FHIT promoter methylation status, low protein and high mRNA levels in patients with non-small cell lung cancer

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Abstract. *FHIT* is a tumor suppressor gene that is frequently silenced in non-small cell lung cancer (NSCLC) and also in preneoplastic lesions. Promoter hypermethylation was previously observed in NSCLC, and its epigenetic silencing, observed on mRNA or protein level, was proposed to predict NSCLC outcome. In the present study we evaluated the relationship between *FHIT* expression on mRNA level and promoter methylation, or immunoexpression level. The aim of this study was to analyze the usefulness of *FHIT* as early differentiating biomarker in NSCLC patients. Lung tissue specimens were obtained from 59 patients with diagnosed NSCLC (SCC=34, AC=20, LCC=5). *FHIT* promoter methylation was assessed in methylation-specific PCR. Relative expression analysis of *FHIT* was performed in real-time PCR (qPCR) and protein immunoexpression by ELISA assay. Significant differences in *FHIT* expression between NSCLC histopathological groups (SCC, AC, LCC) were observed (p=0.000009), with the lowest level in SCC. *FHIT* expression was significantly higher (p=0.034) in men vs. women. Methylated *FHIT* alleles were present both in NSCLC and control specimens. Mean MI value was higher in control tissue vs. neoplasm, and in men vs. women and it increased with patient age. Significant increase in MI level was observed in N0 group vs. N1 and N2, according to the TNM staging (p=0.0073). Differences in *FHIT* expression levels between AC, LCC and SCC indicated the usefulness of this gene as a diagnostic marker for NSCLC subtype differentiation. *FHIT* promoter hypermethylation both in cancer and control tissue indicated the presence of epigenetic alterations in early stage of NSCLC development. Differences in gene promoter methylation between cancer patients with and without node infiltration might be considered as a prognostic marker. Significantly lower *FHIT* protein immunoexpression was revealed in the group with long and intense history of smoking assessed as PYs (PY<40 vs. PY≥40, p=0.01). These results suggest the need of further study on *FHIT* as a potential biomarker.

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Introduction

Lung cancer is one of the most prevalent cancers (1.6 million new cases yearly) and one of the most common cause of deaths (more than million per year) with the mortality to incidence ratio 0.86. Lung cancer stands for 21.2% of all diagnosed cancers in men, and 8.6% among women (after breast and colorectal cancers) (1,2). In clinical classification two main types of lung cancer are distinguished: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) accounting for almost 80% of lung cancers (3). The three main NSCLC subtypes are squamous cell carcinoma (SSC), adenocarcinoma (AC) and large cell cancer (LCC) (4). Late detection of lung tumor (AJCC stage III /IV) drastically reduces the chance for a cure; the 5-year survival rate is ~6.6% (2). Detection of lung cancer in stage I increase the survival up to 83% (5). For this reason, it is important to search for candidate biomarkers, which will enable to recognize NSCLC on early stage and will help to distinguish its subtypes.

Tumor suppressor genes (TSG) are potential cancer markers because their expression in tumor tissues is suppressed or lost. Loss of function can occur as an effect of genetic instability (inactivating mutations, loss of heterozygosity) or by the promoter region hypermethylation (epigenetic mechanism). In lung cancer TSG inactivation frequently occurs in critical regions on 3p, such as 3p21 covering the *loci* of *RASSF1A, RARB, MLH1* (6). One of the potential biomarker is *FHIT* located in 3p14.2 described as FRA3B fragile site, the region frequently altered in lung carcinogenesis. *FHIT* (member of the histidine triad gene family) encodes a diadenosine 5',5'''-P1,P3-triphosphate hydrolase involved in purine metabolism. FHIT protein is homologous to Ap4A hydrolase from the yeast *Schizosaccharomyces pombe* and also exhibits

Ap3A activity in enzymatic assays (7). FHIT inhibits the serine/threonine kinase Akt, a key effector in PI3K pathway, promoting survival and cell growth in response to extracellular signals (8). The TSG function of this gene is reflected by regulation of programmed cell death and suppression of tumor metastasis (8). FHIT protein also plays a role in the modulation of response to DNA damage, for example, preventing the replication of stress-induced DNA damage. *FHIT* interacts with C-terminal domain of β-catenin, inhibiting the Wnt signaling pathway and its target genes, including cyclin D1, MMP14 and survivin (9). *FHIT* protein with Chk1 kinase plays important role in S phase checkpoint. Introduction of a wild-type *FHIT* gene suppresses tumorigenicity and *FHIT* transfection in '*FHIT-lacking*' human cancer cells appears to induce apoptosis and inhibit cell growth (10,11). Several investigators have shown that loss of *FHIT* function in preneoplastic lesions can lead to the accumulation of DNA damage and cell transformation; therefore it is defined as the guardian of the preneoplastic genome (12-14).

Aberrant *FHIT* expression caused by truncated transcripts or promoter region hypermethylation has been found in esophageal, stomach, and colon carcinomas (7,15). Lack of *FHIT* expression in several studies was demonstrated to have impact on tumor aggressiveness (16). In addition, decreased *FHIT* expression present in preneoplastic lesions of the lung has been proposed to predict NSCLC outcome (12). LOH-dependent *FHIT* decreased expression have been linked with high proliferation and low apoptotic index in tumor cells, particularly in SCC (17). It has also been proven that co-hypermethylation of p16 and *FHIT* genes in early stage of NSCLC is poor prognostic factor and can confer cisplatin resistance in NSCLC cells (18).

The aim of this study was to assess the relationship between *FHIT* gene promoter methylation level and *FHIT* gene expression, both in lung cancer tissue and macroscopically unchanged tissue from the operational margin. *FHIT* protein expression level was also evaluated. The obtained results were correlated with the clinical features of patients, tobacco addiction and histopathological characteristics of lung tumors.

