RESEARCH ARTICLE

Small suitability of the *DLEC1*, *MLH1* and *TUSC4* mRNA expression analysis as potential prognostic or differentiating markers for NSCLC patients in the Polish population

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Abstract

According to the latest data, lung cancer is one of the most common cancer worldwide, men contributing nearly 21.2% and women 8.6% of all diagnosed cancers. Late detection of tumour drastically reduces the chance for a cure. Thus, it is important to search for candidate biomarkers for screening of early stage nonsmall cell lung carcinoma (NSCLC). Tumour suppressor genes, *DLEC1*, *TUSC4* and *MLH1*, localized on 3p21 are recognized to play a role in NSCLC carcinogenesis. The aim of this study was to assess the relationship between the *DLEC1*, *TUSC4* and *MLH1* mRNA expression, and clinical features of NSCLC patients, tobacco addiction, and tumour histopathological characteristics. The *DLEC1*, *TUSC4* and *MLH1* expression was analysed in lung tumour tissue samples obtained from 69 patients diagnosed with NSCLC: squamous cell carcinoma ($n = 34$), adenocarcinoma ($n = 24$), large cell carcinoma ($n = 5$), carcinoma adenosquamosum ($n = 5$). A decreased gene expression (RQ < 0.7) was observed for *DLEC1* in 60.9% of tumour samples, for *MLH1* in 50.7% and for *TUSC4* in 26% of NSCLC samples. *DLEC1* was decreased in more aggressive subtypes: large cell carcinoma and adenocarcinoma-squamous cell carcinoma. The simultaneous downregulation of two of the studied genes, *DLEC1* and *MLH1*, was observed in 30.4% of NSCLC samples, highlighting the importance of these two genes in lung carcinogenesis. We found no correlation between the *DLEC1*, *TUSC4* and *MLH1* gene expression and NSCLC patient characteristics (gender, age and smoking) or cancer histopathology. No significant differences in the gene expression among NSCLC subtypes indicate the weakness of *DLEC1*, *TUSC4* and *MLH1* expression analysis as potential differentiating markers of NSCLC subtypes in the Polish population.

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Introduction

Lung cancer represents 22.5% of all cancers and is a leading cause of cancer deaths for both genders. The incidence and mortality due to lung cancer in well-developed countries remain at a very high and constant level, particularly among men, however, the incidence is also rising in women (Alberg and Samet 2003; Jemal *et al.* 2011). The survival rate of lung cancer patients is still poor, mainly because of the cancer detection in advanced stages. The development of new strategies of lung cancer treatment or new targeted therapies has elevated the survival rate, although early cancer detection still is an important factor affecting the effectiveness of treatment and patient survival. New molecular diagnostic biomarkers improving the early

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detection of lung cancer may also be useful in evaluation of possible treatment and as predictors of potential metastases (Hassanein *et al.* 2012).

According to histopathological verification, lung cancer is classified into two major groups based on its biological phenotype, therapy and prognosis: small cell lung cancer (SCLC, accounts for about 15% of primary lung cancers) and nonsmall cell lung cancer (NSCLC, accounts for ~85% of cases). Among NSCLC, the following subtypes can be distinguished: squamous cell carcinoma (SSC); adenocarcinoma (AC), large cell carcinoma (LCC), and carcinoma adenosquamosum (rare subtype of NSCLC containing AC and SCC cells, characterized by worse prognosis) (Alberg and Samet 2003; Tochigi *et al.* 2011).

Tumour suppressor genes (TSGs) play a crucial role in the development tumours in human. In lung cancer, the expression of TSGs localized in critical regions on the short arm of 3rd chromosome (3p) is frequently altered by loss of heterozygosity (LOH), homozygous deletions and epigenetic modifications (Zabarovsky *et al.* 2002; Hesson *et al.* 2007; Senchenko *et al.* 2010). These alterations are observed not only in advanced cancer stages but also in early cancerous stages and preneoplastic lesions of lung epithelia (Lerman and Minna 2000; Wistuba *et al.* 2000; Hesson *et al.* 2007).

