Med Oncol (2016) 33:75
DOI 10.1007/s12032-016-0791-3

ORIGINAL PAPER

Expression level and methylation status of three tumor suppressor genes, *DLEC1*, *ITGA9* and *MLH1*, in non-small cell lung cancer

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Received: 9 May 2016 / Accepted: 4 June 2016 / Published online: 10 June 2016

Abstract Despite therapeutic advances, lung cancer remains one of the most common causes of cancer-related death in the world. There is a need to develop biomarkers of diagnostic and/or prognostic value and to translate findings in basic science research to clinical application. Tumor suppressor genes (TSGs) represent potential useful markers for disease detection, progression and treatment target. We tried to elucidate the role of three 3p21.3 TSGs: *DLEC1*, *ITGA9* and *MLH1*, in non-small cell lung cancer (NSCLC). We assessed their expression pattern by qPCR in 59 NSCLC tissues and in the matched macroscopically unchanged lung tissues. Additionally, we analyzed gene promoter methylation status by methylation-specific PCR in NSCLC samples. We did not find significant correlations between gene expression and methylation. In case of *DLEC1* and *ITGA9*, expression levels were decreased in 71–78 % of tumor samples and significantly different between tumor and normal tissues ($P = 0.0001$). It could point to their diagnostic value. *ITGA9* could also be regarded as a diagnostic marker differentiating NSCLC subtypes, as its expression level was significantly lower in squamous cell carcinoma ($P = 0.001$). The simultaneous down-regulation of *DLEC1* and *ITGA9* was observed in 52.5 % of NSCLCs. MSPs revealed high frequencies of gene promoter methylation in NSCLCs: 84 % for *DLEC1* and *MLH1* and 57 % for *ITGA9*. Methylation indexes reflected moderate gene methylation levels: 34 % for *ITGA9*, 27 % for *MLH1* and 26 % for *DLEC1*. However, frequent simultaneous methylation of the studied genes in more than 50 % of NSCLCs suggests the possibility of consider them as a panel of epigenetic markers.

Keywords Non-small cell lung cancer · Gene expression · Promoter methylation · Tumor suppressor gene · Biomarker

Introduction

Lung cancer is representing 22.5 % of all cancer, as one of the leading causes of cancer deaths [1]. Although substantial progress has been recently achieved toward developing methods and strategies to lung cancer treatment, the survival rate of lung cancer patients is still poor. New molecular technologies used in the field of genomics, epigenomics, proteomics, metabolomics and transcriptomics greatly affect cancer-related research. Their aim is to develop biomarkers for early cancer diagnosis, prediction of potential metastases and evaluation of possible treatment and to translate selected promising findings in basic science research to clinical application [2].

Tumor suppressor genes (TSGs) play crucial role in blocking the development of human tumors. In lung cancer, frequent loss of heterozygosity (LOH) on short arm of chromosome 3 (3p) in multiple critical regions and homozygous deletions and epigenetic modifications are

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observed in early cancerous stages and even in preneoplastic lesions [3–5]. This indicates the presence and significance of genes located in 3p. Indeed, LOH analyses in 3p found two frequently affected regions (FARs) within 3p21.3: LUCA (lung cancer region) in the centromeric region (3p21C) and AP20 (Alu-PCR clone 20 region) in the telomeric region (3p21T) [6]. These regions contain loci of multiple TSGs [6–8]. In our study, we analyzed three consecutive TSGs from AP20 region: *DLEC1*, *ITGA9* and *MLH1*. It should be stressed that this study was a part of a more comprehensive research project, which included TSGs also from LUCA region, namely *FUS1*, *NPRL2/G21* and *RASSF1A* [9].