Table I. Patient profile and tumor characteristics.

Characteristics	Mean age ± SD	No. of patients
Gender		
Women	63.08±7.820	24 (40.7%)
Men	65.78±7.315	35 (59.3%)
NSCLC histopathological verification		
SCC	67.46±6.13	34 (58%)
AC	65.93±5.13	20 (34.4%)
LCC	60.91±3.54	5 (8.6%)
Tobacco addiction and consumption		
Current smokers		31 (53.5%)
Former smokers		23 (43%)
Non-smokers		4 (6.75%)
Pack Years[a] (PYs)		
<40 PYs		26 (48%)
≥40 PYs		28 (52%)
Lung cancer staging		
AJCC Staging[b]		
IA/IB		14 (25%)
IIA/IIB		23 (41%)
IIIA/IIIB		18 (32%)
pTNM staging		
pT1		15 (27%)
pT2		23 (41%)
pT3/pT4		17 (30%)

[a]PYs, Pack Years were calculated according to the NCI Dictionary of Cancer Terms: 1 Pack Year is equal to 20 cigarettes smoked per day for one year (http://www.cancer.gov/dictionary?CdrID=306510). [b]https://cancerstaging.org/references-tools/quickreferences/Documents/LungMedium.pdf (19).

Materials and methods

Clinical characterization of patients and the NSCLC tissue samples. The study received the approval of the Ethics Committee of the Medical University of Lodz, Poland, agreement no. RNN/140/10/KE. All patients were informed and written consent was obtained from each patient.

The lung tissues were obtained from 65 patients who underwent lobectomy or pneumonectomy between July 2010 and March 2013 in the Department of Thoracic Surgery, General and Oncologic Surgery, Medical University of Lodz, Poland. Patients selected for the study had primary tumors and were preoperatively cytologically and histopathologically assessed. Patients did not undergo chemo- or radiotherapy treatment prior to the surgery. The resected tumors were post-operatively subjected to the histopathological analysis. Based on the histopathological results, the NSCLC diagnoses were confirmed for 59 patients, and those patients qualified for further studies. NSCLC samples in the histopathological evaluation were classified as: squamous cell carcinoma (SCC, n=34), adenocarcinoma (AC, n=20), and large cell carcinoma (LCC, n=5). The tumor samples were classified according to the AJCC staging (19) as well as TNM classification (pTNM) post-operative tumor node metastasis classification according to the WHO Histological Typing of Lung Tumors). The study group comprised of 24 women and 35 men. The smoking history was obtained from each patient. Detailed clinicopathological information on NSCLC patients is presented in Table I.

For the study purposes a pair of lung tissue samples was collected from each patient: from the center of the lesion and from the operational margin (obtained from the most distant site from the resected lesion) - the macroscopically unchanged lung tissue, that served as control tissue. The resected lung tissues (100-150 mg) were immediately placed in RNA stabilizing buffer (RNAlater®, Qiagen, Hilden, Germany), cut into smaller parts and stored in -80˚C until further use.

Genomic DNA and total RNA isolation. Genomic DNA and total RNA from NSCLC samples and macroscopically unchanged lung tissues were isolated using the column

Table II. MS-PCR primer sequences and expected product length.

	Primer sequence (5'-3')
Meth F	AAAAGAAATTTAGTTAGTGGGAAGTC
Meth R	AAAAAAATTTAAAACATAAATCGCA
Unmeth F	AGAAATTTAGTTAGTGGGAAGTTGT
Unmeth R	AAAAAAATTTAAAACATAAATCACA

Meth, Methylated; Unmeth, Unmethylated; F, forward; R, reverse. Methylated product length, 170 bp; unmethylated product length, 167 bp. bp, base pairs.

methods, QIAamp DNA Mini kit (Qiagen) and Universal RNA Purification kit (Eurx Ltd., Gdansk, Poland), according to the manufacturer's protocol. After the isolation, quality and quantity of DNA and RNA was spectrophotometrically assessed (BioPhotometer™ Plus; Eppendorf, Hamburg, Germany). For further analysis only high quality DNA samples with a 260/280 nm ratio in the range of 1.8-2.0 and DNA concentration over 50 ng/μl were used. RNA was additionally submitted to qualitative and quantitative assessment in automated capillary electrophoresis on Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) using RNA 6000 Pico/Nano LabChip kit (Agilent Technologies) in order to estimate the 18S/28S rRNA ratio.

Evaluation of FHIT gene promoter methylation. Bisulfite conversion: In order to distinguish methylated from unmethylated cytosine in DNA sequence, the bisulfite conversion reaction was performed (20). The conversion was performed with commercially available kit CpGenome™ Turbo Bisulfide Modification kit (Chemicon International, Millipore, USA), according to the manufacturer's protocol. For the reaction with sodium bisulfite 1 μg of genomic DNA was used. After conversion, the quality and quantity of DNA was spectrophotometrically assessed at 260/280 nm in biophotometer (BioPhotometer™ Plus; Eppendorf).

Methylation specific PCR. In order to assess the methylation status of the studied gene, the methylation-specific polymerase chain reaction (MS-PCR) was performed using two pairs of primers (methylated and unmethylated) and bisulfite converted DNA. MS-PCR was performed in a total volume of 12.5 μl and the mix contained: 2.5 μM dNTPs mix, 2.5 μM $MgCl_2$, Hot Start AmpliTaq Gold® 360 Polymerase (5 U/μl), 10X Universal PCR buffer, nuclease-free water (Applied Biosystems, Foster City, CA, USA), 0.7 μM of each primer (Sigma-Aldrich, Poznań, Poland) and 1000 ng of converted DNA. The set of primers for the studied gene was flanking the 1 kb 5' region upstream from the translation start point. Two pairs of primers for MS-PCR were designed to amplify the same fragment of *FHIT* promoter region, according to the criteria described by Feltus *et al* (21) using the online MethPrimer tool (22). The primer sequences and product length are presented in Table II.