In this study, we analysed three consecutive TSGs from 3p21.3 region: *DLEC1*, *MLH1* and *TUSC4*. *DLEC1* (deleted in lung and oesophageal cancer 1) is frequently lost in lung, oesophageal and kidney cancers. Decreased expression of *DLEC1*—on mRNA and protein levels—was observed in lung cancer cell lines and primary tumours. Silenced *DLEC1* expression by promoter hypermethylation was confirmed in lung cancer, but was not present in normal lung tissues (Seng *et al*. 2005; Zhang *et al.* 2010). DLEC1 protein plays a role in tumour cell growth and metastasis suppression. Restoration of *DLEC1* expression suppressed tumour growth or reduced the invasiveness of lung cancer cells (Zabarovsky *et al.* 2002; Zhang *et al.* 2010). The second analysed TSG was MutL homolog 1 (*MLH1*), a gene playing an important role in maintaining genome stability due to its function in the mismatch repair system. Decreased or lost expression of *MLH1* in cancers can lead to accumulation of mutations or loss of heterozygosity. Decreased *MLH1* expression in lung cancers was found to be associated with heavy smoking and with nodal metastasis in SCC (Xinarianos *et al*. 2000). In many studies, hypermethylation of the *MLH1* promoter region was linked to expression silencing, however, the frequency of detected methylation varied a lot (Wang *et al.* 2003; Seng *et al.* 2008). The next analysed gene tumour suppressor candidate 4 (*TUSC4*) encodes protein with nitrogen permease regulator activity. It is suggested that *TUSC4* is involved in the mismatch repair, cell cycle checkpoint signalling and activation of apoptotic pathway as its downregulation was observed in NSCLC (Li *et al.* 2004; Senchenko *et al.* 2010). *TUSC4* downregulation was reported in NSCLC, more often in SCC than in AC, however, in AC, the decrease correlated with tumour progression, as it was more frequent in advanced stages and in samples with metastases (Senchenko *et al.* 2010).

Genetic alterations, like promoter methylation, loss of heterozygosity, single-nucleotide polymorphism (SNP), posttranscriptional degradation can affect gene expression. A decreased expression of chosen genes has been previously linked with worse prognosis, metastasis or heavy smoking. However, these finding were not confirmed in further studies, e.g. on different populations. Besides, there were no previous studies regarding the expression of these genes in NSCLC in the Polish or central European population. Due to the facts stated above, we decided to assess the usefulness of *DLEC1*, *MLH1* and *TUSC4* expression analysis as potential prognostic or differentiating markers for NSCLC patients in the Polish population.

Materials and methods

The biological material (lung tumour tissue) was obtained from 72 patients admitted to the Department of Thoracic Surgery, General and Oncologic Surgery, Medical University of Lodz, Poland, between July 2010 and August 2012. The study was performed in accordance with the Helsinki declaration. The procedures used in the study have been approved by the ethical committee of the medical university of Lodz (RNN/140/10/KE). Written consent was received from each patient.

Based on the results of preoperative cytological assessment, the patients were qualified for surgery and were treated by either lobectomy or pneumonectomy. Patients did not undergo the chemotherapy or radiotherapy treatment prior to the surgery. Immediately after resection, lung tissue samples (100−150 mg) were placed in a stabilization buffer RNAlater. All samples were homogenized and frozen at −80°C until further analysis.

The resected NSCLC specimens were histhopathologically evaluated postoperatively. NSCLC was characterized using the international system of clinico-morphological classification of tumours (TNM, tumour node metastasis) according to the WHO histological typing of lung tumours, and American joint committee on cancer staging (AJCC staging) and the 7th edition (2010) of the IASCLC cancer staging project. Based on the histopathological results, the NSCLC diagnoses have been confirmed for 69 patients (mean age: 64.88 ± 8.424), and those patients were qualified for further studies.