Cancer development is also linked with aberrant DNA methylation patterns, which can be characterized as global genome hypomethylation accompanied by regional hypermethylation [10]. Primary non-small cell lung cancer and NSCLC cell lines show multiple aberrantly methylated genes, including *RASSF1A*, *DAPK*, *MGMT*, *RARβ*, *TIMP3*, *p16*, *CDH1*, *GSTP1* and *p14ARF* [11–13]. The studies point to gene hypermethylation as a molecular biomarker in early detection of lung neoplasms (e.g., *p16*), or associated with late stage of lung cancer (e.g., *ASC/TMS1*) [14]. It is suggested that modified methylation patterns could be used for diagnosis, for prognosis and even for direct treatment of cancer.

The pre-specified hypothesis tested in the study was altered expression and methylation status of selected TSGs (*DLEC1*, *ITGA9*, *MLH1*) from AP20 region in primary non-small cell lung cancer. The results of such analysis and correlation with NSCLC histotypes, tumor and patient characteristics should give an answer on the importance of these genes in lung carcinogenesis. Additionally, together with our previous findings [9], they will expand our knowledge on lung cancer biology and fit into the trend of recent studies looking for biomarkers in early lung carcinogenesis and cancer progression.

Materials and methods

The study has been approved by the Ethical Committee of the Medical University of Lodz, Poland, no. RNN/140/10/KE. Written informed consent was obtained from each patient.

Lung tissue samples and patients clinical characteristics

The study was performed in the group of patients admitted to the University Clinic of Pneumology and Allergology of 1st Chair of Internal Diseases of Medical University of Lodz and in the Department of Thoracic Surgery, General and Oncologic Surgery, Medical University of Lodz, Poland, between July 2010 and March 2013. Patients, with diagnosed NSCLC, were treated by lobectomy or pneumonectomy. During the surgery, lung tissue samples (100–150 mg) were obtained from the center of primary lesion and 10 cm distant from it (the adjacent non-cancerous specimen, macroscopically unchanged). Immediately after resection, lung tissue samples were placed in a stabilization buffer RNAlater®, then divided into smaller parts (30–50 mg) for further analysis and frozen at −80 °C.

The tissue samples were postoperatively histopathologically evaluated by pathologist and classified according to AJCC staging as well as TNM classification (pTNM). The final study group consisted of 59 patients (24 women and 35 men), with a mean age of 61 ± 7.62. For correlation analyses, they were divided into three groups: aged under or equal to 60 years ($n = 14$), 61–70 years ($n = 30$) and over 70 years ($n = 15$). The history of smoking was obtained for all patients: 54 were smokers (current smokers, $n = 31$, and former smokers, $n = 23$) and five non-smokers. For further correlation analyses, smokers were divided into two groups according to pack-year (PY) values (1 pack-year = 20 cigarettes smoked per day for 1 year): <40 PYs ($n = 26$) and ≥40 PYs ($n = 28$). Based on histopathological assessments, the group of patients was subdivided in relation to NSCLC subtypes: squamous cell carcinoma (SCC, $n = 34$), adenocarcinoma (AC, $n = 20$) and large cell carcinoma (LCC, $n = 5$). All cases were primary tumors without chemo- or radiotherapy treatment.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from primary lung cancer and macroscopically unchanged lung tissues using Universal RNA Purification Kit (Eurx, Poland), according to the manufacturer's recommendations. The quality and quantity of RNA samples was assessed in Agilent 2100 Bioanalyzer (Agilent, USA) using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies, USA).

Reverse transcription (RT) reactions were performed using 100 ng of total RNA and High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). RT master mix contained: 10× RT buffer, 25× dNTP Mix (100 mM), 10× RT Random Primers, MultiScribe™ Reverse Transcriptase, RNase Inhibitor and nuclease-free water, in a total volume of 20 µl. Negative control was included in each RT reaction, containing no RNA (No RT control). RT reactions were performed in the following conditions: 10 min at 25 °C, 120 min at 37 °C, then 5 s at 85 °C and 4 °C hold.