The amplification was conducted in a Thermocycler SureCycler 8800 (Agilent Technologies). MS-PCR conditions were as follows: initial denaturation at 95˚C for 5 min, followed by 35 cycles involving denaturation at 95˚C for 45 sec, annealing temperature 52.5˚C for 45 sec and elongation at 72˚C for 1 min; the final elongation at 72˚C for 10 min. In order to evaluate the methylation-specific PCR, positive and negative MS-PCR controls were included. In each experiment, blank sample with nuclease-free water instead of DNA was used as a control for PCR contamination. 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). CpGenome universal methylated and unmethylated DNA were submitted to the bisulfide conversion.

MS-PCR products analysis. In order to analyze the MS-PCR products, the electrophoretic separation was conducted on 2% agarose gel and visualized in UV transilluminator. Products were also analyzed in automated capillary electrophoresis, using DNA1000 LabChip kit on Agilent 2100 Bioanalyzer (Agilent Technologies). Concentrations (ng) and length of MS-PCR products, U and M, were spectrophotometrically estimated using DNA size marker (DNA Ladder; Agilent Technologies). Based on the concentrations results the Methylation Index (MI) was assessed for each sample, using the following formula: peak height of methylated products / (peak height of methylated products + peak height of unmethylated product), MI=(M)/(M+U).

Evaluation of FHIT expression. In order to analyze the *FHIT* gene expression reverse transcription was performed first, using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). 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 RT reaction 100 ng of total RNA was transcribed to complementary DNA (cDNA). RT reaction, in a total volume of 20 μl, was performed in a Thermocycler SureCycler 8800 (Agilent Technologies). The RT reaction conditions were as follows: 10 min at 25˚C, followed by 120 min at 37˚C, then the samples were heated to 85˚C for 5 sec, and held at 4˚C.

The relative expression of *FHIT* gene was conducted on Micro Fluidic Cards - TLDA (TaqMan® Low Density Arrays, Applied Biosystems) in Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). The qPCR mix contained: 50 ng of cDNA diluted to 50 μl in RNAse/DNAse free water, and 50 μl TaqMan Universal Master Mix (Applied Biosystems). The selected assays: Hs00179987_m1 for *FHIT* and Hs00382667_m1 for ESD (esterase D - reference gene) were pre-loaded on the Micro Fluidic Cards. Real-time PCR reaction was processed in program containing 2 min of initial incubation at 50˚C, 10 min at 94.5˚C for polymerase activation, followed by 40 cycles of 30 sec denaturation at 97˚C and 1 min elongation step at 59.7˚C. The *FHIT* relative expression was assessed using the comparative delta-delta CT method in TaqMan Relative Quantification Assay software (Applied Biosystems). ESD RNA expression level served as the reference gene to adjust the gene of interest expression value (RQ - relative quantity). Normal lung tissue RNA was

