

Respiratory Research

Quantitative Analysis of mRNA Expression Levels and DNA Methylation Profiles of Three Neighboring Genes: FUS1, NPRL2/G21 and RASSF1A in Non-small Cell Lung Cancer Patients --Manuscript Draft--

Manuscript Number:	RERE-D-15-00051R1
Full Title:	Quantitative Analysis of mRNA Expression Levels and DNA Methylation Profiles of Three Neighboring Genes: FUS1, NPRL2/G21 and RASSF1A in Non-small Cell Lung Cancer Patients
Article Type:	Research
Abstract:	<p>Background. Tumor suppressor gene (TSG) inactivation plays a crucial role in carcinogenesis. FUS1, NPRL2/G21 and RASSF1A are TSGs from LUCA region at 3p21.3, a critical chromosomal region in lung cancer development. The aim of the study was to analyze and compare the expression levels of these 3 TSGs in NSCLC, as well as in macroscopically unchanged lung tissue surrounding the primary lesion, and to look for the possible epigenetic mechanism of TSG inactivation via gene promoter methylation.</p> <p>Methods. Expression levels of 3 TSGs and 2 DNA methyltransferases, DNMT1 and DNMT3B, were assessed using real-time PCR method (qPCR) in 59 primary non-small cell lung tumors and the matched macroscopically unchanged lung tissue samples. Promoter methylation status of TSGs was analyzed using methylation-specific PCRs (MSP method) and Methylation Index (MI) value was calculated for each gene.</p> <p>Results. The expression of all three TSGs were significantly different between NSCLC subtypes: RASSF1A and FUS1 expression levels were significantly lower in squamous cell carcinoma (SCC), and NPRL2/G21 in adenocarcinoma (AC). RASSF1A showed significantly lower expression in tumors vs macroscopically unchanged lung tissues. Methylation frequency was 38-76%, depending on the gene. The highest MI value was found for RASSF1A (52%) and the lowest for NPRL2/G21 (5%). The simultaneous decreased expression and methylation of at least one RASSF1A allele was observed in 71% tumor samples. Inverse correlation between gene expression and promoter methylation was found for FUS1 ($rs=-0.41$) in SCC subtype. Expression levels of DNMTs were significantly increased in 75-92% NSCLCs and were significantly higher in tumors than in normal lung tissue. However, no correlation between mRNA expression levels of DNMTs and DNA methylation status of the studied TSGs was found.</p> <p>Conclusions. The results indicate the potential role of the studied TSGs in the differentiation of NSCLC histopathological subtypes. The significant differences in RASSF1A expression levels between NSCLC and macroscopically unchanged lung tissue highlight its possible diagnostic role in lung cancer in situ recognition. High percentage of lung tumor samples with simultaneous RASSF1A decreased expression and gene promoter methylation indicates its epigenetic silencing. However, DNMT overexpression doesn't seem to be a critical determinate of its promoter hypermethylation.</p>
Response to Reviewers:	<p>We thank the Reviewers for their time in reviewing the Manuscript and their suggestions. Our responses to each of these, which we believe strengthen the manuscript, are as follows</p> <p>Reviewer #1: Comments to the Editor,</p> <p>This manuscript studied the quantitative mRNA and DNA methylation profile of three tumor suppressor genes which located in the famous LUCA region in Non-small cell lung cancer in a large Poland population. In the present study, the DNA methylation and gene expression of FUS1, NPRL2/G21 and RASSF1A were measured in same lung cancer and adjacent tissues simultaneously, meanwhile, the gene expression level of DNMT1 and DNMT3B were also detected with RT-PCR. And the relationship among gene expression, DNA methylation and sample states (cancer, normal and/or subtype) were estimated with perfect statistic method. The study was performed rigorously and the findings are interesting. In general, I'd recommend publication if the authors can address the following concerns.</p>

1, what's the degree of the estimated LOH frequency of LUCA region in NSCLC and this should be provided in the background section.

Answer: We have included now information on the frequency of loss of heterozygosity in LUCA region in NSCLC, in the background section. Thus, two additional articles have been included and this has resulted in a change in the numbering of literature.

2, Reference should be provided at line 39 of page 3.

Answer: The reference number [11] has been added in the suggested place.

3, Please provide the counts of the sample in each cell of Table 3.

Answer: The number of samples has been now included in Table 3.

4, why set RQ equal to 0.5 in Table 3, rather than other values?

Answer: We consider RQ value significantly decreased when it represents a minimum of two-fold down change, i.e., when it is equal to or less than 0.5.

5, How to determine the annealing temperature in the process of MSP?

Answer: The annealing temperatures were experimentally determined in a set of gradient PCRs, for each MSP primer.

6, the genomic position of CpG site in primers and the PCR production should be provided in the Table 2.

Answer: CpG sites are now indicated in primers listed in Table 2, as well as primer positions and PCR product length.

7, The font size of the legends in Figure 1 is too small.

Answer: The font size in Fig. 1 has been changed. Additionally, the results have been grouped according to the studied genes – in order to better visualize and compare the results.

8, Please change the Table 5 to a figure so that readers can get more information from the data.

Answer: Data contained in Table 5 are now presented in the form of graph (Figure 2). We hope that in this way we have improved the clarity of their presentation. Due to the introduction of this new figure, the previous figure No. 2 is now No. 3.

9, Is there any evidence showed the mutation and the hypermethylation of these three genes can be considered as diagnostic biomarker for NSCLC? And Do you think the primer in present study can be used in the methylation detection in plasma samples?

Answer: Human chromosome 3p21.3 region is frequently disturbed by genomic abnormalities such as loss of heterozygosity and homozygous deletions found in the early stages of the development of various human cancers, including lung cancer [Lerman & Minna, The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res. 2000;60:6116–33; Zabarovsky et al., Tumor suppressor genes on chromosome 3p involved in the pathogenesis of lung and other cancers. Oncogene. 2002;21(45):6915–35]. The studied tumor suppressor genes, RASSF1A, NPRL2/G21 and FUS1, are located in 3p21.3C region (LUCA region). Not surprisingly, they are subjected to mutations recognized in cancers. In lung cancer and lung cancer cell lines, NPRL2/G21 inactivation mechanisms via aberrant splicing transcripts, homozygous deletions in the 3' end, or frameshift mutation producing a stop codon, nonsense deletions and missense mutations have been found [Calvo et al., Altered HOX and WNT7A expression in human lung cancer. Proc Natl Acad Sci U S A. 2000;97(23):12776–81; Lerman & Minna, The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer

Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res. 2000;60:6116–33; Li et al. Functional characterization of the candidate tumor suppressor gene NPRL2/G21 located in 3p21.3C. Cancer Res 2004; 64:6438–43]. In case of FUS1 gene, single allele loss is commonly found, however, only a few missense mutations and C-terminal deletion mutations have been identified in primary lung cancer samples [Ji & Roth, Tumor suppressor FUS1 signaling pathway. J Thorac Oncol. 2008; doi: 10.1097/JTO.0b013e31816bce65]. Nevertheless, there are no reports on those gene significance – based on their specific mutations – as diagnostic biomarkers in lung cancer.

Mutational analysis of RASSF1A has revealed that tumor-associated mutations are rare and the principal cause of RASSF1A loss of expression in tumors is gene promoter hypermethylation, including lung cancer [Dammann et al., Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. Nat Genet. 2000;25(3):315-9; Agathangelou et al., Role of the Ras-association domain family 1 tumor suppressor gene in human cancers. Cancer Res. 2005; doi: 10.1158/0008-5472.CAN-04-4088; Lerman & Minna, The 630-kb lung cancer homozygous deletion region on human chromosome 3p21.3: identification and evaluation of the resident candidate tumor suppressor genes. The International Lung Cancer Chromosome 3p21.3 Tumor Suppressor Gene Consortium. Cancer Res. 2000;60:6116–33; Liu et al., Associations between RASSF1A promoter methylation and NSCLC: a meta-analysis of published data. Asian Pac J Cancer Prev. 2013;14(6):3719-24]. However, as mentioned in our manuscript, the results of studies assessing the utility of RASSF1A epigenetic inactivation as a prognostic marker in NSCLC are still divergent [Hesson et al., The role of RASSF1A methylation in cancer. Dis Markers. 2007; 23(1-2):73–87; Choi et al., RASSF1A is not appropriate as an early detection marker or a prognostic marker for non-small cell lung cancer. Int J Cancer. 2005;115(4):575-81; Niklinska et al., Prognostic significance of DAPK and RASSF1A promoter hypermethylation in non-small cell lung cancer (NSCLC). Folia Histochem Cytobiol. 2009;47(2):275-80]. Nevertheless, there are many research in which RASSF1A gene is included in a panel of DNA methylation-based biomarkers [Nikolaidis et al., DNA methylation biomarkers offer improved diagnostic efficiency in lung cancer. Cancer Res. 2012; doi: 10.1158/0008-5472.CAN-12-2309; Zhang et al., Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. Cancer Lett. 2011; doi: 10.1016/j.canlet.2010.12.011].

Regarding FUS1 and NPRL2/G21, there are no reports on their hypermethylation in NSCLC. So, it was interesting to analyze this potential mechanism of their silencing in lung cancer.