The relative expression of the studied genes was assessed in qPCRs performed in 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with RQ software (TaqMan Relative Quantification Assay software). Micro Fluidic Cards, the so-called TLDA (TaqMan® Low-Density Custom Arrays) plates, were used in qPCRs. The selected assays (Applied Biosystems, USA), preloaded on the plates, were: Hs00201098_m1 (DLEC1), Hs00979865_m1 (*ITGA9*), Hs00179866_m1 (MLH1) and Hs00382667_m1 (*ESD*) which served as a reference gene. RNA from normal lung tissue (Human Lung Total RNA, Ambion®, USA) was used as calibrator. The reaction mixture contained: 50 µl cDNA (50 ng) and 50 µl TaqMan® Universal Master Mix (Applied Biosystems, USA). For negative control, No RT control sample was used. The PCR program was as follows: initial incubation 2 min at 50 °C, AmpliTaq Gold® DNA polymerase activation at 94.5 °C for 10 min, then 40 two-step cycles 30 s at 97 °C and 60 s at 59.7 °C. Each reaction was performed in triplicate.

DNA extraction, bisulfite conversion and methylation-specific PCRs

Genomic DNA was obtained using QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. The quality and quantity of DNA was spectrophotometrically assessed (Eppendorf BioPhotometer™ plus, Germany), and DNA samples with a 260/280-nm ratio in the range 1.8–2.0 were considered as high quality and selected for further analysis.

Bisulfite conversion was performed using genomic DNA (1 µg) and CpGenomeTM Turbo Bisulfide Modification Kit (Chemicon International, Millipore, USA), according to the manufacturer's recommendations. The concentration and purity of modified DNA was spectrophotometrically estimated (Eppendorf BioPhotometer™ plus, Germany).

Next, methylation-specific polymerase chain reactions (MSPs) were performed to assess methylation status of the studied genes. Primers for MSPs were designed according to the criteria described by Feltus et al. [15]. Promoter region sequences for the studied genes were taken from NCBI database. Then, sequences were analyzed using a computer tool (methPrimer v1.1 beta, Li Lab, Department of Urology, USCF) [16] that enabled the prediction of bisulfite modified sequence (with cytosines in sites of methyl cytosines and uracils in sites of unmethylated cytosines). Table 1 presents the sequences for methylated and unmethylated promoter regions of the studied genes. MSP master mix contained: 1000 ng DNA, 0.7 µM of each primer (Sigma, Germany), 2.5 µM dNTPs mix, 2.5 µM $MgCl_2$, Hot Start AmpliTaq Gold® 360 DNA Polymerase (5U/µl), 10× Universal PCR buffer and nuclease-free water, in a total volume of 12.5 µl. PCR conditions were as follows: initial denaturation at 95 °C for 5 min, followed by 40 three-step cycles involving denaturation at 95 °C for 45 s, specific annealing temperature (see Table 1) for 45 s and elongation at 72 °C for 1 min; the final elongation step was done at 72 °C for 10 min.

In each PCR, CpGenome Universal Methylated DNA (enzymatically methylated human male genomic DNA) served as a positive methylation control and CpGenome Universal Unmethylated DNA (human fetal cell line) was used as a negative control (CHEMICON International, Millipore, USA). Blank samples with nuclease-free water instead of DNA were used as a control for PCR contamination.

The products of MSPs were electrophoretically separated (2 % agarose gel) and their concentrations (ng) were spectrophotometrically estimated (for U and M DNA alleles), using DNA1000 LabChip Kit, on Agilent 2100 Bioanalyzer (Agilent Technologies, USA). For each sample, methylation index (MI) was assessed, according to the formula: peak height of methylated products/(peak height of methylated products + peak height of unmethylated product), $MI = (M)/(M + U)$.