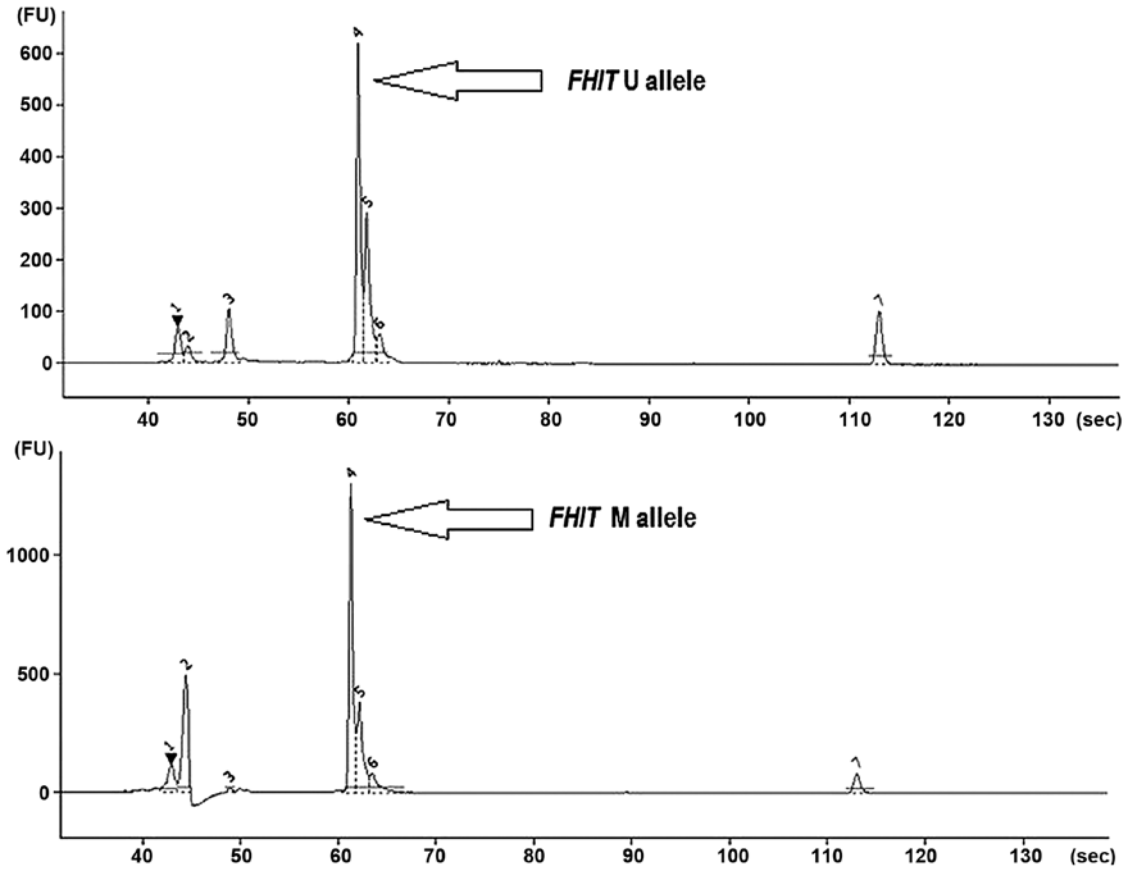

Figure 1. The examples of presence of methylated and unmethylated alleles in cancer tissue of NSCLC patient, SCC subtype (MI=0.79) (Agilent 2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA, USA).

used as a calibrator - Human Lung Total RNA (Ambion®, Life Technologies, CA, USA).

Evaluation of FHIT protein expression. Lung tissue samples (10-40 mg) from 51 patients were rinsed in ice-cold PBS buffer (0.01 mol/l, pH 7.0-7.2) and homogenized in 5 ml of PBS buffer. The resulting suspension was subjected to two cycles of freezing and thawing. Then, the homogenates were centrifuged for 5 min at 5000 x g, the supernatant was removed and the suspension was aliquoted and stored at -80˚C until further analysis. *FHIT* immunoexpression levels in lung tissue homogenates were assessed using ELISA kit for Fragile Histidine Triad Protein (Aviva Systems Biology Corp., San Diego, CA, USA) according to the manufacturer's procedure. The intensity of the final colorimetric reaction, in proportion to the amount of protein bound, was measured in a plate reader (ELx800; BioTek Instruments Inc., Winooski, VT, USA) at the wavelength 450 nm. The obtained results were compared to the standard solutions of known concentrations (100-1000 ng/ml).

Statistical analysis. Statistical analysis was performed using the Statistica for Windows 10.0 software (StatSoft, Cracow, Poland) (v.10). Nonparametrical statistical tests: ANOVA Kruskal-Wallis, Mann-Whitney U test and Spearman's rank correlation coefficient were used in order to evaluate the relationships between gene expression level (RQ), immunoexpression level, gene promoter methylation level (MI) and patient characteristics: age and gender, smoking status (current/former/never smoker), history of smoking measured in pack years (PYs) and clinical features of the tumor (staging according to TNM, AJCC, histopathological NSCLC subtype). The results of relative expression analysis (RQ value), immunoexpression level and gene promoter methylation level (MI) are presented as mean ± SEM and mean ± SD values. Statistical significance for all tests were set at $p<0.05$, and assessed by calculating the p-value.

To identify the parameters associated with *FHIT* immunoexpression, RQ and MI level stepwise logistic regression analysis with backward selection were performed using patient gender, age, history of smoking measured in pack-years (PY), AJCC and histopathological NSCLC subtype as independent variables selected after exclusion of autocorrelated covariates.

Results

Evaluation of FHIT gene promoter methylation. The MS-PCR analysis (gel electrophoresis) revealed the presence of methylated (M) and unmethylated (U) *FHIT* alleles both in NSCLC and control specimens (Fig. 1).

Due to degradations of several DNA samples after bisulfite conversion, methylation levels of 52 NSCLC samples and 31 control specimens (macroscopically unchanged tissues) were assessed in automated capillary electrophoresis (Agilent 2100 Bioanalyzer). The co-presence of U and M alleles was the most common, and this was observed for 43 cancer tissue

Table III. The presence of methylated (M) and unmethylated (U) alleles in histopathological subtypes (SCC, AC, LCC) and paired macroscopically unchanged tissues.

NSCLC subtype	Control	SCC	Control	AC	Control	LCC
n	18	29	9	18	5	5
MI=1 (only M alleles)	5 (28%)	2 (7%)	0	4 (22%)	1 (20%)	-
0<MI<1 (U and M alleles)	11 (61%)	25 (86%)	7 (78%)	13 (72.5%)	4 (80%)	5 (100%)
MI=0 (only U alleles)	2 (11%)	2 (7%)	2 (22%)	1 (5.5%)	-	-
MI value	0.506	0.370	0.315	0.433	0.638	0.314
p-value[a]	p>0.05		0.024		p>0.05	

[a]Spearman's rank correlation coefficient.

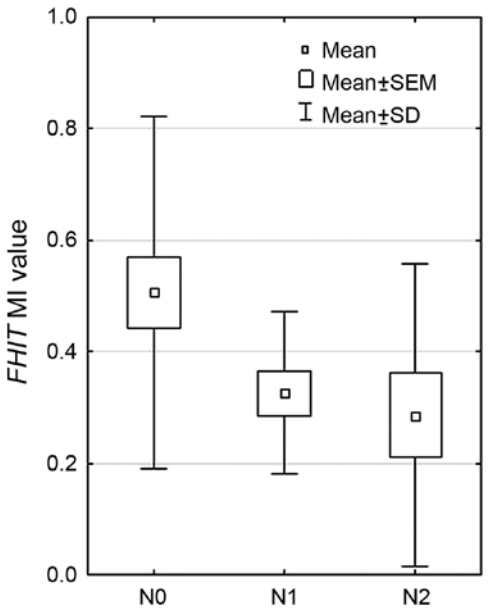

Figure 2. Box and whisker plots representing mean *FHIT* MI values in the groups according to the 'N' value (pTNM staging).