With regard to the second part of the question, our study was designed for NSCLC tissue analysis. Plasma and serum contain cancer DNA in high dilution, and it could be the reason of insufficient amount of DNA for bisulfite conversion. Additionally, it also represents the whole body, and the question raises: can DNA methylation patterns found in plasma provide information on tumor specific methylation pattern? On the other hand, it could be related with lower specificity of lung cancer detection. We believe that because methylation pattern is tissue-specific (changed in cancerous tissue) the first step should be to ensure that the DNA analyzed actually comes from a tumor - then the use of primers designed for MSP would be reliable. However, there are reports describing tumor-specific genetic and epigenetic alterations in circulating DNA and the increasing evidence points to the potentials of cell-free epigenetic biomarkers in the blood for cancer detection [Ponomaryova et al., Potentialities of aberrantly methylated circulating DNA for diagnostics and post-treatment follow-up of lung cancer patients. Lung Cancer. 2013; doi: 10.1016/j.lungcan.2013.05.016; Ramirez et al., Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. Cancer Lett. 2003; 193(2):207-16]. Unfortunately, in our study cohort we are unable to check it out as we don't have blood plasma from the studied NSCLC patients.

10, why human fetal cell line could be taken as negative control? They do not have any methylation in such genomes?

Answer: We used CpGenome Universal Unmethylated DNA (Millipore, USA) as a negative control for MSP, because this kit contains unmethylated genomic DNA controls and is recommended for gene methylation studies and methylation-specific PCR (MSP).

11, Please provide the sensitivity, specificity with such three genes (DNA methylation or/and gene expression) in the prediction or distinguish of the NSCLC from normal tissues in result or discussion section.

Answer: In Results section we have provided now sensitivity and specificity for RASSF1A gene expression as a factor distinguishing lung cancer tissue (NSCLC) from macroscopically unchanged lung tissue. RASSF1 gene was selected as its expression was the lowest and significantly different as compared with other studied TSGs and, additionally, significantly lower in tumor than in adjacent lung tissue.

Additionally, we performed ROC curve analysis for RASSF1A and FUS1 (separately and combined) to distinguish SCC and NSCC groups, as we found statistically significant differences for these genes between the NSCLC histotypes.

12, Is there any possibility to discrete continuous MI to binary status (methylation or unmethylation), so that, the present data can be re-used in the future meta-analysis?

Answer: In our opinion, MI values – spectrophotometrically estimated concentrations (ng) of MSP products (U and M DNA alleles) in Agilent 2100 Bioanalyzer (Agilent Technologies, USA) and calculated as peak heights of methylated products/(peak heights of methylated products + peak heights of unmethylated product), $MI = (M)/(M+U)$ - could be transformed into binary status. For this purpose, we would recommend to recognize samples with MI values ≥ 0.5 as methylated, and those with $MI < 0.5$ as unmethylated.

13, why the authors believe DNA methylation can be used as diagnostic biomarkers/models? Can you provide some lasted/promising diagnostic model or biomarkers which are based on DNA methylation in the background section? What's the best diagnosis accuracy can be achieved by DNA methylation based biomarkers/models?

Answer: Epigenetic biomarkers, including DNA methylation, have unique properties that allow to study them as potential diagnostic and/or prognostic biomarkers. They are stable and easily handled in PCR-based measurements. As such, they have been analyzed and described widely in literature. Accordingly to the Reviewer's suggestion, we have listed some of the most frequently reported methylation biomarkers in NSCLC and quoted the latest (2015) review focused on epigenetic modifications in lung carcinogenesis.

Regarding lung cancer diagnosis accuracy (sensitivity, specificity, and area under the curve) based on DNA methylation, it is much higher for multi-marker panels [Shicheng Guo et al., Identification and validation of the methylation biomarkers of non-small cell lung cancer (NSCLC). Clin Epigenetics. 2015; doi: 10.1186/s13148-014-0035-3; Jiang et al., A panel of sputum-based genomic marker for early detection of lung cancer. Cancer Prev Res (Phila). 2010; doi: 10.1158/1940-6207.CAPR-10-0128]. However, as found by others, also single markers reveal good sensitivity and specificity in NSCLC tissue (APC gene: sensitivity: 55%, specificity: 78%) [Shicheng Guo et al., Quantitative assessment of the diagnostic role of APC promoter methylation in non-small cell lung cancer. Clinical Epigenetics 2014; doi:10.1186/1868-7083-6-5] or bronchial aspirates (SHOX2 gene: sensitivity: 79%, specificity: 96%) [Dietrich et al., Performance evaluation of the DNA methylation biomarker SHOX2 for the aid in diagnosis of lung cancer based on the analysis of bronchial aspirates. Int J Oncol. 2012 Mar;40(3):825-32. doi: 10.3892/ijo.2011.1264].

Reviewer #2: In this paper the authors aimed to determine compare the expression levels of these 3 Tumor suppressor gene (TSGs) in NSCLC, as well as in macroscopically unchanged lung tissue surrounding the primary lesion, and to look for the possible epigenetic mechanism of TSG inactivation via gene promoter methylation. Fifty-nine primary non-small cell lung tumors and the matched macroscopically unchanged lung tissue samples were collected, and retrospectively matched with histopathological data. The results indicate differences between cancer cells and surrounding normal tissue in TSGs expression and different value according to histology in the context of malignant cells.

The current review requires a series of revisions, mostly related to the adopted methodology, as follows:

*Methods [page 5, line 7]: Quantitation of DNA only by spectrophotometer is nowadays considered partially reliable, taking into account the risk of contamination by RNA as well. Authors should indicate this possibility as a limitation of the performed analysis.
Answer: We agree with Reviewer, however in the procedure of DNA isolation (QIAamp DNA mini Kit, Qiagen, Germany) RNase A was used to obtain RNA-free genomic DNA, thus quantitation of DNA was reliable. We have added the information on this step in the DNA isolation procedure in Methods section.

*Methods [page 5, line 17]: The quantitative determination after treatment with bisulfite may drive to unreliable results and there is no consensus with regard to how much DNA will degrade and how much it will be generically amplifiable. The author should consider to make a gel (as reported in literature) and a real-time PCR with 'generic' primers to see if and when the signal is present.

Answer: In our laboratory practice, the level of ≥ 10 ng/ μ l bisulfite converted DNA is sufficient for successful MSPs. Because of fact that methylated (M) and unmethylated (U) products were analyzed in pairs, in case of samples with no M or U product, DNA from patients was re-treated with bisulfite. If still no product could be detected, the sample was rejected.

At the stage of method setting and prior to performing MSPs with the primers designed for our study, we controlled the completeness of DNA bisulfite conversion. For this purpose, we used bisulfite converted DNA and CpG WIZ® p16 Amplification Kit (CHEMICON International, Millipore, USA), which contained W (wild-type), M (methylated) and U (unmethylated) primers. We considered DNA samples completely converted when no products were observed with W primers. Then, the set conditions of DNA bisulfite conversion were further applied in our research reported in the manuscript.

*Methods [page 5, line 33]: with regard to the methylation specific PCR, the authors go straight to 40 cycles without first checking that there is not saturation. Then the ratio between methylated and un-methylated DNA is determined. It would be more correct and accurate to perform some different cycles (30, 35, and 40) to verify that the signal is not saturated and then evaluate only the presence or absence of the amplified DNA band.

Answer: We agree with Reviewer's opinion. However, the described procedure is based on the experiments previously performed with different numbers of cycles. The selection of 40 cycles was associated with clear visualization of control DNA bands (both methylated and unmethylated). In the manuscript we reported the optimal conditions. Additionally, it should be emphasized that MSP is qualitative analysis and the ratio between methylated and un-methylated DNA was determined in automatic electrophoresis in Agilent 2100 Bioanalyzer.

*Results [page 6, from line 23]: The authors have isolated both tumor than the adjacent normal tissue, and analyzed separately for the analysis of gene expression, by using commercial DNA as a normalizer. In this regard, the authors should explain (or indicate a reference) on why it would not be sufficient to compare the tumor DNA with the corresponding normal. For this purpose, the paper by Malpeli et al. (Methylation-associated down-regulation of RASSF1A and up-regulation of RASSF1C in pancreatic endocrine tumors. BMC Cancer 2011, 11:351) should be quoted.

Answer: Our study was designed to compare two groups of tissue: lung cancer tissue (obtained from the center of primary lesion) and macroscopically unchanged lung tissue surrounding the tumor (the so called "normal" tissue, taken at least 10 cm away from the neoplastic lesion). Accumulating evidence point to genetic and epigenetic changes occurring in normal tissue adjacent to tumor. This process, called field cancerization, is widely described in literature in various cancer types and highlights the importance of early abnormalities in carcinogenesis. Regarding lung cancer,

molecular changes in histologically normal airway epithelium were confirmed [Gomperts et al., Enriching the molecular definition of the airway "field of cancerization:" establishing new paradigms for the patient at risk for lung cancer. Cancer Prev Res (Phila). 2013; doi: 10.1158/1940-6207.CAPR-12-0470; Kadara & Wistuba, Field cancerization in non-small cell lung cancer: implications in disease pathogenesis. Proc Am Thorac Soc. 2012; doi: 10.1513/pats.201201-004MS; Kadara et al., Transcriptomic architecture of the adjacent airway field cancerization in non-small cell lung cancer. J Natl Cancer Inst. 2014; doi: 10.1093/jnci/dju004; Tsay et al., Molecular characterization of the peripheral airway field of cancerization in lung adenocarcinoma. PLoS One. 2015; doi: 10.1371/journal.pone.0118132]. That is why we couldn't assume our macroscopically unchanged lung tissue group as "completely normal" and regarded it as additional study group. However, there are also reports on no significant differences between tumor and adjacent normal tissue, with respect to, inter alia, the level of some TSG expression. In this regard, we agree with the remark of Reviewer and quote the work of Malpei et al. [12].