Histopathological assessments of tumour specimens were obtained from pathomorphological reports, and were as follows: SCC, $n = 35$; AC, $n = 24$; LCC, $n = 5$; AC-SCC, $n = 5$. The studied group consisted of 23 women (mean age: 62.52 ± 8.86) and 46 men (mean age: 66.04 ± 8.03). Patients were divided into groups according to their smoking habits (the smoking history was available for 67

Table 1. Clinicopathological features of the studied NSCLC group.

		No. of patients (*n*)	%
Patients' demographic characteristics			
Age groups	≤60 years	19	27.5
	61–70 years	34	49.3
	>70 years	16	23.2
Histopathological type of NSCLC	SCC	35	51
	AC	24	35
	LCC	5	7
	AC-SCC	5	7
Lung cancer staging			
AJCC classification[a]	AJCC IA/IB	27	39
	AJCC IIA/IIB	20	29
	AJCC IIIA/IIIB	22	32
Tumour size (pT) according to pTNM classification[b]	pT1 (T1a + T1b)	20	29
	pT2	31	45
	pT3/T4	18	26
Node involvement (N) according to pTNM classification[b]	N0 stage (no node metastasis)	36	52
	N1 stage (with node metastasis)	23	33.5
	N2 stage (with node metastasis)	10	14.5
Tobacco addiction and consumption			
Smoking addiction	Nonsmokers	5	7.5
	Current smokers	42	62.7
	Former smokers	20	29.8
Smoking period	<40 years	36	59
	≥40 years	26	41
Pack years[c] (PYs)	Up to 30 PYs	18	26.8
	30–45 PYs	26	38.8
	>45 PYs	18	26.8

[a]AJCC, American Joint Committee on Cancer Staging according to the IASCLC Cancer Staging Project 7th ed. (2010).
[b]pTNM, postoperative tumour node metastasis classification according to the WHO histological typing of lung tumour.
[c]PYs were calculated according to the NCI dictionary of cancer terms: pack year is equal to 20 cigarettes smoked per day for one year (http://www.cancer.gov/dictionary?CdrID=306510).

patients). Patients who admitted to smoking cessation in the period of < 1 year prior to the surgery were qualified in the group of current smokers. Detailed data on the clinicopathological features of the studied NSCLC patients are presented in table 1.

Experimental protocols

RNA isolation: Total RNA was extracted from lung tissues using Universal RNA Purification Kit (EURx, Poland) according to the manufacturer's recommendations. The quality and quantity of RNA samples were spectrophotometrically assessed with absorbance measured at a wavelength of 260/280 nm using an Eppendorf BioPhotometer Plus (Eppendorf, Germany). Next, the quality was additionally determined by minielectrophoresis in polyacrylamide gel (Agilent 2100 Bioanalyzer, Agilent, USA), using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies, USA).

Reverse transcription: Complementary DNA (cDNA) was transcribed from 1000 ng of total RNA, using high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, USA) in a total volume of 20 μL per reaction. Reverse transcription (RT) master mix contained: 10× RT buffer, 25× dNTP mix (100 mM), 10× RT random primers, MultiScribeTM reverse transcriptase, RNase inhibitor and nuclease-free water. RT reactions were conducted in Stratagene Thermo Cycler (Perlan Technologies, USA) in the following conditions: 10 min at 25°C, followed by 120 min at 37°C, then, the samples were heated to 85°C for 5 s and held at 4°C.

Relative gene expression analysis: Gene expression was assessed in qPCR reactions in the ABI PRISM 7900HT real-time PCR system (Applied Biosystems) using TaqMan probes (selected on Applied Biosystems' web site): Hs00201098_m1 (*DLEC1*), Hs00179866_m1 (*MLH1*), Hs00198012_m1 (*TUSC4*), respectively. The TaqMan

MGB probes were labelled with FAM™ (6-carboxyfluorescein) at the 5′ end as the reporter dye and with a nonfluorescent quencher (TAMRA, 6-carboxytetramethylrhodamine) at the 3′ end. The *ACTB* gene (Hs99999903_m1) was selected as the reference for real-time PCR. The relative expression levels were assessed using the comparative delta–delta C_T method (TaqMan Relative Quantification Assay software, Applied Biosystems) and presented as RQ values, adjusted to the *ACTB* expression. Macroscopically unchanged lung tissue served as a calibrator sample for which RQ value equaled 1.