Statistical analysis

The results of relative expression analysis were compared between cancer and non-cancer specimens using Mann–Whitney *U* test and Kruskal–Wallis test. The same non-parametric tests were applied to compare gene expression and methylation patterns between NSCLC subtypes (SCC, AC and LCC). To evaluate the relationship between gene expressions and clinicopathological parameters (patients' age and gender, history of smoking, tumor staging according to pTNM and AJCC classifications), Spearman's rank correlation coefficient, Mann–Whitney *U* test and Kruskal–Wallis test were used. Nonparametric Spearman's criterion was used to calculate the coefficient of correlation between the levels of mRNA expression or promoter methylation for pairs of studied TSGs.

P values <0.05 were considered statistically significant. All statistical procedures were performed using Statistica for Windows 10.0 software.

Results

Relative expression levels of the studied TSGs

For relative expression analysis, delta–delta C_T method was used, and the obtained results were presented as RQ values adjusted to the expression of *ESD* (endogenous control) and in relation to the expression level of calibrator (normal lung tissue), for which $RQ = 1$. Table 2 indicates

Table 1 MSP primers (M: methylated; U: unmethylated) used in the study

Gene	MSP primers	Forward primer	Reverse primer	Product length (bp)	Annealing temp. (°C)
DLEC1	M	GATTATAGCGATGACGGGATTC	ACCCGACTAATAACGAAATTAACG	193	57.5
	U	AATGATTATAGTGATGATGGGATTTG	ACCCAACTAATAACAAAATTAACACC	196	55.0
ITGA9	M	TTTTTGATAAGTTTTTAGATGACGT	ATTATTCATAACAATAATAACCACGTA	141	44.5
	U	TTTTTGATAAGTTTTTAGATGATGT	TATTCATAACAATAATAACCACATA	139	44.5
MLH1	M	GGGAGGTTATAAGAGTAGGGTTAAC	TACCCGCTACCTAAAAAAATATACG	246	50.5
	U	GGAGGTTATAAGAGTAGGGTTAATGT	TACCCACTACCTAAAAAAATATACACT	245	57.5

RQ values (medians) of the studied genes and the frequency of samples with importantly decreased expression (RQ < 0.5, indicating a minimum of twofold down change in gene expression level) in individual tissue groups. Among the studied genes, *DLEC1* and *ITGA9* showed decreased expression in 77.9 and 71.2 % of NSCLC samples, respectively, and *MLH1*—in 18.6 %. The simultaneous down-regulation of *DLEC1* and *ITGA9* was observed in 52.5 % of NSCLCs and regarding histopathological subtypes: in 76.5 % of SCCs and 15 % of ACs.

The obtained RQ values in lung cancer tissues were correlated with histopathological NSCLC subtypes (SCC, AC, LCC), tumor staging (pTNM, AJCC), patients' age, gender and smoking history, as well as with RQ values of the studied genes in macroscopically unchanged lung tissue samples. The expression levels of *ITGA9* and *MLH1* were significantly different between NSCLC subtypes: lower in SCC than in NSCC ($P < 0.05$). Presenting the exact numerical values, the median RQ values of *ITGA9* in SCC versus NSCC were 0.50 versus 1.50 ($P = 0.0001$, Mann–Whitney *U* test) and of *MLH1* were 1.21 versus 1.57 ($P = 0.03$, Mann–Whitney *U* test).

For all studied genes, significant differences in expression levels were observed between tumor samples and macroscopically unchanged lung tissues (in total NSCLC group and/or in NSCLC histopathological subtypes). *DLEC1* expression was significantly lower in NSCLC samples (0.36 vs. 1.11, $P = 0.0001$, Mann–Whitney *U* test), as well as in NSCLC subtypes: SCC (0.31 vs. 1.13, $P = 0.00002$, Mann–Whitney *U* test) and AC (0.55 vs. 1.09 $P = 0.01$, Mann–Whitney *U* test). Similarly, *ITGA9* expression was significantly decreased in tumors as compared to normal lung tissues (0.68 vs. 1.50, $P = 0.0001$, Mann–Whitney *U* test) and in SCC subgroup (0.50 vs. 1.55, $P = 0.0001$, Mann–Whitney *U* test). *MLH1* gene revealed significantly higher expression level in NSCLC samples than in macroscopically unchanged lung tissues (1.29 vs. 1.17, $P = 0.03$, Mann–Whitney *U* test) and similarly in NSCC subgroup (1.57 vs. 1.28, $P = 0.04$, Mann–Whitney *U* test).