In our study, similarly to Malpeli et al., qPCR method was performed according to the comparative method following the User Bulletin #2 (Applied Biosystems).

*Results [page 7, lines 1-35]: a series of correlation are reported, and this way is actually confusing for the average reader. It should be clearer to have an additional table in this regard. Did the analyses take into account the multiple comparisons? If not (as it actually seems) the authors should acknowledged that as a potential limitation of their conclusions.

Answer: The results from page 7 are now included in a table (Table 4). In manuscript body, the text concerning these results has been shortened so as not to duplicate the information contained in the table.

The previous Table No 4 is now Table No. 5.

The performed statistical analyses involved – as a post-hoc test – the Newman-Keuls method to identify significant differences between the studied genes. However, in case of gene expression analysis, the P values haven't been reported (although the test has been mentioned). Now the detailed information on significant correlation between studied genes, along with P values, are incorporated into manuscript. Regarding methylation analysis, the results obtained from Newman-Keuls test have already been in the Result section.

Quantitative Analysis of mRNA Expression Levels and DNA Methylation Profiles of Three Neighboring Genes:
***FUS1, NPRL2/G21 and RASSF1A* in Non-small Cell Lung Cancer Patients**

Dorota Pastuszek-Lewandoska (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
dorota.pastuszek-lewandoska@umed.lodz.pl)

Jacek Kordiak (Department of Chest Surgery, General and Oncological Surgery, University Hospital No. 2, Medical
University of Lodz, Poland; jacek.kordiak@umed.lodz.pl)

Monika Migdalska-Sęk (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
monika.migdalska-sek@umed.lodz.pl)

Karolina H. Czarnecka (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
karolina.czarnecka@umed.lodz.pl)

Adam Antczak (Department of General and Oncological Pulmonology, Medical University of Lodz, Poland;
adam.antczak@umed.lodz.pl)

Paweł Górski (Department of Pneumology and Allergology, Medical University of Lodz, Poland;
pawel.gorski@umed.lodz.pl)

Ewa Nawrot (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
ewa.nawrot@umed.lodz.pl)

Justyna M. Kiszalkiewicz (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
justyna.kiszalkiewicz@gmail.com)

Daria Domańska (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
daria.domanska@umed.lodz.pl)

Ewa Brzezińska-Lasota (Department of Molecular Bases of Medicine, Medical University of Lodz, Poland;
ewa.brzezianska@umed.lodz.pl)

Corresponding author

Dorota Pastuszek-Lewandoska, address: Department of Molecular Bases of Medicine, Medical University of Lodz,
Pomorska 251, C-5, 92-213 Lodz, Poland; E-mail: dorota.pastuszek-lewandoska@umed.lodz.pl

ABSTRACT

Background. Tumor suppressor gene (TSG) inactivation plays a crucial role in carcinogenesis. *FUS1*, *NPRL2/G21* and *RASSF1A* are TSGs from LUCA region at 3p21.3, a critical chromosomal region in lung cancer development. The aim of the study was to analyze and compare the expression levels of these 3 TSGs in NSCLC, as well as in macroscopically unchanged lung tissue surrounding the primary lesion, and to look for the possible epigenetic mechanism of TSG inactivation *via* gene promoter methylation.

Methods. Expression levels of 3 TSGs and 2 DNA methyltransferases, DNMT1 and DNMT3B, were assessed using real-time PCR method (qPCR) in 59 primary non-small cell lung tumors and the matched macroscopically unchanged lung tissue samples. Promoter methylation status of TSGs was analyzed using methylation-specific PCRs (MSP method) and Methylation Index (MI) value was calculated for each gene.

Results. The expression of all three TSGs were significantly different between NSCLC subtypes: *RASSF1A* and *FUS1* expression levels were significantly lower in squamous cell carcinoma (SCC), and *NPRL2/G21* in adenocarcinoma (AC). *RASSF1A* showed significantly lower expression in tumors *vs* macroscopically unchanged lung tissues.

Methylation frequency was 38-76%, depending on the gene. The highest MI value was found for *RASSF1A* (52%) and the lowest for *NPRL2/G21* (5%). The simultaneous decreased expression and methylation of at least one *RASSF1A* allele was observed in 71% tumor samples. Inverse correlation between gene expression and promoter methylation was found for *FUS1* ($rs=-0.41$) in SCC subtype. Expression levels of DNMTs were significantly increased in 75-92% NSCLCs and were significantly higher in tumors than in normal lung tissue. However, no correlation between mRNA expression levels of DNMTs and DNA methylation status of the studied TSGs was found.

Conclusions. The results indicate the potential role of the studied TSGs in the differentiation of NSCLC histopathological subtypes. The significant differences in *RASSF1A* expression levels between NSCLC and macroscopically unchanged lung tissue highlight its possible diagnostic role in lung cancer *in situ* recognition. High percentage of lung tumor samples with simultaneous *RASSF1A* decreased expression and gene promoter methylation indicates its epigenetic silencing. However, DNMT overexpression doesn't seem to be a critical determinate of its promoter hypermethylation.

Key words: non-small cell lung cancer; gene expression; promoter methylation; tumor suppressor gene; DNA methyltransferase

1. BACKGROUND

The development of non-small cell lung cancer (NSCLC) is associated with molecular changes in more than 20 genes localized in different chromosomal regions. The most frequent molecular event in lung cancer pathogenesis is allele loss (loss of heterozygosity, LOH) on short arm of chromosome 3 (3p), in multiple, so called critical regions. LOH analyses in 3p indicate 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), which contain loci of multiple tumor suppressor genes (TSGs) [1]. Inactivation of TSGs is the key event in carcinogenesis and involves two steps, each of them affecting one allele. Most frequently, one TSG allele is lost due to LOH and the other – to mutation, or, alternatively, epigenetic inactivation *via* gene promoter hypermethylation. The studies on LOH analysis in lung cancer indicated that 3p allele loss is nearly universal, shows a "discontinuous LOH pattern" involving multiple, discrete sites, and appears to occur firstly in the 600-kb 3p21.3 region [2]. The reported incidence of LOH in LUCA region varied, ranging from 10% to 74%, however, it was always higher in squamous cell carcinoma (SCC) than in adenocarcinoma (AC), up to 96% and 50%, respectively [1-3]. Additionally, low mutation rate, below 10%, found in lung cell lines and primary tumors, highlighted the significance of epigenetic modifications of TSGs in lung carcinogenesis [4,5].

Epigenetic mechanisms, which include DNA methylation, post translational modifications to core histones, microRNA (miRNA) and long non-codingRNA (lncRNA) regulation [6], play a crucial role in regulation of gene expression by affecting chromatin accessibility. The best known epigenetic modification in human is DNA methylation. Cancer-related aberrant DNA methylation pattern, e.g., hypermethylation of promoter sequences of TSGs, provide a range of opportunities for risk assessment, early detection, disease progression and prognosis, as well as therapeutic stratification and post-therapeutic monitoring of cancer. In lung carcinogenesis, among the potential DNA methylation biomarkers, the hypermethylation of *APC*, *CDH1*, *CDH13*, *DAPK1*, *FHIT*, *MGMT*, *p16INK4a*, *RARβ*, *RASSF1A*, *RUNX3* and *SHOX2* is the most frequently reported [5,7]. However, among the ever increasing number of new studies there are also those that negate the previous conclusions [8].

DNA methylation is an enzymatic process mediated by DNA methyltransferases (DNMTs). DNMT1, possessing a 7- to 21- fold preference for hemimethylated DNA than unmethylated DNA, is the primary enzyme responsible for copying methylation patterns, i.e., for its maintenance [9]. DNMT3 family enzymes (-3A and -3B) exhibit *de novo* methylation activity, as they have similar affinities for both unmethylated and hemi-methylated DNA substrates, and affect the methylation status of normally unmethylated CpG sites [9]. Additionally, a cooperation between DNMT3 family and DNMT1 has been shown in cancer [10]. DNMTs not only play a pivotal role in carcinogenesis, being responsible for DNA methylation, but also seem to be promising molecular bio-markers for cancer diagnosis and therapy [11].

The pre-specified hypothesis tested in the study was that the expression levels of 3 selected TSGs from LUCA region (*FUS1*, *NPRL2/G21* and *RASSF1A*) were decreased in primary non-small cell lung cancer with promoter hypermethylation as the responsible epigenetic mechanism of their silencing. To investigate the factors involved in TSG hypermethylation in NSCLC, we attempted to determine whether *DNMT1* and *DNMT3B* RNA expression levels correlated with the hypermethylation of the promoters of the studied tumor suppressor genes. We tried to elucidate the role of the studied TSGs in early lung carcinogenesis and cancer progression.