The qPCR mixture contained cDNA (1–100 ng), 20× TaqMan® Gene Expression Assay, 2× TaqMan® Gene Expression Master Mix and RNase free water in total volume of 20 μL. The PCR conditions were as follows: initial incubation at 50°C for 2 min, AmpliTaq Gold® DNA polymerase activation at 94.5°C for 10 min, real-time PCR amplification was processed in 40 cycles composed of 30 s denaturation at 97°C, followed by 1 min elongation step at 59.7°C. The qPCRs were repeated thrice for each sample.

Statistical analysis: To compare the levels of relative expression values (RQs) of *DLEC1, MLH1* and*TUSC4* among NSCLC histotypes, Kruskal–Wallis ANOVA test and Mann–Whitney U test were used. The Spearman's rank correlation coefficient, Mann–Whitney U test and Kruskal–Wallis ANOVA test were performed to evaluate the relationship between the expression levels (RQ values) of the studied genes (*DLEC1*, *MLH1* and *TUSC4*) and the examined parameters (patient characteristics: age, gender and tumour staging according to pTNM and AJCC classification, tobacco addiction and consumption). The, statistical significance level was estimated at $P < 0.05$. The results of relative expression levels of the studied genes are presented as RQ (relative quantification) median ± SEM and RQ median ± SD values. Statistica for Windows 10.0 program was applied for calculations.

Results

The results of the qPCR analysis, presented as RQ values were calculated using the delta–delta C_T method. The obtained C_T values of the studied genes were adjusted to the expression of *ACTB* (endogenous control) and related to the expression level of calibrator (macroscopically unchanged lung tissue), for which RQ = 1. The results indicating median RQ values for each studied gene and the number of samples with the significantly decreased gene expression (RQ < 0.7) are presented in tables 2 and 3.

Among the studied genes, *DLEC1* showed the most pronounced under-expression, followed by *MLH1*. The number of samples with the significantly importantly decreased *DLEC1* and *MLH1* expression was 59–63% and 40–60%, respectively, depending on the histotype. The *TUSC4* expression level was similar to that observed in normal lung tissue (calibrator), as it was near 1 or slightly above 1. The number of NSCLC samples with the significantly decreased *TUSC4* expression ranged from 20 to 40%. The simultaneous downregulation of all three genes was observed in 14 NSCLC samples (20.3%). Considering two genes showing more decreased expression (*DLEC1* and *MLH1*), the simultaneous downregulation was observed in 21 cases—30.4% of NSCLC samples. The analysis of *DLEC1* and *MLH1* simultaneous silencing among NSCLC histotype revealed the higher downregulation in LCC, 40% (2/5 cases) and AC, 37.5% (9/24), followed by 25% (9/35) in SCC and 20% (1/5) in AC–SCC. The statistical analysis (Kruskall–Wallis test) did not reveal any correlations between the expression levels of the studied genes and NSCLC subtypes ($P > 0.05$). The gene coexpression analysis (the Spearman's rank correlation coefficient) showed no statistically significant correlations between *DLEC1*, *MLH1* and *TUSC4* (see table 2).

Gene expression levels were also correlated with tumour staging (pTNM, AJCC), patients' age, gender and smoking history. Median RQ values were slightly lower in women than in men for *DLEC1* and *MLH1* genes.

Table 2. Relative expression (median RQ values) of the studied genes and the frequency of gene downregulation in all studied samples.