There were not any significant correlations between expression levels of the studied genes and patients' gender, and smoking history ($P > 0.05$). Regarding patients' age, a negative correlation was found in SSC group for *DLEC1* ($P = 0.04$, Spearman's rank correlation coefficient). In case of *MLH1* gene, statistically significant differences were observed between tumor stages II and III (according to AJCC classification) in SCC (1.34 vs. 1.07, $P = 0.01$, Mann–Whitney *U* test) and AC (1.19 vs. 1.82, $P = 0.02$, Mann–Whitney *U* test). No significant differences were found between tumor groups classified according to pTNM ($P > 0.05$).

Table 2 Expression levels of the studied *TSGs and the frequency* of samples with their importantly decreased expression

Relative expression in the studied tissue groups			*DLEC1*	*ITGA9*	*MLH1*
Median *RQ* values	NSCLC total group		0.36	0.68	1.29
	NSCLC subtypes	SCC	0.31	0.50	1.21
		NSCC	0.54	1.07	1.57
		(AC; LCC)	(0.55; 0.27)	(1.50; 0.52)	(1.61; 1.29)
	Macroscopically unchanged lung tissue		1.11	1.50	1.17
Frequency of *RQ* < 0.5	NSCLC total group		56 %	35.6 %	3.4 %
	NSCLC subtypes	SCC	64.7 %	50 %	5.9 %
		NSCC	44 %	16 %	0 %
	Macroscopically unchanged lung tissue		20.3 %	1.7 %	1.7 %

Methylation status of the studied TSG genes

Due to DNA degradation in several samples, i.e., 10 in case of *DLEC1* and *MLH1* and 12 in case of *ITGA9*, the results on methylation status of the studied genes were obtained for different numbers of patients.

Qualitative assessment of TSG methylation

In all studied tumor tissue groups, the presence of unmethylated (U) and/or methylated (M) alleles was revealed after electrophoretic separation of MSP products. M alleles were observed in 57–84 % of samples, depending on the gene. The frequencies of methylated and unmethylated alleles in the studied tissue groups, for all TSGs, are summarized in Table 3. The highest frequency of both TSG methylated alleles was observed for *ITGA9* (19 %); however, the same gene revealed the highest number of samples without any methylated allele (43 %). In case of *DLEC1*, there were 84 % of NSCLC samples with the presence of at least one methylated allele. The simultaneous presence of methylated alleles of all three genes was found in 53.5 % of NSCLC samples.

Quantitative assessment of TSG methylation (methylation index)

Based on spectrophotometric estimation (Agilent 2100 Bioanalyzer), fluorescence units (FU) of MSP products were quantified (ng/µl), according to DNA size marker (DNA ladder, Agilent Technologies, USA), and MI was calculated for each gene in all tissue samples. MIs indicated gene methylation level, which in NSCLC samples was 26–34 %, depending on the gene. Table 4 summarizes TSG methylation levels in the studied lung cancer tissue groups.

Statistical analysis did not reveal any correlations between gene methylation levels in NSCLC or its histopathological subtypes. Concerning tumor histopathological characteristics, the following correlations were found: *DLEC1* methylation level in SCC group was significantly higher in T2 versus T3/T4 tumors ($P = 0.017$, Mann–Whitney U test); in NSCC group, *DLEC1* was significantly higher methylated in stage I vs stage II tumors ($P = 0.04$, Mann–Whitney U test); in SCC group, *MLH1* methylation status was significantly higher in stage I/II vs stage III tumors ($P = 0.02$, Mann–Whitney U test).