2. 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.

2.1. Characterization of the lung tissue samples and patients clinical characteristics

The study involved the group of 59 patients with diagnosis of NSCLC, treated in the University Clinic of Pneumology and Allergology of Ist 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 – March 2013. In NSCLC patients, during the planned surgery (lobectomy or pneumonectomy), tissue fragments (100-150 mg) were obtained from the center of primary lesion and the adjacent noncancerous (10 cm distant from the primary lesion), macroscopically unchanged tissue (conventional “normal” sample). Immediately after resection, lung tissue samples were placed in a stabilization buffer RNeasyLater®. Each tissue sample was divided into smaller parts (30-50 mg) for individual analysis, and frozen at -80°C.

The resected tissue samples were post-operatively histopathologically evaluated and classified according to the AJCC staging as well as TNM classification (pTNM). Based on histopathological assessments, the group of patients was subdivided in relation to NSCLC subtypes, i.e., squamous cell carcinoma (SCC), adenocarcinoma (AC) and large cell carcinoma (LCC). Relevant clinical and pathological characteristics of the patients with NSCLC included in this study are summarized in Table 1. All cases were primary tumors without chemo- or radiotherapy treatment.

The design of our study assumed the analysis of gene expression in two study groups: the tumor and the surrounding normal lung tissue, to compare the both type of tissue. The more so, because there are reports indicating no significant differences between cancerous and non-cancerous tissue in regard to some TSG expression [12]. On the other hand, accumulating evidence point to genetic/epigenetic changes occurring in normal tissue adjacent to tumor (the so called “field cancerization” process), which are widely described in literature in various cancer types and highlights the importance of early abnormalities in carcinogenesis [13,14].

2.2. RNA extraction, real-time PCR (qPCR method)

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 qualitative and quantitative assessments of RNA samples were determined using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies, USA) in Agilent 2100 Bioanalyzer (Agilent, USA).

Complementary DNA (cDNA) was transcribed from 100 ng of total RNA, using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA). Reverse transcription (RT) master mix contained: 10x RT buffer, 25x dNTP Mix (100 mM), 10x RT Random Primers, MultiScribe™ Reverse Transcriptase, RNase Inhibitor and nuclease-free water, in a total volume of 20 µl. RT reactions were performed in the following conditions: 10 minutes at 25°C, 120 minutes at 37°C, then 5 seconds at 85°C for, and 4°C hold.

The relative expression of the studied genes was assessed in qPCRs using Micro Fluidic Cards, the so called TLDA (TaqMan® Low Density Custom Arrays) plates, with pre-loaded selected assays (Applied Biosystems, USA): *FUS1*-Hs00200725_m1, *NPRL2/G2I*-Hs00198012_m1, *RASSF1A*-Hs00200394_m1, *DNMT1*-Hs00945875_m1, *DNMT3B*-Hs00171876_m1. *ESD* (Hs00382667_m1) was used as a reference gene and RNA isolated from normal lung tissue (Human Lung Total RNA, Ambion®, USA) served as calibrator samples.

All qPCRs were performed using 7900HT Fast Real-Time PCR System (Applied Biosystems, USA) with RQ software (TaqMan Relative Quantification Assay software). The reaction mixture contained: 50 µl cDNA (50 ng) and 50 µl TaqMan® Universal Master Mix (Applied Biosystems, USA). The PCR program was as follows: initial incubation 2 minutes at 50°C, AmpliTaq Gold® DNA polymerase activation at 94.5°C for 10 minutes, then 40 two-step cycles 30 seconds at 97°C and 60 seconds at 59.7°C.

2.3. DNA extraction, bisulfite conversion and methylation-specific PCRs (MSP method)

The extraction of genomic DNA from NSCLC specimens was performed using a QIAamp DNA Mini Kit (Qiagen, Germany), according to the manufacturer's protocol. To obtain RNA-free genomic DNA, RNase A (100 mg/ml) was used as optionally indicated in the kit procedure. The quality and quantity of DNA was spectrophotometrically assessed, measuring absorbance at 260/280 nm (Eppendorf BioPhotometer™ plus, Germany). 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.

Genomic DNA (1 µg) was modified with sodium bisulfite, using the CpGenome™ Turbo Bisulfide Modification Kit (CHEMICON International, Millipore, USA), according to the manufacturer's protocol. Its concentration and purity was spectrophotometrically estimated (Eppendorf BioPhotometer™ plus, Germany).

Bisulfite converted DNA was used for methylation-specific polymerase chain reactions (MSPs) to assess methylation status of the studied genes. Primers for MSPs were designed according to the criteria described by Feltus et al. [15], using computer tool (methPrimer v1.1 beta, Li Lab, Department of Urology, USCF) [16]. The set of primers for the studied genes were flanking the 1 kb 5' region upstream from the translation start point Primer sequences for

1 methylated and unmethylated promoter regions of the studied genes are included in Table 2. MSP master mix contained:
2 1000 ng DNA, 0.7 µM of each primer (Sigma-Aldrich, Germany), 2.5 µM dNTPs mix, 2.5 µM MgCl₂, Hot Start
3 AmpliTaq Gold® 360 DNA Polymerase (5U/µl), 10x Universal PCR buffer and nuclease-free water, in a total volume
4 of 12.5 µl. PCR conditions were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 three-step cycles
5 involving denaturation at 95°C for 45 seconds, specific annealing temperature – (see Table 2) – for 45 seconds and
6 elongation at 72°C for 1 minute; the final elongation step was done at 72°C for 10 minutes.
7

8
9
10 Positive and negative MSP controls were included in each PCR reaction. CpGenome Universal Methylated
11 DNA (enzymatically methylated human male genomic DNA) served as a positive methylation control and CpGenome
12 Universal Unmethylated DNA (human fetal cell line) was used as a negative control (CHEMICON International,
13 Millipore, USA). As a control for PCR contamination, blank samples with nuclease-free water instead of DNA were
14 used.
15

16
17 The MSP products were electrophoretically separated on 2% agarose gel and concentrations (ng) of MSP
18 products (U and M DNA alleles) were spectrophotometrically estimated, using DNA1000 LabChip Kit, on Agilent 2100
19 Bioanalyzer (Agilent Technologies, USA). For each sample, Methylation Index (MI) was assessed using the following
20 formula: peak height of methylated products/(peak height of methylated products + peak height of unmethylated
21 product), $MI = (M)/(M+U)$.
22

23 2.4. Statistical analysis

24
25 The results of relative expression analysis (RQ values) are presented as medians in each studied group. The
26 comparison of RQ values between cancer and non-cancer specimens was performed using Mann-Whitney U test and
27 Kruskal-Wallis test. The same nonparametric tests were applied to compare the differential expressions and methylation
28 status of TSGs between NSCLC subtypes, i.e., SCC, AC and LCC. Spearman's rank correlation coefficient, Mann-
29 Whitney U test and Kruskal-Wallis test were performed to evaluate the relationship between gene expression and
30 methylation levels and clinicopathological parameters (patients' characteristics: age, gender, history of smoking and
31 tumor staging according to pTNM and AJCC classifications). The Newman-Keuls method was used to identify
32 significantly different samples, regarding gene expression and methylation levels. Nonparametric Spearman's criterion
33 was used to calculate the coefficient of correlation between the levels of mRNA expression or promoter methylation for
34 pairs of studied TSGs and in pairs with methyltransferases. Receiver operating characteristic (ROC) curve was
35 established to discriminate NSCLC and adjacent normal tissue.
36

37
38 P-values < 0.05 were considered statistically significant. All statistical procedures were performed using
39 Statistica for Windows 10.0 software.
40

41 3. RESULTS

42 3.1. Relative expression levels of the studied TSGs

Relative expression levels of the studied tumor suppressor genes in NSCLC and macroscopically unchanged lung tissue samples were determined using delta-delta C_T method, and expressed 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.

The obtained RQ values, for the studied TSGs in individual NSCLC samples, are presented in Figure 1. The simultaneous down regulation of all 3 TSGs was found only in 5.9% specimens. Simultaneous decreased expression of *RASSF1A* and *FUS1* was observed in 23.5% SCC samples, and paired *RASSF1A* and *NPRL2/G21* were simultaneously decreased in 20.9% SCC and 16% NSCC samples. Generally, *RASSF1A* gene showed decreased expression in the majority of lung tumors (especially in SCC subtype), while the expression levels of *NPRL2/G21* and *FUS1* in most cases were similar to the level of calibrator. Table 3 shows the frequency of NSCLC samples with importantly decreased expression.

The obtained RQ values 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. Additionally, correlations between all studied genes were analyzed.