		DLEC1	*MLH1*	*TUSC4*
NSCLC total group ($n = 69$)	Median RQ value	0.425	0.696	0.945
	↓ Expression[a] (%[b])	43 (62%)	35 (50.7%)	18 (26%)
Adenocarcinoma ($n = 24$)	Median RQ value	0.468	0.667	0.787
	Decreased expression	15 (62.5%)	13 (54%)	8 (33.3%)
SCC ($n = 35$)	Median RQ value	0.424	0.768	1.328
	Decreased expression	22 (63%)	17 (48.6%)	7 (20%)
LCC ($n = 5$)	Median RQ value	0.279	0.631	0.835
	Decreased expression	3 (60%)	2 (40%)	2 (40%)
AC–SCC ($n = 5$)	Median RQ value	0.399	1.586	0.948
	Decreased expression	3 (60%)	3 (60%)	1 (20%)

[a]↓ expression, no. of samples with decreased expression.
[b]% Samples with decreased expression.

Table 3. Relative expression (median RQ values) of the studied genes and the frequency of gene downregulation according to gender, age, AJCC and TNM classifications.

			DLEC1	*MLH1*	*TUSC4*
Gender	Woman	Median RQ value	0.399	0.789	0.696
		↓ Expression[a] (%[b])	14 (61%)	13 (56.5%)	8 (35%)
	Man	Median RQ value	0.440	1.166	0.682
		↓ Expression	29 (63%)	23 (50%)	13 (283%)
Age	≤60 years	Median RQ value	0.484	1.329	0.894
		↓ Expression	12 (63%)	8 (42%)	6 (31.5%)
	61–70 years	Median RQ value	0.412	0.838	0.838
		↓ Expression	22 (64%)	23 (67%)	10 (29%)
	>70 years	Median RQ value	0.370	1.114	0.804
		↓ Expression	10 (62.5%)	7 (44%)	6 (37.5%)
AJCC classification[c]	AJCC IA/IB	Median RQ value	0.360	0.957	0.697
		↓ Expression	17 (63%)	14 (52%)	7 (26%)
	AJCC IIA/IIB	Median RQ value	0.402	0.814	0.711
		↓ Expression	13 (65%)	12 (60%)	6 (30%)
	AJCC IIIA/IIIB	Median RQ value	0.467	0.975	0.598
		↓ Expression	11 (50%)	12 (54.5%)	6 (27%)
pTNM staging[d]	T1	Median RQ value	0.889	1.524	0.834
		↓ Expression	8 (40%)	9 (45%)	5 (25%)
	T2	Median RQ value	0.360	0.789	0.697
		↓ Expression	23 (75%)	16 (51.6%)	9 (29%)
	T3/T4	Median RQ value	0.400	0.933	0.527
		↓ Expression	11 (61%)	11 (61%)	5 (28%)
	N0	Median RQ value	0.392	0.669	0.885
		↓ Expression	21 (58%)	21 (58%)	8 (22%)
	N1	Median RQ value	0.544	0.726	0.917
		↓ Expression	14 (61%)	11 (48%)	8 (35%)
	N2	Median RQ value	0.455	0.691	1.217
		↓ Expression	6 (60%)	5 (50%)	2 (20%)

[a]↓ Expression, no. of samples with decreased expression.
[b]% of samples with decreased expression.
[c]AJCC, American Joint Committee on Cancer Staging according to the IASCLC Cancer Staging Project 7th ed. (2010).
[d]pTNM, postoperative tumour node metastasis classification according to the WHO histological typing of lung tumour 1.

However, the differences were not statistically significant ($P > 0.05$, Mann–Whitney U test). The most decreased expression among the analysed genes was observed for *DLEC1* (in 61 and 63% of samples for women and men, respectively). Regarding age groups (≤60 years; 61–70 years; >70 years), the *DLEC1* and *TUSC4* expression levels decreased with increasing patients' age, with the lowest level in patients older than 70 years. However, the obtained results were not statistically significant ($P > 0.05$, Kruskall–Wallis test). We have not found significant differences for the *MLH1* expression, either ($P > 0.05$, Kruskall–Wallis test). The frequency of samples with the reduced *TUSC4* expression was higher in patients older than 70 years when compared with other age groups, however, not significantly ($P > 0.05$, Kruskall–Wallis test).