Regarding patients' characteristics, statistically significant correlations between *DLEC1* methylation status and patient age were found in NSCLC group (patients aged ≤60 vs. 61–70 years, $P = 0.02$; 61–70 vs. >70 years, $P = 0.001$, Mann–Whitney U tests), in AC subgroup (≤60 vs. 61–70 years, $P = 0.03$, Mann–Whitney U test) and in NSCC subgroup (≤60 vs. 61–70 years, $P = 0.02$, Mann–Whitney U test). In all those groups, the highest methylation level of *DLEC1* was observed in patients aged 61–70 years.

Correlation between gene expression level and methylation status

The decreased expression of *DLEC1* with simultaneous promoter methylation was found in 63.3 % of NSCLC samples, in case of *ITGA9*—in 40.4 % and in case of *MLH1*—in 6.1 %.

We did not find negative correlations between the RQ and MI values of the studied genes. Positive correlation was revealed for *MLH1* ($P = 0.03$, Spearman's rank correlation coefficient, $rs = 0.31$).

Discussion

Lung cancer is recognized as a complex and heterogeneous disease, not only at clinical but also at biochemical and molecular level, considering metabolites, proteins and genes. There is a need to collect findings from multiple disciplines into one model that will integrate genomic and clinical features of NSCLC to improve the understanding of this disease.

Table 3 Presence of methylated (M) and unmethylated (U) alleles in the studied tumor tissue groups

MI values in the studied tissue groups			*DLEC1* ($n = 49$)	*ITGA9* ($n = 47$)	*MLH1* ($n = 49$)
$MI = 1$	NSCLC total group		1 (2 %)	9 (19 %)	5 (10 %)
(both M alleles)	NSCLC subtypes	SCC	1 (3 %)	6 (22 %)	2 (7 %)
		NSCC	0 (0 %)	3 (15 %)	3 (15 %)
$MI = 0$	NSCLC total group		8 (16 %)	20 (43 %)	8 (16 %)
(both U alleles)	NSCLC subtypes	SCC	3 (10 %)	10 (37 %)	5 (17 %)
		NSCC	5 (26 %)	10 (50 %)	3 (15 %)
$0 < MI < 1$	NSCLC total group		40 (82 %)	18 (38 %)	36 (74 %)
(M and U allele)	NSCLC subtypes	SCC	26 (87 %)	11 (41 %)	22 (76 %)
		NSCC	14 (74 %)	7 (35 %)	14 (70 %)

Table 4 MI values (%) of the studied TSGs

	NSCLC total group	DLEC1 26 %	ITGA9 34 %	MLH1 27 %
NSCLC subtypes	SCC	30	37	23
	NSCC	19	30	33
	AC	20	25	36

In our study, we analyzed the expression levels of three genes located in AP20 region in 3p21.3, which is frequently disturbed in lung cancers. We also analyzed gene expression levels in macroscopically unchanged lung tissue, surrounding the primary lesion. The studied genes, *DLEC1*, *ITGA9* and *MLH1*, are functional TSGs. However, the reports on their expression in lung cancers are ambiguous and the mechanisms underlying their decreased expression remain unclear. We looked for gene promoter hypermethylation as the possible epigenetic mechanism of gene silencing. Epigenetic alterations are regarded as equally important as genetic lesions in the carcinogenesis and cancer progression [10]. As found in several studies, aberrant gene promoter methylation represents an ideal candidate for diagnostic and prognostic markers in cancer [17].

In our analysis, among the studied genes, the highest frequency of decreased expression was found for *DLEC1*, followed by *ITGA9*. *DLEC1* was importantly decreased in 56 % of NSCLC samples, more often in SCC subtype (in nearly 65 %) than in NSCC (44 %). Similarly, *ITGA9* showed lower expression in SCC samples. The simultaneous down-regulation of *DLEC1* and *ITGA9* was observed in more than 50 % of total NSCLCs and in nearly 77 % of SCCs. Methylation frequencies of all studied genes in total NSCLC samples were 57–84 %, with equally higher methylation frequency for *DLEC1* and *MLH1*. The simultaneous methylation of all three genes was found in 53.5 % of NSCLC samples.