Comparing NSCLC histopathological subtypes, the expression levels of *RASSF1A* and *FUS1* were significantly different, while in case of *NPRL2/G21* gene, only a tendency was observed. Regarding patients' age, *NPRL2/G21* showed significantly lower expression in older patients. Concerning patients' sex, *RASSF1A* showed significantly lower expression level in females in NSCC group, and significantly lower expression in males in SCC group. In relation to tumor size (pTNM classification), *RASSF1A* expression was the lowest in T2 group in total NSCLC group, and similarly in NSCC subgroup. Regarding AJCC classification, statistically significant differences were observed for *FUS1* gene in NSCC subtype. Comparing tumor and macroscopically unchanged lung tissue samples, statistically significant differences were found for all genes, however only in case of *RASSF1A* gene its expression level was significantly lower in tumors. No significant differences were observed in correlation with cigarette smoking. The results are summarized in Table 4.

Newman-Keuls test documented statistical significances between the expression levels of the studied genes in pairs in NSCLC group, as well as in NSCLC histopathological subtypes: statistically significant differences were found between *RASSF1A* vs *NPRL2/G21* (P=0.001) and *RASSF1A* vs *FUS1* (P=0.00002) in total study group, and similarly in SCC subtype, where significant differences were found between *RASSF1A* vs *NPRL2/G21* (P=0.001) and *RASSF1A* vs *FUS1* (P=0.0003); in AC and NSCC subtypes, the expression levels of *RASSF1A* and *FUS1A* were significantly different (P=0.03 and P=0.005, respectively). In all mentioned cases, *RASSF1A* expression level was significantly lower than those of the other genes. Spearman's rank correlation coefficient revealed positive correlations between genes: *RASSF1A* and *NPRL2/G21* (P=0.0001, rs=0.38), *RASSF1A* and *FUS1* (P=0.001, rs=0.34) in NSCLC group; and also

between *RASSF1A* and *NPRL2/G21* in AC (P=0.02, rs=0.52) and NSCC (P=0.002, rs=0.60) group.

ROC curve analysis of *RASSF1A* expression level as a marker to distinguish NSCLC tissue from macroscopically unchanged lung tissue demonstrated a sensitivity of 61% , specificity of 73% and accuracy of 67% (AUC=0.695). Additionally, we analyzed the utility of *RASSF1A* and *FUS1* as potential markers distinguishing NSCLC histopathological subtypes: NSCC and SCC. Analyzed together, the genes yielded a sensitivity of 69%, specificity of 66% and accuracy of 68% (AUC=0.714). Separately, ROC curve analysis illustrated for *RASSF1A*: a sensitivity of 79%, specificity of 64% and accuracy of 73% (AUC=0.729); and for *FUS1*: a sensitivity of 88%, specificity of 56% and accuracy of 74% (AUC=0.729).

3.2. Relative expression levels of the studied DNMTs

Relative expression of two DNA methyltransferases, DNMT1 and DNMT3b, were also analyzed on mRNA level. The expression levels of both DNMTs were increased in tumor samples: median RQ value for *DNMT3B* was 3.81 and for *DNMT1* 1.38. Regarding the frequency of samples with increased *DNMT* expression, *DNMT1* was overexpressed in 75% NSCLCs (68% SCC, 76% NSCC) and *DNMT3B* in 92% NSCLCs (91% SCC, 92% NSCC).

Correlations were analyzed between the expression levels of the studied *DNMTs* and histopathological NSCLC subtypes (SCC, AC, LCC), tumor staging (pTNM, AJCC), patients' age, gender and smoking history, as well as in relation to RQ values of the studied *DNMTs* found in macroscopically unchanged lung tissue samples. *DNMT3B* expression level was significantly higher in younger patients (≤ 60 vs 61-70 years old) in AC subtype (7.14 vs 2.43, P=0.04, Mann-Whitney U-test) and in NSCC subtype (7.37 vs 2.43, P=0.006, Mann-Whitney U-test), where also the negative correlation was observed (rs= -0.50, P=0.01, Spearman's rank correlation coefficient). No statistically significant differences were found in relation to patients' gender and smoking, or tumor size (pTNM classification) and grade (AJCC classification). However, in SCC group *DNMT1* revealed a trend (P=0.05, Mann-Whitney U-test) toward higher expression in grade I/II vs grade III tumors (1.53 vs 1.10).

The expression levels of both DNMTs were significantly higher in tumors when compared with macroscopically unchanged lung tissue samples, in total NSCLC group and in NSCLC histopathological subgroups, as shown in Table 5.

Additionally, the expression levels of the studied DNA methyltransferases were positively correlated (Spearman's rank correlation coefficient) in all tissue groups: in total NSCLC cohort (rs=0.51, P=0.0001), in SCC (rs=0.51, P=0.002), in AC (rs=0.49, P=0.03), in NSCC (rs=0.55, P=0.004).

There weren't any negative correlations between the levels of expression of the methyltransferases and TSGs.

3.3. Methylation status of the studied TSG genes: the qualitative and quantitative assessments

The results on methylation status of the studied genes were obtained for different numbers of patients, due to DNA degradation in several samples: 17 in case of *RASSF1A*, 3 in cases of *NPRL2/G21* and *FUS1*.

Promoter methylation analysis of the studied genes, performed as MSP reactions, distinguished unmethylated (U) from methylated (M) DNA alleles after electrophoretic separation. Both U and M alleles were found in all studied tumor tissue groups. Next, 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 Methylation Index (MI) was calculated for each gene in all tissue samples.

The MSP reactions revealed the presence of M alleles for all genes, in 38-76% samples, depending on the gene. The frequencies of methylated and unmethylated alleles of all studied TSGs, are shown in Figure 2. The highest frequency of both methylated alleles was observed for *RASSF1A* gene (20%) and the same gene revealed the lowest number of samples with no methylated alleles (4%). Generally, the most abundant were samples with the presence of both methylated and unmethylated alleles (38%-76%, depending on the gene).

The calculated MIs indicated gene methylation level, which in tumor samples ranged from 3% to 66%, depending on the gene. Among the studied genes, *RASSF1A* showed the highest methylation level: 52% in total NSCLC group, 50% in SCC, and 66% in AC; *NPRL2/G21* – the lowest: 5% in total NSCLC group, 3% in SCC, and 10% in AC. Methylation level of *FUS1* was 17% in total NSCLC group, also 17% in SCC and 10% in AC.

Statistical analysis (Newman-Keuls test) revealed significantly higher methylation status of *RASSF1A* than those of other studied genes in all tissue groups. In NSCLC: *RASSF1A* vs *FUS1* ($P=0.0001$), *RASSF1A* vs *NPRL2/G21* ($P=0.00002$); MI values of *FUS1* were also significantly higher than *NPRL2* methylation status ($P=0.01$) in this tissue group. The results are shown in Figure 3. Similarly in SCC group: *RASSF1A* vs *NPRL2/G21* ($P=0.00002$), *RASSF1A* vs *FUS1* ($P=0.0001$); and also *FUS1* vs *NPRL2/G21* ($P=0.04$). In AC: *RASSF1A* vs *NPRL2/G21* ($P=0.0001$), *RASSF1A* vs *FUS1* ($P=0.0001$).

Concerning tumor histopathological characteristics, in SCC group *FUS1* was significantly higher methylated in stage II vs stage III tumors ($P=0.03$, Mann-Whitney U test). No other significant correlations were found between gene methylation and clinicopathological parameters.

3.4. Correlation between gene expression level and methylation status

The decreased expression of *RASSF1A* was accompanied by promoter methylation in 71% NSCLC samples, in case of *FUS1* it was found in 20% NSCLC specimens, and only in 5% NSCLC samples for *NPRL2/G21*. Statistical analysis revealed the negative correlation between the RQ and MI values for only one gene, *FUS1* ($P=0.02$, Spearman's rank correlation coefficient, $rs=-0.41$) in SCC subtype.

Spearman's rank correlation coefficient didn't reveal statistically significant correlations between TSG methylation and DNMT expression ($P>0.05$).

4. DISCUSSION

The aim of our study was to analyze by qPCR and compare the expression levels of 3p TSGs in non-small cell

lung cancer samples, as well as in macroscopically unchanged tissue surrounding the primary lesion. To look for the possible epigenetic mechanism of TSG inactivation, gene promoter methylation was assessed, as well as the levels of DNMT1 and DNMT3B RNA expression.

To the best of our knowledge, only few studies were performed to compare expression of several 3p TSGs in the same lung tumor samples [17-21]. In order to investigate it further we chose 3 TSGs and analyzed their expression in lung primary tumors: non-small cell lung cancer (NSCLC), adenocarcinoma (AC), squamous cell lung cancer (SCC) and large cell cancer (LCC). Similarly, few studies have focused so far on simultaneous analysis of methylation status of more than two genes at 3p21.3 locus in primary lung cancer [20,22,23].

The chosen TSGs, namely *FUS1*, *NPRL2/G21* and *RASSF1A*, are located in proximity, in 3p21.3 subregion called LUCA. Such close position could suggest the tendency toward simultaneous decrease of their expression. However, this was not the case in our study. Only few tumor samples showed decreased expression of all three genes. The low levels of promoter methylation of *NPRL2/G21* and, to some extent, of *FUS1* also didn't designate their epigenetic inactivation in the population studied.