We also evaluated the association between the gene expression and histopathological features of tumours (pTNM staging, AJCC classifications). Taking into account TNM classification, namely, the size of the tumour (pT), median RQ values of all genes were lower in pT2 and pT3/T4 stages when compared with the pT1a + T1b group. However, the differences were not significantly different ($P > 0.05$; Kruskal–Wallis test). The analysis of gene expression in groups according to lymph node metastasis (TNM classification: N0 vs N1 vs N2) did not reveal statistical significance ($P > 0.05$; Kruskal–Wallis test), although the expression levels of all studied genes were the lowest in N0 group.

The analysis of gene expression performed in groups according to the AJCC classification (IASCLC 2010) revealed the lowest median RQ value for *TUSC4* in the most advanced tumour stage (AJCC IIIA/IIIB) in comparison to AJCC IA/IB and AJCC IIA/IIB stages. Median RQ values increased with tumour aggressiveness for *DLEC1* (the lowest median RQ value in AJCC IA/IB stage). In case of *MLH1*, the lowest median RQ value was observed in AJCC IIA/IIB stage. However, in none of the analysed cases, the differences were statistically significant ($P > 0.05$, Kruskall–Wallis test).

Table 4. Gene expression levels (median RQ values) according to the tobacco addiction and consumption.

	DLEC1	*MLH1*	*TUSC4*
Smoking addiction			
Current smokers	0.454	0.670	1.038
Former smokers	0.358	0.761	0.819
Never smokers	0.257	0.435	0.917
Duration of tobacco addiction			
<40 years	0.483	1.038	0.829
40 years and more	0.412	1.201	0.599
Long-life tobacco intake			
30 PYs or less	0.527	0.617	0.934
30–45 PYs	0.350	1.288	1.352
More than 45 PYs	0.442	0.458	0.975

The gene expression analysis in relation to the tobacco addiction revealed the most pronounced downregulation of *DLEC1* in nonsmokers when compared to former and current smokers. Similarly, the expression of *MLH1* was the lowest in never smoking patients. The highest expression was observed in *TUSC4* in current smokers group. However, the differences between the individual groups were not statistically significant ($P > 0.05$, Kruskall–Wallis test). The results are summarized in table 4. The analysis where patients were grouped according to the duration of tobacco addiction and the amount of cigarettes smoked in a lifetime calculated as pack years (PYs), revealed the lowest expression of *DLEC1* (see table 4).

Discussion

Lung cancer incidence and mortality is the leading cause of cancer deaths for both sexes (Alberg and Samet 2003; Jemal *et al.* 2011). Because of the fact that many lung cancers are still detected at an advanced stage and the survival rate of lung cancer patients is poor, there is a strong need for the development of new biomarkers. Studies on molecular background of lung cancerogenesis have not led to the development of a compatible genetic diagnostic/prognostic panel. Advances in proteomics and metabolomics have given promising results, but the most requested are simple biomarkers whose analysis could be performed in nonspecialized laboratories with the use of simple tests. Genes selected for this study, *MLH1*, *TUSC4* and *DLEC1*, possess the tumour suppressor function. All three genes are localized on 3p, a frequently altered chromosomal region in lung cancers (Zabarovsky *et al.* 2002; Senchenko *et al.* 2010). Loss of heterozygosity, homozygous deletions and promoter hypermethylation are a frequent mechanism of silencing of 3p genes (Lerman and Minna 2000; Wistuba *et al.* 2000; Hesson *et al.* 2007). However, this region is widely studied, until now, there have been no studies on the Polish population regarding the expression analysis of those genes. In this study, we observed altered expression of the selected 3p genes in all four subtypes: AC, SCC, LCC and AC–SCC. This is the first study on the *MLH1*, *TUSC4* and *DLEC1* expression in adenosquamous carcinoma of the lung, a rare NSCLC subtype.