Table IV. *FHIT* expression levels (mean RQ values) in NSCLC subtypes (SCC, AC, LCC) and paired macroscopically unchanged tissues.

	Tissue	n	Mean RQ	p-value
NSCLC group	Cancer	59	1.83	>0.05[a]
	Control tissue	58	1.57	
SCC subtype	Cancer	34	1.40	>0.05[a]
	Control tissue	34	1.59	
NSCC subtype	Cancer	25	2.41	**0.001073[a]**
(AC + LCC)	Control tissue	24	1.55	
AC subtype	Cancer	20	2.27	**0.000153[a]**
	Control tissue	19	1.61	
LCC subtype	Cancer	5	1.81	>0.05[a]
	Control tissue	5	1.30	

[a]Mann Whitney U test.

samples (83%) and for 21 control specimens (67.5%). The presence of only M alleles was detected in 6 cancer tissue samples (11.5%) and 6 controls (19.5%). No methylation (only U alleles) was observed in 4 cancers (5.5%) and 3 controls (11%). Regarding SCC and LCC subtypes, methylated *FHIT* alleles were present more often in macroscopically unchanged tissue when compared to cancer. Only in AC subtype *FHIT* methylation was more frequent in cancer tissue, and the difference was statistically significant (p=0.024, Spearman's rank correlation coefficient). The MI value reflects the observation on the M and U allele frequency ratio in cancer and control groups. Methylation level of *FHIT* (mean MI value) was higher in control tissue (0.472) than in cancer (0.382), but without statistical significance (p>0.05, Spearman's rank correlation coefficient). Data on the presence of M and U alleles in NSCLC subtypes are shown in Table III.

The analysis of MI level between control and cancer tissues in gender groups revealed that higher levels of MI were observed in men vs. women in both tissues: cancer and control (N: 0.416 vs. 0.343; C: 0.555 vs. 0.347, respectively), however the differences did not reach statistical significance (p>0.05, Mann-Whitney U test). No statistically significant difference was observed between gender groups, In NSCLC subtypes (SCC, AC, LCC) the differences in MI values according to the gender groups were also evaluated. Mean MI values in men vs. women were higher in SCC (0.382 vs. 0.302) and AC (0.593 vs. 0.256), and lower in LCC (0.201 vs. 0.389) (p>0.05, Mann-Whitney U test). MI values in cancer tissues were increasing with the patient age (in years), however without statistical significance (p>0.05, Spearman's rank correlation coefficient).

Analysis of MI values in groups according to the AJCC classifications demonstrated higher MI in AJCC I (0.481) than in AJCC II (0.326) or AJCC IIIA/B (0.366), (p>0.05, Kruskal-Wallis test). Mean MI values in groups according to TNM staging were similar in pT1 and pT3/4 (0.446 and 0.442, respectively), and lower in pT2 (0.338) (p>0.05, Kruskal-Wallis test). According to the presence of metastasis, it was observed that mean MI value was decreasing with lymph node involvement (pTNM staging, according to the 'N' value): the highest MI was observed in patient with N0 (0.526), lower in N1 (0.271) and the lowest in N2 (0.222) (p=0.0073, Kruskal-Wallis test),

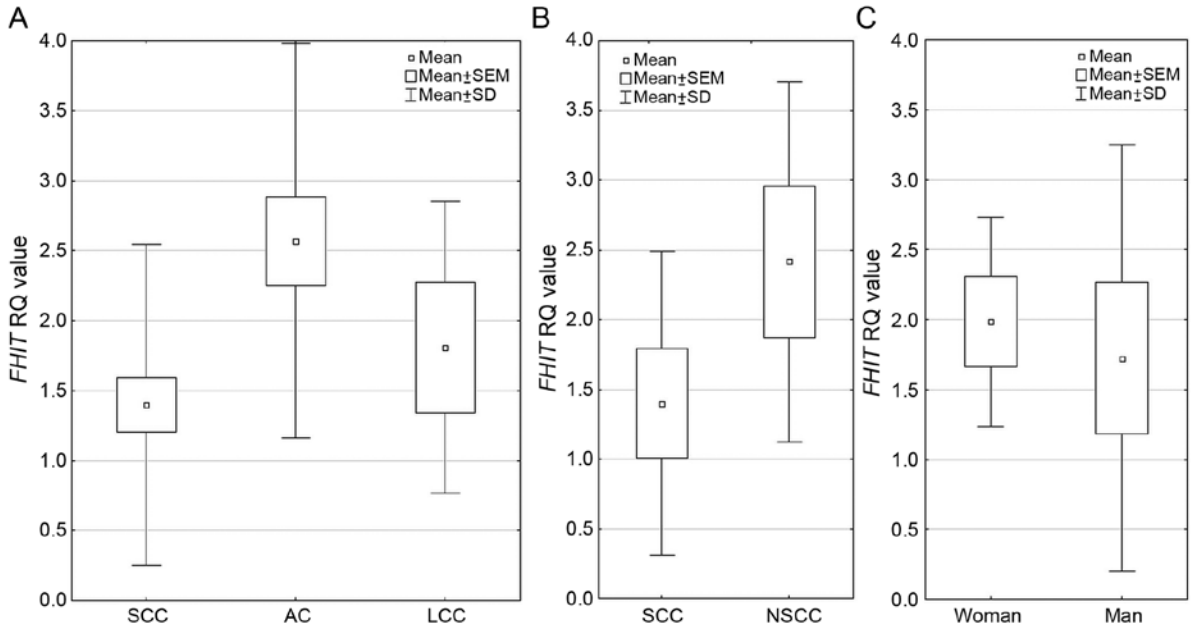

Figure 3. Box and whisker plots representing mean *FHIT* RQ values in the histopathological subtypes (A and B) of the NSCLC and in gender groups (C).