The highest frequency of decreased expression and increased promoter methylation we found for *RASSF1A* gene. The simultaneous decreased expression and methylation of at least one *RASSF1A* allele was found in 71% tumor samples. Tumor suppressor gene, *RASSF1A* (*Ras association domain family member 1*), is involved in development or progression in the vast majority of cancers [4,24,25]. In NSCLC, *RASSF1A* decreased expression was found in 67% samples, as reported by Senchenko et al. [20], although it is usually reported at the level of about 30% [26]. In our study we confirmed decreased expression of *RASSF1A* ($RQ < 1$), and the important gene downregulation ($RQ < 0.5$) was observed in more than 20% of NSCLC samples, with higher frequency in SCC group (reaching nearly 30%). In paired "normal" lung tissue specimens *RASSF1A* decreased expression was found only in 3-8% samples. The differences between the studied groups, i.e., between NSCLC subtypes and between primary tumor specimens and the surrounding macroscopically unchanged lung tissue samples, were statistically significant (in total study group and in SCC subtype). The highest *RASSF1A* expression level – similar to that observed in "normal" lung tissue – was found in AC group. Similarly, the increased level of *RASSF1A* mRNA in lung cancer was also observed by others, who found even 3-to-4 fold increase in AC subtype [20]. *RASSF1A* gene – as the only one in our analysis – revealed correlations with gender: significantly lower gene expression was found in men in SCC group and in women in NSCC group. Regarding TNM classification, significantly lower *RASSF1A* expression was observed in T2 tumors as compared with T3/4 NSCLC. It could confirm its role at earlier stages of lung carcinogenesis. However, in other studies, no statistically significant differences were observed with relation to age, smoking history and other cytological and pathological characteristics [20].

Significant differences found between tumor and "normal" lung samples indicate the possible clinical utility of

RASSF1A gene expression level as a diagnostic marker. In most studies, the principal cause of *RASSF1A* loss of expression was tumor-specific *RASSF1A* promoter hypermethylation, while tumor-associated mutations were rare [24,25,27]. In our study, the frequency of *RASSF1A* methylated alleles in total NSCLC group was 76% and gene methylation level was equal to or exceeded 50%, depending on the histotype. The observed methylation frequency of *RASSF1A* was in the top of the reported by others 26-87% range [22,24,28-33].

In several reports, *RASSF1A* methylation was associated with poor prognosis in NSCLC patients [29-31,34]. We didn't observe any associations with tumor clinicopathological features, such as TNM or AJCC staging, or patients' smoking habits. Similar results were obtained by others [7,33,34]. Meta-analysis performed in 2013 confirmed a significant association between *RASSF1A* methylation and NSCLC, however there were no significant differences in *RASSF1A* methylation in relation to gender, pathology, TNM stage and smoking behavior among NSCLC cases [35].

It is true that of all the genes in the 3p21.3 critical region *RASSF1* has been the most comprehensively studied at the genetic, epigenetic and functional level, and its role as a tumor suppressor gene has been proven. However, the results regarding the utility of *RASSF1A* epigenetic inactivation as a prognostic marker in NSCLC are still divergent [8,26,30,32]. In our study, *RASSF1A* methylation level was significantly higher than that of other studied genes, however, lack of any other associations does not entitle us to decisive conclusions.

Another gene localized in LUCA region, *FUS1* (*fused in sarcoma*), also known as *TUSC2* (*tumor suppressor candidate 2*) is recently identified TSG, involved in apoptosis. Loss or reduction of *FUS1* protein expression, associated with poorer prognosis, was observed in lung cancers [36-38]. We didn't observe importantly decreased expression of *FUS1* on mRNA level in lung tumor samples. Statistically significant differences between NSCLC histotypes and the lowest gene expression level in SCC might suggest *FUS1* significance predominantly in squamous cell carcinoma. In NSCC subtype, significantly decreased *FUS1* expression in stage II vs stage III tumors could indicate its role at early stage of carcinogenesis. However, generally, *FUS1* expression level was similar to that observed for calibrator, i.e., normal lung tissue. No aberrant *FUS1* mRNA level was also found by other researchers [27]. *FUS1* promoter methylation analysis in our study revealed the presence of methylated alleles in 58% NSCLC samples and rather moderate gene methylation status, ranging from 10 to 22%, depending on histopathological subtype. However, as the only gene in our analysis, *FUS1* revealed negative correlation between its expression level and methylation status. Additionally, significantly higher gene promoter methylation level in II vs III tumors in SCC subgroup might support *FUS1* possible role at early stage of lung cancer development.

So far, there have not been published any reports on *FUS1* promoter hypermethylation in lung cancer, although it was found to be partially methylated in head and neck squamous cell carcinoma [39]. Little is known on molecular mechanisms involved in the regulation of *FUS1* expression in primary lung cancer cells. *FUS1* mRNA transcripts were found in both normal lung and some lung cancer cell lines, but *FUS1* protein was absent in a majority of lung cancer

cell lines and NSCLC samples [37]. Researchers indicate the role of 3'-untranslated regions (UTRs) of the FUS1 gene transcript [40] or the influence of another epigenetic mechanisms, e.g., the role of several miRNAs in the down-regulation of FUS1 protein expression in lung cancer cells [41] or aberrant histone modifications [42]. Thus, the inactivation of FUS1 may occur through abnormal translational control of *FUS1* mRNA or aberrant post-translational protein modification resulting in its inactivation despite the absence of mutations and in the presence of high levels of transcript mRNA [43].

The physiological role of FUS1 is still poorly known and further investigation of this interesting TSG in lung cancer is required.

NPRL2/G21 (nitrogen permease regulator-like 2 gene), also known as *TUSC4* (tumour-suppressor candidate 4), is a tumor suppressor gene commonly expressed in normal tissues, including lung tissue. The study results indicate that *NPRL2/G21* is involved in DNA repair, cell cycle control and apoptosis [44]. Senchenko et al. [20] showed significantly decreased gene expression level in 73% NSCLC cases, but the earlier report described normal *NPRL2/21* mRNA expression in most lung cancers [24]. Stop mutations, nonsense deletions and missense mutations were found in this gene [17,44]. Epigenetic analyses failed to show gene promoter hypermethylation in cancer cell lines [42,44], and no such studies have been performed so far in primary lung tumors.

In our study, we didn't confirm decreased expression of *NPRL2/G21* in lung tumor samples. However, the differences between NSCLC histopathological subtypes were significant, with lower expression level in SCC samples. Although, it was still at the level of calibrator (normal lung tissue). In some studies, increased expression of *NPRL2/G21* on mRNA or protein level was also observed [20,45]. Interestingly, *NPRL2/G21* gene – as the only TSG in our study group – showed correlation with age: in SCC samples its expression level was significantly lower in elder patients (>70 years old).

The exact mechanism involved in the possible inactivation of *NPRL2/G21* in human cancers has not been elucidated. In our study we didn't observe hypermethylation of *NPRL2/G21* promoter region. The frequency of gene methylation in NSCLC samples was very low and its methylation status was significantly lower than that of other studied TSGs.

According to the results obtained by Lerman & Minna [27], *NPRL2/G21* may be one of the haploinsufficient genes that predispose to cancer in a hemizygous state and do not show a second mutation – or promoter hypermethylation – in the other allele (wild-type allele). Alternatively, as in the case of other 3p21.3 tumor suppressors, like *RASSF1A* and *FUS1*, such mechanisms as chromosome instability, aneuploidy, altered RNA splicing, or defects in transcriptional, translational, and posttranslational modifications that are common in the 3p region, may play a role in the inactivation of *NPRL1/G21* in lung tumors.

Although the function of *NPRL2/G21* is still unknown, it revealed interesting association in lung cancer: gene

decreased expression was conversely correlated with cisplatin sensitivity in NSCLC cell lines [45]. This finding, of significant clinical value, could indicate the possible therapeutic role of *NPRL2/G21* for enhancing and re-sensitizing non-responders to cisplatin. Therefore, *NPRL2/G21* deserves attention and further study in lung cancers, regarding its expression and underlying mechanisms of its possible silencing.

None of the genes revealed correlations with smoking history. It is in accordance with the results of Senchenko et al. [20]. Although smoking is a known risk factor in NSCLC, the lack of correlations with expression levels and methylation status of the studied TSGs could indicate that tobacco smoke targets other than studied genes.

We tested whether the methylation status of tumor suppressor genes was associated with the mRNA expression levels of DNA methyltransferases (DNMTs). The role of DNMT mediated epigenetic alterations in lung cancer development has been the focus of increasing interest in recent years. In our study both enzymes were highly expressed in a coordinate manner in lung tumors – positive correlation was observed in all NSCLC histopathological types, as well as in total NSCLC group. In case of both studied DNMTs, we found significantly higher expression levels in lung tumor specimens as compared with macroscopically unchanged tissue samples.

Regarding NSCLC histopathological subtypes, *DNMT3B* expression was higher in SCC than in NSCC, although it didn't reach statistical significance. *DNMT1* revealed trend toward higher expression in grade I/II tumors in SCC group, suggesting its role at early stages of lung carcinogenesis. However, in other studies, deregulated expression of *DNMT1* was an independent prognostic factor in SCC [46,47]. Interestingly, in AC and NSCC subtypes, *DNMT3B* expression levels were significantly higher in patients aged 60 and below as compared with older patients. Elevated DNMT expression in younger patients was more frequent also in other study [47].