Previous studies demonstrated that *MLH1*, *TUSC4* and *DLEC1* were downregulated in lung cancer (Zabarovsky *et al.* 2002; Anedchenko *et al.* 2008; Senchenko *et al.* 2010). In our study, we confirmed the decreased expression of, especially, *DLEC1*, which was importantly downregulated in 60.9% of NSCLC samples and *MLH1* importantly downregulated in 50.7% of NSCLC samples. The simultaneous downregulation of these two genes was observed in 30.4% of lung tumour samples. In the analysis performed by Seng *et al.* (2008) on Australian population, the inactivation of *DLEC1* by promoter methylation was an indicator of poor survival (especially for SCC), but concordant *DLEC1* and *MLH1* genes inactivation was a poor prognostic marker (independent of AJCC stage) for all NSCLC histotypes. However, the observed promoter hypermethylation in Seng *et al.* (2008) study did not have direct impact on expression silencing. In our study, *DLEC1* downregulation was observed in 63% of SCC samples and it was the highest frequency of the decreased gene expression among NSCLC histotypes. Nevertheless, it did not reach statistical significance. In other study, performed by our group, on similar NSCLC cohort, it was demonstrated that the decreased expression was simultaneously occurring with promoter methylation was found in 63.3% of NSCLC samples for *DLEC1* and 6.1% for *MLH1* (Pastuszak-Lewandoska *et al.* 2016). Regarding *MLH1* expression, the decrease was observed in half of the analysed population, occurring more frequently in AC and AC–SCC. The *MLH1* level was decreased in 40% of LCC samples, and it was the lowest frequency of its downregulation. Unfortunately, the number of LCC and AC–SCC samples in our cohort was low. Additionally, we did not find any correlation with poor prognosis. Nevertheless, the simultaneous decrease of the *DLEC1* and *MLH1* expression, might be an interesting marker of pathogenic process in lung tumour cells.

DLEC1 function in normal cells is the suppression of tumour growth. Decreased *DLEC1* expression—on mRNA and protein levels—was observed in primary tumours and lung cancer cell lines (no expression detected) (Zabarovsky *et al.* 2002; Seng *et al.* 2008). Additionally, its expression alterations were observed not only in NSCLC, but also in preneoplastic lesions of the lung epithelia, as a process leading to cancer (Zhang *et al.* 2010). In our study performed on malignant lesions, of all the three analysed genes, *DLEC1* revealed the most pronounced downregulation, with twice or more than twice decrease in the expression level in all NSCLC subtypes. In the recent study performed by our group, high percentage (78%) of samples with *DLEC1* decrease and the significantly decreased expression in NSCLC samples was observed

in comparison to macroscopically unchanged lung tissues (Pastuszak-Lewandoska *et al.* 2016). The lowest *DLEC1* expression was found in the subtypes characterized by poor prognosis and aggressiveness. We demonstrated that the *DLEC1* downregulation was more frequent in more advanced stages of lung cancer (pT2 and pT3/4) when compared with pT1a + T1b (61–75% vs 40%). It is in line with the results of Seng *et al.* (2008), who observed correlations between the decreased *DLEC1* expression and poorer prognosis in NSCLC patients and lymph node metastasis, as well as shorter survival time. This can lead to the conclusion that gene expression decreases with the increasing tumour staging and the aggressiveness of the histopathological subtypes. On the other hand, the *DLEC1* expression was decreasing with age, indicating that silencing of the expression might be due to processes accumulating with the age, not because of aggressiveness of the tumour. Similar results were observed in the study by Pastuszak-Lewandoska *et al.* (2016), where *DLEC1* expression level in SCC patients negatively correlated with age, and significantly elevated methylation was observed among patients aged 61–70 years.

Smoking is a well-known risk factor in lung cancer development (Mao *et al.* 1997). Indeed, in our study, a stronger *DLEC1* expression decrease was observed in groups of patients with stronger tobacco addiction, i.e. persons smoking more than 31 PY and smoking longer than 40 years (both current and former smokers). Although, the lowest *DLEC1* expression level was observed in patients who had never smoked, no information on second-hand smoking in nonsmokers was available, and the tobacco impact cannot be excluded. This inconsistency can also be explained as an effect of accumulation of genetic alterations in the advanced stage of rapidly developing tumour (Diaz-Cano 2012). Besides, the group of nonsmokers were very small, which could also affect the observed differences.