Table V. FHIT immunoexpression levels assessed by ELISA method and percentage of samples with decreased and without decreased expression level in all studied histopathological NSCLC subtypes.

Histopathological NSCLC subtype	Median IE value (ng/ml)	Decreased IE value (range) (ng/ml)	Samples with:	
			Decreased IE value (%)	Non-decreased IE (%)
SCC (n=30)	326	307 (118-493)	18 (60)	12 (40)
AC (n=16)	246	262 (126-409)	14 (88)	2 (12)
LCC (n=5)	235	275 (128-469)	4 (80)	1 (20)
Total (n=51)	246	290 (118-493)	36 (71)	15 (29)

the results are presented in Fig. 2. Significant differences were observed between N0 vs. N1, and N0 vs. N2 groups (p=0.0113 and p=0.008, respectively, Mann-Whitney U test).

No significant relationships were found between *FHIT* MI values (total NSCLC group, NSCLC subtypes, cancer and control tissues) and smoking history (the length of addiction in years, or tobacco intake in PYs) (p>0.05, Kruskal-Wallis test, Mann-Whitney U test, followed by Spearman's rank correlation coefficient). Mean MI value was lower in current smokers (0.345) than in former (0.431) or never-smokers (0.435), but in all control tissue groups the MI values were higher than in cancer (0.381, 0.552, 0.704, respectively) (p>0.05, Kruskal-Wallis test).

FHIT gene expression analysis. The expression of *FHIT* gene, in relation to calibrator sample (RNA from the normal lung tissue), was elevated in all analyzed NSCLC subtypes, and also in macroscopically unchanged tissue. Higher *FHIT* expression level was detected in the tumor (1.83) than the control (1.57) (p>0.05, U-Mann Whitney test). Statistically significant higher RQ levels in cancer when compared to control tissue were observed in AC and NSCC subtypes (p=0.000153 and p=0.001073, respectively, U-Mann Whitney test). The obtained results are presented in Table IV.

Regarding *FHIT* expression among the three NSCLC histopathological subtypes, the difference was statistically significant p=0.000009 (Kruskal-Wallis test) and mean RQ value was the highest in AC group. Analysis performed between 2 histological subtypes SCC vs. NSCC (non-squamous cell carcinoma, comprising of AC and LCC) revealed statistically significant increase in *FHIT* expression in NSCC group (p=0.00001, U-Mann Whitney test). In gender groups, *FHIT* expression was significantly higher in women than in men (1.984 vs. 1.723, respectively; p=0.0351 U-Mann Whitney test). These results are presented in Fig. 3. With increasing age of patients, the relative expression level of *FHIT* was decreasing in cancer tissues (p>0.05, Spearman's rank correlation coefficient).

According to TNM classification, in total NSCLC group, the RQ value increased with tumor size: pT1 (1.457), pT2 (1.789), pT3/4 (2.25), and similar observation was made in NSCLC subtypes - in SCC and NSCC groups, however the

Table VI. Multivariate logistic regression model for *FHIT* immunoexpression coefficients and summary.

Factor	B	Std. Error	Beta	t	p-value	R	R^2	Adjusted R square	Std. Error of the estimate
PY	-0.34	0.15	-80.637	-2.28	0.02	0.34	0.12	0.09	111.69

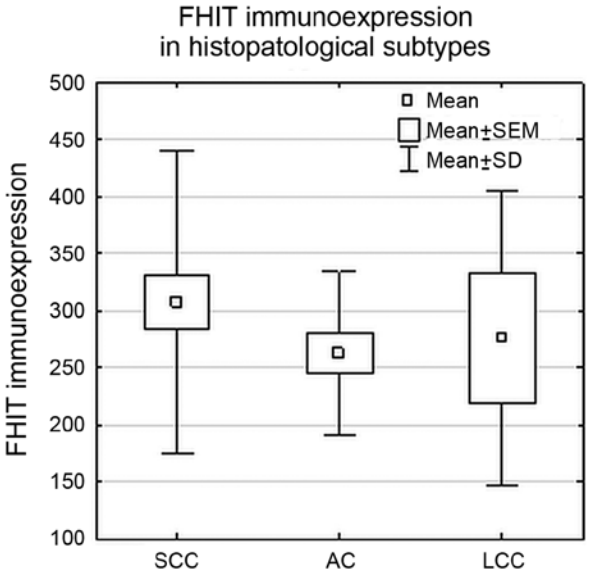

Figure 4. Box-and-whisker plot, representing the FHIT immunoexpression in histopathological subtypes (p>0.05; Mann-Whitney U test) in total NSCLC cohort.