We didn't observe any negative correlations between the expression levels of the studied TSGs and methyltransferases. The results of others are also conflicting. Similarly to our findings, the elevated mRNA levels of *DNMT1* and *DNMT3B* were not significantly associated with hypermethylation of the several TSGs, including *RASSF1A*, in lung cancer cell lines [47] and in lung primary tumors [47,49]. Others found correlations between TSG promoter hypermethylation and DNMT overexpression, but on protein level [46]. Overexpression of *DNMT* was found to occur earlier than the methylation modifications and complex interactions between several factors in hypermethylation process was highlighted [47,49]. So, there is still a need to elucidate the clinicopathological significance of DNA methyltransferases in primary non-small cell lung cancer.

CONCLUSIONS

The results of our study indicate the potential role of the studied genes in the differentiation of NSCLC histopathological subtypes: *RASSF1A* and *FUS1* could be regarded as markers differentiating SCC and NSCC, as their expression levels were significantly lower in squamous cell carcinoma subtype; similar role could be assign to *NPRL2/G21* in differentiating SCC and AC subtypes. Although, the most prominent role was documented in case of

RASSF1A. The significant differences in its expression level between NSCLC and macroscopically unchanged lung tissue surrounding the primary lesions highlight its possible diagnostic role in lung cancer *in situ* recognition. High percentage of lung tumor samples with simultaneous *RASSF1A* decreased expression and gene promoter methylation indicates its epigenetic silencing. However, overexpression of methylating enzymes (DNMT1 and DNMT3B) enzymes was not a critical determinate of tumor-specific promoter hypermethylation of *RASSF1A* and *FUS1*, which revealed, respectively, high and moderate methylation frequency in our study.

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CONFLICTS OF INTEREST STATEMENT

Authors declare no conflicts of interest in relation to this article.

AUTHORS' CONTRIBUTION

AA and EB-L conceived the idea of this project. DP-L and JK participated in experimental design, coordination and execution of the experiments and preparation of the manuscript. JK and AA contributed in biological material sampling. DD, EN and JMK participated in sample handling and performed experiments (MSPs, qPCRs). DP-L, JK, and KHC analyzed and interpreted data. MM-S performed the statistical analysis. EB-L revised the manuscript critically for important intellectual content. PG gave final approval for the submission.

Dorota Pastuszek-Lewandoska and Jacek Kordiak have equally contributed to this work.

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Fig. 1. Relative expression levels of the three TSGs, presented as log₂RQ, in the studied NSCLC histopathological subtypes

Fig. 2. The frequencies of methylated (M) and unmethylated (U) alleles of the studied genes, based on MI values.

Fig. 3. Box and whisker plot representing mean MI values of the studied genes in NSCLC group

Table 1. Clinicopathological features of the studied NSCLC group.

Analyzed variables	NSCLC patients	
Gender (n, %)	Women (24, 41%)	
	Men (35, 59%)	
Age (n, %)	Mean 61 ± 7.62	≤ 60 yrs (14, 24%)
		61-70 yrs (30, 51%)

>70 yrs (15, 25%)

Smoking status and smoking history (n, %)	Smokers (54, 92%)	< 40 Pack Years ¹ (PYs) (26, 48%)
	current smokers (31, 57%)	≥ 40 PYs (28, 52%)
	former smokers (23, 43%)	
	non-smokers (5, 8%)	
Histopathological type of NSCLC (n, %)	SCC (34, 58%)	
	NSCC (25, 42%)	AC (20, 80%)
		LCC (5, 20%)
AJCC classification ² (n, %)	I (11, 18%)	
	II (21, 36%)	
	III (27, 46%)	
Tumor size according to pTNM classification ³ (n, %)	T1 (12, 20%)	
	T2 (33, 56%)	
	T3/T4 (14, 24%)	

¹ PYs were calculated according to the NCI Dictionary of Cancer Terms: 1 Pack Year is equal to 20 cigarettes smoked per day for 1 year (<http://www.cancer.gov/dictionary?Cdrid=306510>)

² AJCC – American Joint Committee on Cancer Staging according to the IASCLC Staging Project 7th ed. (2010) Cancer

³ pTNM – post-operative Tumor Node Metastasis classification according to the WHO Histological Typing of Lung Tumor

Table 2. Characterization of MSP primers (M – methylated; U – unmethylated) used in the study. **The underlined nucleotides in forward and reverse primers indicate the presence of methylated cytosines in DNA sequence.**

Gene	Forward primer	Primer position	Reverse primer	Primer position	Product length [bp]	Annealing temp. [°C]
<i>FUS1</i>	M: TGTTAT <u>CG</u> TGGAT TAGATATTGTT <u>C</u>	-559 to -482	M: ACTATATTTTAC <u>G</u> ATTACCAC <u>G</u> CT	-375 to -349	207	56.5
	U: TTAT <u>T</u> GTGGATTA GATATTGTT <u>T</u> GT	-557 to -480	U: ACTATATTTTAC <u>A</u> ATTACCAC <u>A</u> CT	-375 to -349	205	56.5
<i>NPRL2/G21</i>	M:	+108 to +131	M:	+238 to +261	153	52.5

	GTT <u>C</u> GGTTATTGT		AACCAATTAAACT		
	TATGGGTAG <u>C</u>		CTC <u>G</u> AAAAC <u>G</u> T		
	U:	+107 to +132	U:	+238 to +261	153
	GGTT <u>T</u> GGTTATTG		ACCAATTAAACTC		56.5
	TTATGGGTAG <u>T</u> G		TC <u>A</u> AAAAC <u>A</u> TC		
<i>RASSF1A</i>	M:	-599 to -573	M:	-409 to -384	215
	ATATTTTTT <u>C</u> GATT		CTACACTATAACC		56.5
	TGGAGTTTTTTT <u>C</u>		TACCCATCCTC <u>G</u>		
	U:	-599 to -573	U:	-410 to -385	211
	TTTTTTT <u>T</u> GATTG		TACACTATAACCT		57.5
	GAGTTTTTTT <u>G</u> T		ACCCATCCTC <u>A</u> C		

Table 3. Frequency of importantly decreased TSG expression (RQ<0.5)

Gene	NSCLC samples (T)			Paired „normal” samples (N)		
	Total	SCC	NSCC	Total	SCC	NSCC
	(n=59)	(n=34)	(n=25)	(n=59)	(n=34)	(n=25)
<i>RASSF1A</i>	23.7%	29.4%	16%	5.1%	2.9%	8%
	(n=14)	(n=10)	(n=4)	(n=3)	(n=1)	(n=2)
<i>NPRL2/G21</i>	5.1%	2.9%	8%	3.4%	2.9%	4%
	(n=3)	(n=1)	(n=2)	(n=2)	(n=1)	(n=1)
<i>FUS1</i>	0%	0%	0%	1.7%	2.9%	0%
	(n=0)	(n=0)	(n=0)	(n=1)	(n=1)	(n=0)

Table 4. Correlations analyzed between the expression levels of the studied genes and tumor clinicopathological parameters and patients' characteristics

<i>FUS1</i> (n=59)	<i>NPRL2/G21</i> (n=59)	<i>RASSF1A</i> (n=59)
Median RQ in NSCLC samples		
1.35	1.36	0.77

SCC vs AC vs LCC

1.18 vs 1.83 vs 2.39 P=0.002 ^a	1.28 vs 1.79 vs 0.83 P=0.05 ^a	0.68 vs 1.01 vs 0.86 P=0.01 ^a
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SCC vs AC

1.18 vs 1.83 P=0.004 ^b	1.28 vs 1.84 P=0.03 ^b	0.68 vs 1.01 P=0.002 ^b
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SCC vs NSCC

1.18 vs 2.13 P=0.001 ^b	P>0.05	0.68 vs 1.01 P=0.002 ^b
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Age (≤ 60 yrs vs 61-70 yrs vs >70 yrs) – in total NSCLC group; in NSCLC histopathological subgroups

P>0.05	In SCC: 61-70 yrs vs >70 yrs	P>0.05
	1.55 vs 0.99 P=0.001 ^b	

Gender (females vs males) – in total NSCLC group; in NSCLC histopathological subgroups

P>0.05	P>0.05	In SCC: 1.04 vs 0.64 P=0.02 ^b ;
		in AC: 0.86 vs 1.31 P=0.01 ^b ;
		in NSCC: 0.86 vs 1.31 P=0.02 ^b

pTNM classification (T1 vs T2 vs T3/4) – in total NSCLC group; in NSCLC histopathological subgroups

P>0.05	P>0.05	0.80 vs 0.67 vs 0.94 P=0.04 ^a ;
		T2 vs T3/4 P=0.01 ^b ;
		in NSCC: 1.35 vs 0.75 vs 1.27
		P=0.04 ^a ; T2 vs T3/4 P=0.04 ^b

AJCC classification (I vs II vs III) – in total NSCLC group; in NSCLC histopathological subgroups

In NSCC: 2.44 vs 1.20 vs 2.25	P>0.05	P>0.05
P=0.04 ^a ; I vs II P=0.02 ^b		

Smoking status (current smokers vs former smokers vs never smokers) – in total NSCLC group; in NSCLC histopathological subgroups

P>0.05	P>0.05	P>0.05
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Smoking history (PYs: <40 vs ≥40) – in total NSCLC group; in NSCLC histopathological subgroups

P>0.05	P>0.05	P>0.05
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Tumor (T) tissue vs macroscopically unchanged lung tissue – in total NSCLC group; in NSCLC

histopathological subgroups

in AC: 1.83 vs 1.18 P=0.01 ^b ;	1.36 vs 0.84 P=0.0003 ^b ;	0.77 vs 1.07 P=0.0003 ^b ;
in NSCC: 2.13 vs 1.20 P=0.002 ^b	in SCC: 1.28 vs 0.84 P=0.01 ^b ;	in SCC: 0.68 vs 1.09 P=0.000002 ^b
	in AC: 1.84 vs 0.84 P=0.002 ^b ;	
	in NSCC: 1.79 vs 0.84 P=0.009 ^b	

^a Kruskal-Wallis test; ^b Mann-Whitney U-test.

