analyzed according to the TNM stage of disease, there was no statistically significant differences between I, II and IIIa stages and *DAPK* methylation (p=0.2) and *RASSF1A* methylation (p=0.1). On the other hand, there was a significant association between *RASSF1A* methylation and TNM when stages I and II were considered together *vs*. stage IIIa (p=0.03).

We then analyzed the effect of *DAPK* and *RASSF1A* promoter methylation on the patient survival.

The group of patients with tumors containing *DAPK* promoter methylation had significantly poorer

Table 2. Proportions of positive results of the DAPK and RASSF1A promoter hypermethylation by histological type and TNM stage of non-small cell lung cancer

	DAPK methylation	p-value	RASSF1A methylation	p-value
HP				
SqCC	17/41 (46%)	0.3	7/41 (17%)	0.06
AdC	5/20 (25%)		9/20 (45%)	
LCC	2/9 (22%)		2/9 (22%)	
TNM				
I (Ia+Ib)	9/33 (27%)	0.2	7/33 (21%)	0.1
II (IIa+IIb)	7/22 (32%)		4/22 (18%)	
IIIa	8/15 (53%)		7/15 (47%)	
TNM				
I+II	16/55 (29%)	0.08	11/55 (20%)	0.03
IIIa	8/15 (53%)		7/15 (47%)	
Total	24/70 (34%)			18/70 (26%)

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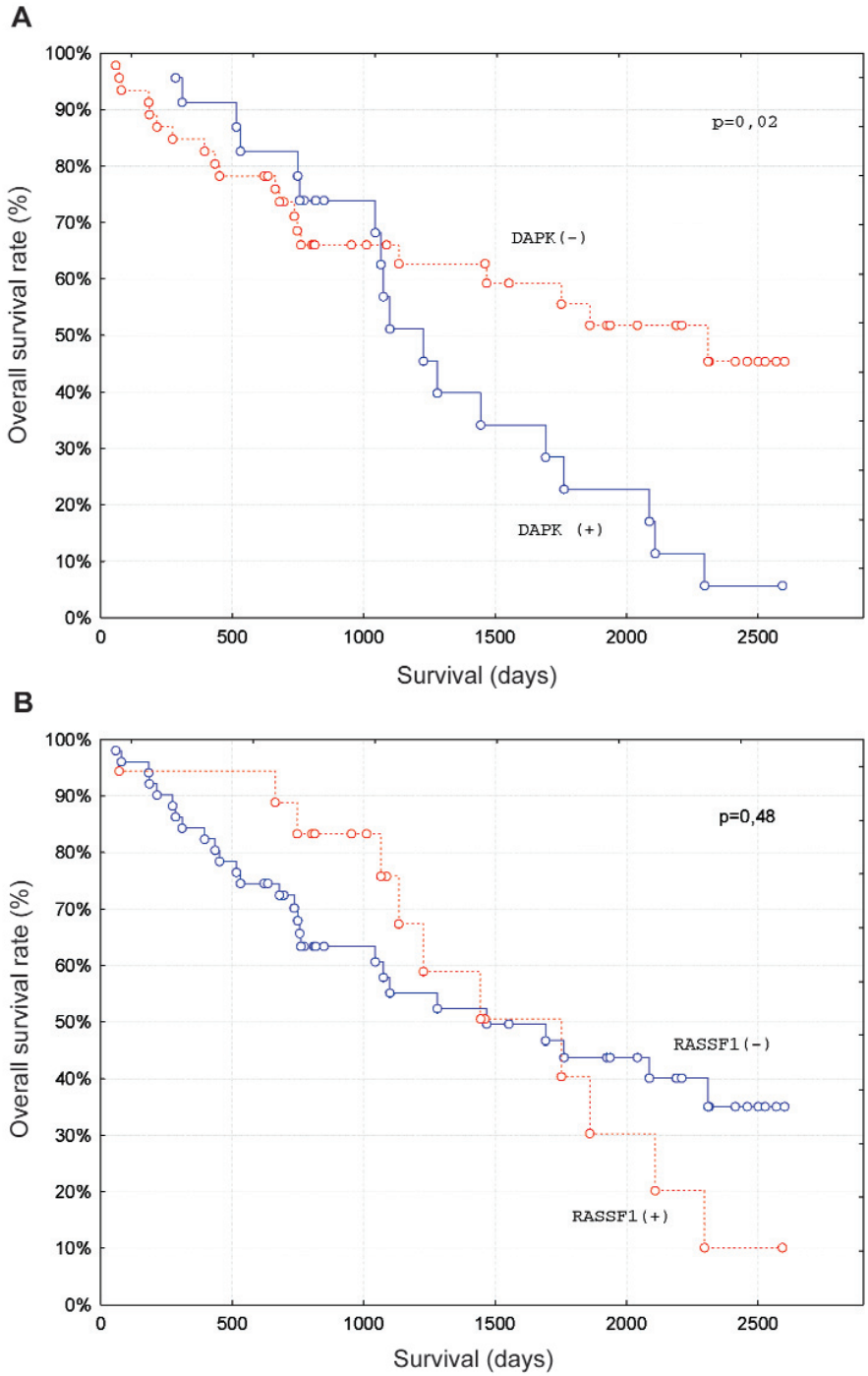