The studies on *MLH1* expression in AC and SCC subtypes demonstrated reduction of its expression, observed both on mRNA and protein levels (Xinarianos *et al.* 2000; Cooper *et al.* 2008; Geng *et al.* 2009). In Geng *et al.* (2009) study, loss of gene expression was observed in 79.3% of cases. The decreased *MLH1* protein expression was found in 53–76.7% of samples, depending on the study (Xinarianos *et al.* 2000; Cooper *et al.* 2008; Geng *et al.* 2009). In our analysis, the mRNA expression was decreased in 50.7% of NSCLC samples. In Cooper *et al.* (2008) study, it was demonstrated that *MLH1* reduced expression occurred in all subtypes of cancer (AC, SCC and LCC), but significantly more frequently in LCC and poorly differentiated tumours. Comparing our results to those of Cooper's, in our study, we confirmed the decreased expression of *MLH1* in AC, SCC and LCC subtypes, but the differences between histopathological groups were not significant.

Xinarianos *et al.* (2000) showed the correlation of *MLH1* expression in 83% of SCC subtype samples with node metastasis and with heavy smoking. We did not observe such a correlation. Despite the fact that *MLH1* expression level was the lowest in the group of patients who smoked more than 45 PYs, we could not confirm the relationship between the *MLH1* expression and the amount of cigarettes smoked in lifetime. These results are not in concordance with Xinarianos' results (Xinarianos *et al.* 2000). The possible reason could be due to a different definition of heavy smokers in the Xinarianos study (more than one pack per day) than in our study as we were analysing the amount of cigarettes smoked in a lifetime. Similar to *DLEC1*, the lowest *MLH1* expression was also found in never smoking patients, but it was also decreased in current/former smokers. This observation can be explained according to the observation of the European prospective investigation into cancer and nutrition (EPIC), where constant smoking was identified as affecting or accelerating the tumourigenesis (effects of smoking on the second mutation rate / second molecular event and the net clonal expansion rate), but having low impact on the first molecular event (Schöllnberger *et al.* 2006).

In our study, we also analysed the new putative marker gene *TUSC4. TUSC4* decreased expression was previously observed in NSCLC and it was proposed that it could affect the resistance to cisplatin (Li *et al.* 2004; Ueda *et al.* 2006). In the study performed on an east European cohort, Senchenko *et al.* (2010) demonstrated that downregulation of *TUSC4* expression was more frequent in SCC than in AC. In our study, we observed only a slight downregulation of *TUSC4*. Small differences in the gene expression profile were observed in groups according to the TNM staging. The *TUSC4* expression decreased with the size of the lesion (pT) according to the TNM stages, but was increasing with the lymph node involvement. This is the first study on *TUSC4* expression in the Polish/Central European population.

In conclusion, this project was intended to analyse the possibility of using those genes as NSCLC biomarkers in the Polish population. Our study concerning *TUSC4*, *DLEC1* and *MLH1* genes is one of the first such studies that focus on the Polish population. In this study, *DLEC1*, *MLH1* and *TUSC4* gene expression was analysed in NSCLC samples to verify whether there is any relationship with the following criteria: demographic features (age, gender), tobacco addiction, TNM and AJCC classifications, and histopathological subtypes of NSCLC samples. No correlations found between the selected gene expression profiles and clinical features of NSCLC patients or cancer histopathology can lead to the conclusion that these genes cannot be taken into consideration as diagnostic/prognostic markers in nonsmall cell lung cancer. Nevertheless, the observed decreased expression of, especially, *DLEC1* and *MLH1*, may be associated with proliferation, accumulation of genetic changes leading to the dedifferentiation or spread of tumour cells. An interesting finding on elevated expression of *TUSC4* in

the group with lymph node involvement requires further studies. The above presented results indicate small suitability of the *DLEC1*, *MLH1* and *TUSC4* mRNA expression analysis as potential prognostic or differentiating markers for NSCLC patients in the Polish population. To confirm/reject the usage of these genes as potential diagnostic biomarkers, the extended study should be performed incorporating in particular, the nonsmoking patients group.

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