DLEC1 (*deleted in lung and esophageal cancer 1*), as a TSG, suppresses tumor growth or reduces the invasiveness of cancer cells. *DLEC1* is expressed in normal lung or kidney tissue, while in tumors gene expression is disturbed [8, 18]. Decreased *DLEC1* expression—on mRNA and protein level—was observed in lung cancer cell lines and primary tumors [18–20]. In our study, *DLEC1* revealed the highest frequency of decreased expression. Additionally, the differences between NSCLC and paired macroscopically unchanged lung tissue were significant: *DLEC1* expression was significantly lower in tumor specimens.

DLEC1 promoter hypermethylation is responsible for its silencing, as found in a variety of human cancers. Indeed, epigenetic silencing of *DLEC1* was found to be specific for cancerous lung tissue, as it was only rarely detected in matched normal lung tissue [18, 20]. In NSCLC primary lesions and cell lines, *DLEC1* methylation was observed in 27–56 % of samples [18–21] and in most cases correlated with decreased gene expression [18–20]. In our study, we observed *DLEC1* methylation in 84 % of NSCLCs, and the simultaneous promoter methylation and gene decreased expression were found in 63 % of lung tumor samples. Gene methylation level was moderate, showing higher level in SCC (30 %) than in NSCC (19 %), although the difference was not statistically significant. In several studies, gene promoter hypermethylation was associated with poorer prognosis in NSCLC patients [18, 20, 21]. The study conducted by Seng et al. [20] showed that epigenetic silencing of the gene was tightly connected with II grade tumors and lymph node metastasis, as well as shorter survival time. However, the findings are conflicting [21]. In our study, significantly higher *DLEC1* methylation level was associated with T2 tumors in SCC group and stage I in NSCC group. It could indicate the role of gene epigenetic modification in lung cancer initiation rather than in progression.

In others' studies, there were not any associations between the methylation status of *DLEC1* and pathological stages, lymph node metastasis, tumor squamous histology, patients' smoking status, age and gender [18, 21]. We found significant correlation between *DLEC1* methylation and patients' age, indicating its higher level in older NSCLC patients. It is in line with negative correlation observed between *DLEC1* expression level and patients' age in SCC subgroup.

Interesting finding regarding *DLEC1* methylation in lung cancer was its good correlation with gene methylation status in patients' plasma, as found by Zhang et al. [18]. Summing up, *DLEC1* methylation could be regarded as a common event contributing to NSCLC initiation and progression. It provides a potential biomarker that could be a constituent of the methylation-based panel for NSCLC diagnosis and prognosis.

Such panel might include also another studied TSG, *ITGA9* (*integrin, alpha 9*). Gene decreased expression in NSCLC was demonstrated, and the possible mechanism responsible for its silencing was methylation and/or deletion [7, 22]. In our study, importantly decreased *ITGA9* expression was observed in 36 % of NSCLC samples and more frequently in SCC (50 %) as compared to NSCC (16 %). This is in accordance with the results of others [7], on the basis of which *ITGA9* was incorporated into the panel of 19 markers that allowed early detection, tumor progression, metastases and discrimination between SCC and AC. In our study, we confirmed statistically significant differences in gene expression between the histopathological subtypes, with significantly lower *ITGA9* expression in

SCC. Gene promoter methylation—which could be responsible for gene silencing in lung tumor—was observed in 57 % of NSCLC samples, and its methylation level ranged from 25 to 37 %, depending on the histotype. The simultaneous gene decreased expression and promoter hypermethylation were revealed in 52 % of SCC samples (data not shown); however, we did not observe statistically significant correlations between RQ and MI values.