Fig. 2. Kaplan-Meier survival curves for overall survival in non-small cell lung cancer patients and DAPK (**A**) and RASSF1A (**B**) promoter methylation status.

overall survival rates (p=0.02) then the patients with tumors that did not contain *DAPK* promoter methylation (Fig. 2A). However, the association between the *RASSF1A* promoter methylation status and overall survival rates was not statistically significant (p=0.48)(Fig. 2B). The results were confirmed using univariate proportional hazards regression model (p=0.05, HR 1.903 for *DAPK* and p=0.81, HR 1.088 for *RASSF1A*) (Table 3).

Table 3. The Cox univariate proportional hazard regression model of survival

Variables	Hazard ratio (HR)	P value
TNM stage I vs II vs IIIa	1.331	p=0.15
TNM stage I+II vs IIIa	1.439	p=0.34
DAPK methylation	1.903	p=0.05
RASSF1A methylation	1.088	p=0.81

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Discussion

In lung cancer, several biological and molecular factors have been used to evaluate tumor progression as a prognostic indicator. We have previously reported the prognostic significance of some molecular factors, such as p53 and VEGF alterations [11].

Promoter DNA methylation plays an important role in tumor development by regulating the expression of specific genes [12].

In lung cancer, methylation has been observed in many tumor suppressor genes, including *DAPK* and *RASSF1A*.

In this study, we investigated the relationship between the promoter methylation of *DAPK* and *RASSF1A* tumor suppressor genes, and clinicopathological parameters and prognosis of patients with respectable NSCLC. We have found that the *DAPK* promoter methylation was more frequently observed in squamous cell carcinoma than in adenocarcinoma and large cell carcinoma, but there were no statistically significant differences. On the other hand, a statistically significant trend was observed between the *RASSF1A* methylation and lung adenocarcinoma. Some previous reports are consistent with these findings [13].

Regarding the TNM stage of disease we found no statistically significant differences between stage I, II and IIIa and the *DAPK* and *RASSF1A* promoter methylation. Similar results were obtained by Kim *et al.* [14,15] who did not show any correlation between the *DAPK* and *RASSF1A* methylation status and the TNM stage.

In this paper, we have also studied the prognostic significance of DAPK and RASSF1A in surgically treated NSCLC.

We found that the patients with tumors showing the *DAPK* promoter methylation had significantly poorer overall survival rates than the patients with tumors that did not show the *DAPK* promoter methylation.

Other reports have also demonstrated an interesting association between the methylation status of *DAPK* tumor suppressor gene and the prognosis.

Lu *et al.* [16] have analyzed the panel of six biomarkers in the population of resected stage I NSCLC patients, and have demonstrated that the *DAPK* promoter methylation was the most statistically significant predictor of survival for these patients. Similar results were shown by Tang *et al.* [17]. They have also found the significant association of the *DAPK* methylation with poorer overall and disease-free survival in stage I NSCLC patients.

Kim *et al.* [18] investigated the role of the *DAPK* methylation in 185 NSCLC patients who underwent surgical resection, including 102 patients with stage I disease. The *DAPK* methylation was significantly correlated with advanced stage, including lymph node involvement. Stage I patients with the *DAPK* methylation had worse overall survival, although this association was not statistically significant.

In our study we have also investigated the prognostic significance of the *RASSF1A* methylation in NSCLC. We have found that the association between the *RASSF1A* promoter methylation status and the overall survival rates was not statistically significant.

Similar results were obtained in two other studies showing no significant correlation between *RASSF1A* methylation and prognosis of NSCLC patients [19,20].

In the study by Wang *et al.* [21] patients whose stage I/II tumors carried *RASSF1A* promoter methylation had poorer 5-year survival rate, but the association was not statistically significant. In patients with stage IIIa disease, however, *RASSF1A* promoter hypermethylation was a stronger independent predictor of survival.

On the other hand, the methylation status of *RASSF1A* has been reported to be significantly associated with a poor survival in some other papers [22-24]. Yanagawa *et al.* [24] additionally showed that patients with *RASSF1A* methylation positive had a shorter survival in squamous cell carcinoma than in adenocarcinoma.

Published reports and our results showed in this paper support the importance of epigenetic gene regulation in lung cancer progression and prognosis. However, additional studies are necessary to prove the prognostic significance of promoter methylation of *DAPK* and *RASSF1A* in patients with NSCLC.

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