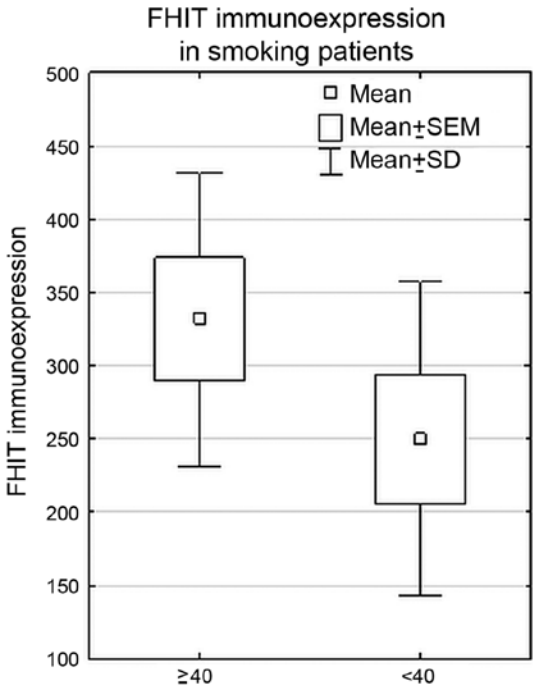

Figure 5. Box-and-whisker plots, representing the FHIT immunoexpression in ≥40 PY and <40 PY groups (p=0.01; Mann-Whitney U test) in total NSCLC cohort.

differences were not significant (p>0.05, Kruskal-Wallis test). In AJCC groups, the mean RQ value was the lowest in AJCC III, and the highest in AJCC II (p>0.05, Kruskal-Wallis test). In relation to smoking history of patients, *FHIT* expression was higher in current smokers (1.96), than in former smokers (1.68) and non-smokers (1.70) (p>0.05, Kruskal-Wallis test). No statistically significant relationships were found between *FHIT* expression (total NSCLC group, NSCLC subtypes) and smoking history (the length of addiction in years, or tobacco intake in PYs) (p>0.05, Kruskal-Wallis test, Mann-Whitney U test). Of note, in the group of active smokers with the highest intake in PYs (>45 PYs) the RQ level was the lowest.

FHIT protein expression analysis. The immunoexpression level of FHIT <350 ng/ml (in tissue homogenates) was considered as decreased. The decreased immunoexpression was observed in 71% of all NSCLC samples, and was in the range of 60-88%, depending on the histotype. The results are presented in Table V.

Statistical analysis did not reveal significant differences in FHIT protein expression levels between studied histopathological subtypes (SCC, AC and LCC) (p>0.05; ANOVA Kruskal-Wallis test) or between SCC and NSCC group (p>0.05; U Mann-Whitney's test), as shown in Fig. 4.

In the whole cohort of NSCLC patients, statistically significant higher FHIT immunoexpression was revealed in the group of heavy smokers (PYs ≥40) (PY <40 vs. PY ≥40, p=0.01, Mann-Whitney U test). Such correlation was also found in SCC subtype (p=0.01, Mann-Whitney U test). The results are shown in Fig. 5.

Multivariate stepwise logistic regression analysis with backward selection revealed that higher protein immunoexpression level was correlated with lower value of PYs smoked in a lifetime. This significant correlation was confirmed in multivariate logistic regression model shown in Table VI. The PYs variable explains only 9% of total immunoexpression variance (R^2=0.09).

There were no statistically significant correlations between FHIT protein levels and the clinical features of the studied NSCLC patients, i.e., patient age, gender, and status of smoking (p>0.05; Mann-Whitney U test, ANOVA Kruskal-Wallis test, followed by Spearman's rank correlation coefficient). Statistical analysis did not reveal any associations between FHIT immunoexpression level and pTNM or AJCC classifications (p>0.05; ANOVA Kruskal-Wallis test).

Correlations between gene expression and methylation or protein expression values. It was observed that in all analyzed groups the mean RQ values were elevated in cancer when compared to control tissue, and MI was lower in cancer, however we did not find any significant correlations (p>0.05, Spearman's rank correlation coefficient). No significant association between MI and RQ values was found among

NSCLC subtypes (SCC, AC, LCC), gender and age groups, tobacco addiction or cancer staging ($p>0.05$, Spearman's rank correlation coefficient). Similarly, there were no statistically significant correlations between *FHIT* expression and protein immunoexpression among NSCLC subtypes (SCC, AC, LCC), gender and age groups, tobacco addiction or cancer staging ($p>0.05$, Spearman's rank correlation coefficient). In addition, there were no statistically significant correlations between FHIT protein immunoexpression and MI levels ($p>0.05$, Spearman's rank correlation coefficient).

Discussion

FHIT, the tumor suppressor gene localized on 3p fragile site (3p14.2), is frequently altered in many human cancers (renal, lung, gastric, lymphomas) (7,12). *FHIT* expression loss was detected frequently during the early onset of disease progression in cancer (14,23). Loss of function of the *FHIT* gene can lead to constitutive accumulation of high levels of intracellular diadenosine tetraphosphate and the stimulation of DNA synthesis and proliferation (25,26). Reduction of *FHIT* expression is consider as poor diagnostic factor correlated with tumor aggressiveness due to the epithelial-mesenchymal transition (EMT) (16,26-28). The EMT is considered as crucial step in the early stage of cancer metastasis. Activation of *FHIT* gene can enhance the cell ability to enter apoptosis and to inhibit cell growth (29). Several studies have underlined the putative function of *FHIT* gene as lung cancer biomarker (12,18). In mouse lung cancer model *FHIT* function was linked with protecting against chemically-induced cancerogenesis (30). In lung cancer cell lines restoration of *FHIT* expression resulted in induction of apoptosis and tumorigenicity suppression, therefore the gene was proposed as potential agent in targeted gene therapy (11).

In the present study, we assessed *FHIT* expression on mRNA and protein level, as well as gene promoter methylation. Analysis was performed in primary lung lesions and in macroscopically unchanged lung tissues to deepen the knowledge of potential significance of *FHIT* as an early diagnostic biomarker. Searching for such biomarkers is very important, especially when lack of effective diagnostic tools at the early stage of the disease can cause up to 85% mortality rate (in 5 years) (2).

In the previously conducted studies, the presence of *FHIT* alteration (LOH, expression alteration) was detected in preneoplastic bronchial lesions (12). Our study is the first one where both mRNA expression level and gene methylation status were analyzed in cancer and macroscopically unchanged lung tissue. We demonstrated that altered gene expression was not only characteristic for cancer, we also observed increased gene expression both in NSCLC tissue and macroscopically unchanged lung tissue from the same patients.