To the best of our knowledge, there are no other available reports on *ITGA9* in lung cancer. *ITGA9* is an interesting gene, and it encodes a component of the α9β1 integrin receptor that plays an integral role in different signal transduction pathways, controlling cellular proliferation and differentiation [23]. However, the data regarding *ITGA9* expression in cancers are conflicting [24, 25]. Our results resemble those obtained by Mostovich et al. [25] in breast cancer, especially in AC group in which *ITGA9* expression was decreased in one half of samples and increased in the second half of specimens (data not shown). In SCC group—in all samples except two—gene expression was reduced. The heterogenous status of *ITGA9* expression in lung tumors could be the basis for their differentiation and gene considered as diagnostic marker. It is strengthened by our finding of its significantly lower expression level in lung tumor specimens as compared to macroscopically unchanged lung tissue.

The third studied gene, *MLH1* (*mutL homolog 1*), belongs to a mismatch repair (MMR) system and plays a pivotal role in maintaining genome stability, including microsatellite instability (MSI) and the accumulation of gene mutations associated with carcinogenesis. The studies indicated LOH and promoter methylation as main mechanisms of *MLH1* decreased expression in various cancers [26, 27]. However, regarding methylation status of *MLH1*, the reported frequencies in NSCLC are very different, ranging from 0 to 72 % [20, 26, 28–30]. Our analysis revealed frequent promoter methylation of *MLH1* (84 % of NSCLC samples) and the methylation level of the gene in the range of 27–34 %. Additionally, significantly higher *MLH1* methylation was found in stage I/II SCC samples as compared to stage III. It could suggest the role of epigenetic modifications of *MLH1* at early stages of lung carcinogenesis. In other studies, analyzing patient survival rates, strong association between gene methylation and poor prognosis in NSCLC was observed [20, 31].

Similarly to *MLH1* methylation results in different studies, the reports on its expression are ambiguous. Geng et al. [26] reported high, almost equal in frequencies, methylation (72 %) and loss of expression (68 %) of *MLH1* in Chinese population. In other studies, despite high (72 % in SCC) or moderate (35 %) methylation level, loss of MLH1 protein expression was rare, suggesting that *MLH1* promoter methylation does not usually lead to gene silencing in lung cancer [20, 32]. Similarly, in our study, *MLH1* expression was not importantly decreased, as found only in 3.4–5.9 % of the tumor samples, depending on the histotype. The simultaneous decreased gene expression and gene promoter hypermethylation occurred only in three NSCLC samples.

In general, we found median values of *MLH1* expression levels similar in tumor and macroscopically unchanged lung tissue groups. The overexpression of *MLH1* on protein level (especially in AC subtype) was also found by Li et al. [33], who concluded that other genetic lesions—such as EGFR mutations—might be earlier events and could occur before MLH1 disturbed expression pattern in lung carcinogenesis.

Conclusions

In our analysis, the expression levels of *DLEC1* and *ITGA9* were prominently decreased in lung tumor samples. Their significant differences—when compared with macroscopically unchanged lung tissue surrounding primary lesions—highlight the role of those TSGs in lung carcinogenesis. Additionally, *ITGA9* could be regarded as diagnostic markers differentiating NSCLC histopathological subtypes, and—similarly to *DLEC1*—distinguishing lung cancerous and macroscopically unchanged tissue.

Promoter methylation analysis pointed to the epigenetic modifications of all three studied genes and their significance in lung carcinogenesis. Their simultaneous methylation in more than 50 % of NSCLC samples might suggest the possibility of considering them as a panel of epigenetic markers. Confirmation of such hypothesis requires further study. Perhaps in the future, these genes become targets for novel possible epigenetic drugs.

Acknowledgments This work was supported by the Grant of the National Science Centre, Poland, No. 2011/01/B/NZ4/04966.

Authors' contribution Dorota Pastuszak-Lewandoska and Jacek Kordiak have equally contributed to this work.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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