Table 5. RQ values (medians) correlated between tumor vs macroscopically unchanged lung tissue samples, with P value (Mann-Whitney U-test)

Gene	total NSCLC group	SCC subgroup	AC subgroup	NSCC subgroup
<i>DNMT1</i>	1.38 vs 0.71 P=0.0001	1.36 vs 0.69 P=0.0001	1.57 vs 0.74 P=0.0001	1.38 vs 0.73 P=0.0002
<i>DNMT3B</i>	3.81 vs 1.63 P=0.0001	5.24 vs 1.61 P=0.0001	3.63 vs 1.72 P=0.002	3.57 vs 1.64 P=0.001

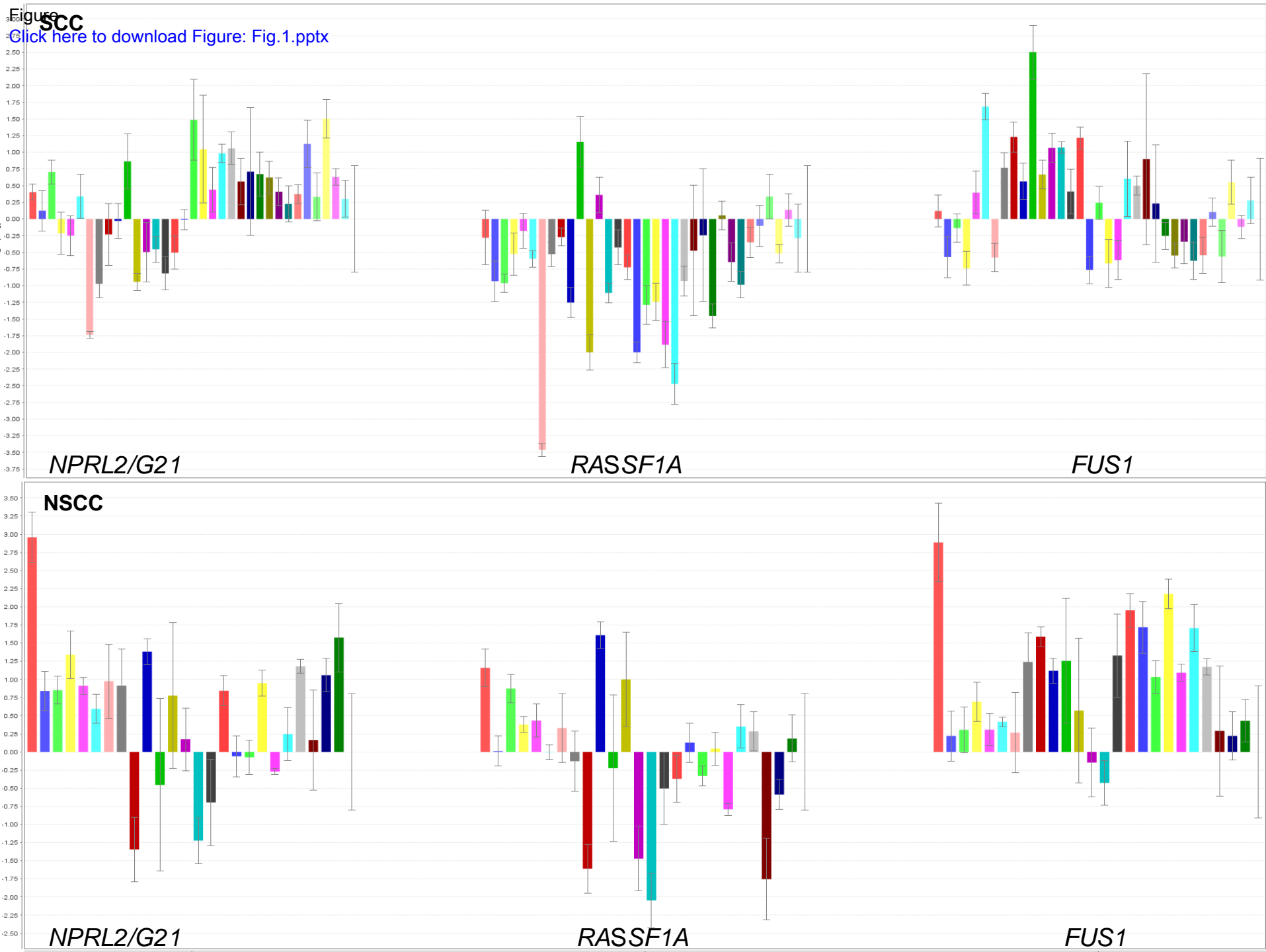


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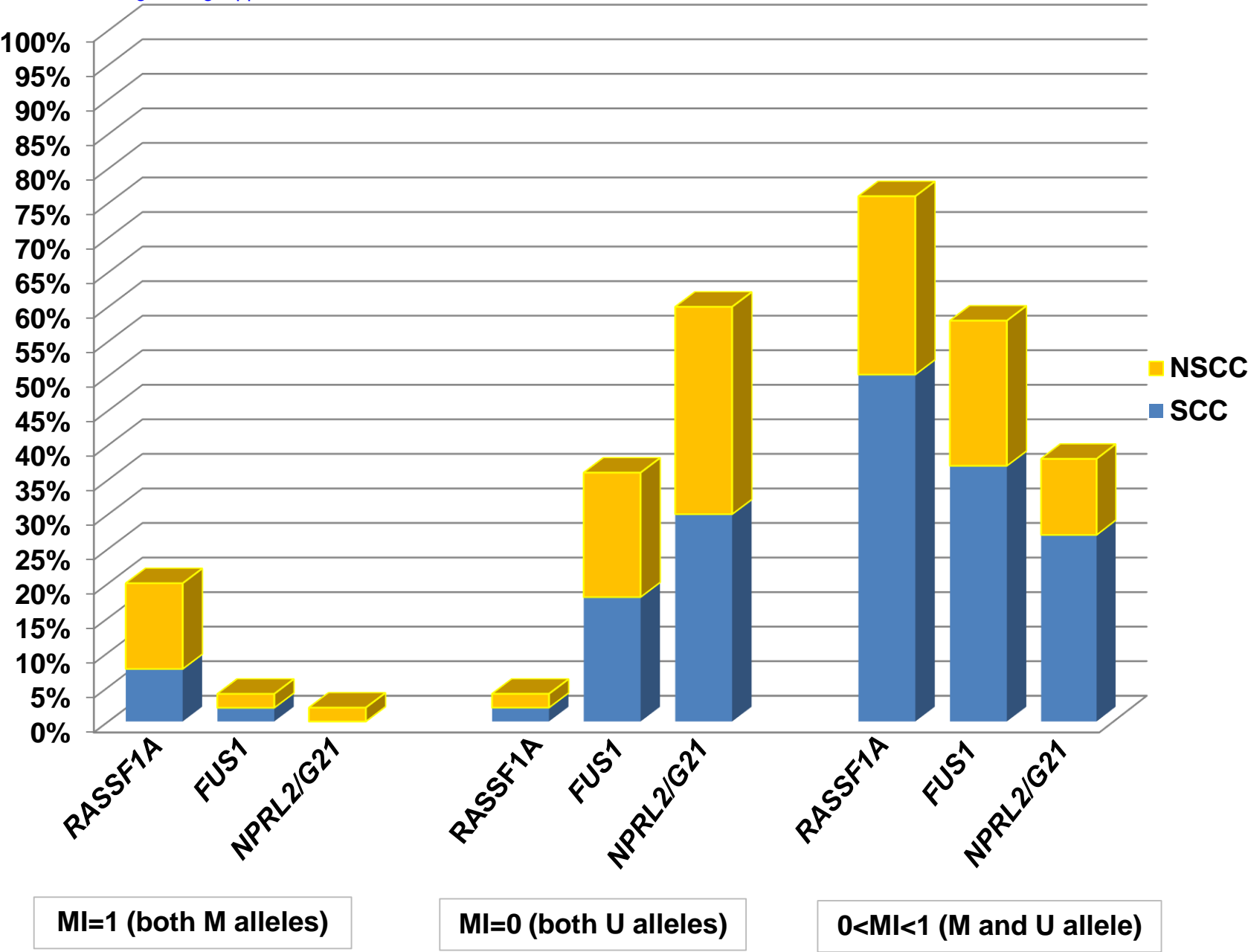


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