In our study we found elevated *FHIT* expression in all NSCLC subtypes which is contrary to the results of other groups (14,23,26,31). Also, in our study, *FHIT* expression level in macroscopically unchanged tissue, regarded as 'normal', although surrounding the primary lesion, was elevated, when compared to calibrator RNA. This can stand for the hypothesis that *FHIT* activation in lung carcinogenesis process is a response to accumulation of genetic changes in the cells (12,32). The differences in *FHIT* expression between samples from the same patient could suggest the important role of *FHIT* gene in a very early stage of lung carcinogenesis.

Also in methylation analysis we demonstrated promoter region hypermethylation in both tissues. This finding is inconsistent with the results of Feng *et al*, who found no methylation in cancerous or non-cancerous tissues (33). In our study, in case of AC subtype, *FHIT* promoter methylation status was significantly higher than in normal tissue.

Statistically significant differences in the *FHIT* expression between histopathological subtypes AC, LCC and SCC could suggest the possibility to deepen the study on the gene as a differentiating marker for NSCLC subtypes. Additionally, it might help in the selection of therapy. Differences between expression in subtypes (the lowest in SCC, the highest in AC) may be under consideration as NSCLC prognostic marker. Unfortunately, these differences between subtypes were not observed in the methylation analysis.

Increased expression of *FHIT* gene, identified in the current work, would suggest the resulting elevation of its product the FHIT protein. However such result was not confirmed. The analysis of immunoexpression revealed the reduction of FHIT protein level in NSCLCs tissue samples, which was generally consistent with earlier results (23,25,31,32,34,35). We observed FHIT protein reduction in 71% of NSCLCs, and there was no difference between the histopathological subtypes (88% AC, 60% SCC). It is contrary to the findings of other authors, who reported significant loss of protein expression mainly in SCC (25,34). Tomizawa *et al* (34) described the decreased level of FHIT expression in only 10% of AC samples in comparison to 86% of SCCs. The possible reason of the inconsistency between our results and the compared reports could be different material and molecular techniques used to evaluate FHIT immunoexpression. We assessed it in tumor tissue homogenates, while others used paraffin-embedded tissue blocks (17), thus the data cannot be compared reliably. On the other hand, such divergent results suggest poor usage of FHIT immunoexpression as a differentiating marker, that has been recently confirmed in the review article by Lindskog *et al* (36).

Many studies revealed that loss of *FHIT* expression or immunoexpression was significantly associated with tumors occurring in heavy smokers (24,25,32,35,37). We have not confirmed this on mRNA level - neither in relation to the length of smoking or to the amount of cigarettes smoked. Interestingly, we found significant differences on protein level: in heavy smokers group FHIT protein level was significantly higher (in total NSCLC group and in SCC group). Sozzi *et al* (25) obtained the opposite results, the protein expression decreased with heavy smoking.

This discrepancy can be explained by the results of multivariate regression model showing that only 9% of FHIT immunoexpression variance can be explained by the number of PYs. Such low contribution of smoking history to the FHIT protein level in our study suggest the impact of other, probably more important factors on FHIT protein reduction. Several other possible explanations could be considered, including the method used. In our study we analyzed FHIT immunoexpression with ELISA method in cancer tissue homogenates, not immunohistochemically stained paraffin-embedded tissues. The methodological difference might be also due to the

contamination of tumor specimens with non-cancerous cells, because the tumors analyzed in this study were macrodissected and not microdissected.

In many studies it was confirmed that *FHIT* activation or enforced expression significantly suppressed metastasis, accompanied by inhibition of EMT (26,27,28). We demonstrated statistically significant differences in gene methylation status according to the node infiltration status (TNM staging) in the group N0 vs. N1 and N2. Lower gene methylation in groups of patients with nodes infiltration could be due to the actions taken by the cell leading to growth inhibition, as FHIT plays role in EMT inhibition. However, these lower methylation levels did not correlate with expression enhancement. In Suh *et al* study (28), the elevated expressions of *FHIT* gene and *FHIT*-dependent miR-30c were proposed as metastasis predictor, as patients with elevated expression had improved metastasis free-survival. In our study the observed difference in *FHIT* methylation level between patients with and without nodes infiltration could be considered as prognostic marker.

In conclusion, we demonstrated the presence of *FHIT* promoter methylation both in cancer and control tissue and altered gene expression in both tissue types. It is worth mentioning, that our results give information on methylation level of gene promoter, not only the presence of methylated gene. This can give deeper insight into epigenetic landscape of the lung cancer tissue. The presence of gene promoter hypermethylation both in cancer and control tissue and in different TNM groups suggests early involvement of epigenetic alterations in the development of NSCLC. Differences in *FHIT* methylation status between NSCLC patients with and without nodes infiltration seems to be considered as prognostic marker. However, these findings do not confirm the observations of other scientist, especially we did not prove the negative correlation between *FHIT* expression and methylation. Reassuming, the results of our study indicate the value of *FHIT* gene expression as a differentiating marker of histopathological subtypes of NSCLC. Ambiguous results concerning relationship between *FHIT* protein level and the amount of cigarettes smoked in a lifetime, suggest unclear impact of smoking on this particular gene. Results of our study indicate that the observed level of *FHIT* promoter methylation was not enough to suppress gene expression. Lack of negative correlation between *FHIT* expression and methylation, or positive correlation between gene expression and immunoexpression suggest the role of another molecular mechanisms regulating FHIT expression on mRNA and protein levels in NSCLC patients.

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