SHORT COMMUNICATION

Expression of selected miRNA, *RARβ* and *FHIT* genes in BALf of squamous cell lung cancer (squamous-cell carcinoma, SCC) patients: a pilot study

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Abstract

Two suppressor genes which often undergo epigenetic silencing during the early stages of lung carcinogenesis are those encoding retinoic acid receptor-β (*RARβ*) and Fhit protein (*FHIT*). *RARβ* expression is regulated by miRNA-34a and miRNA-141, and *FHIT* expression by miRNA-143 and miRNA-217. The aim of the study was to assess how selected miRNAs regulate the expression of their targeted genes in bronchoalveolar lavage fluid (BALf), obtained from patients with SCC of the lung. It also examines the relationship between the genetic findings and the clinical and pathomorphological features of the tumor. A total of 50 BALf samples were taken: 25 from patients with SCC and 25 from healthy donors. The expression (RQ) of the selected genes was analyzed by qPCR, as well as the miRNA level, with a particular emphasis on the relationship between the expression of the genes themselves and their corresponding miRNAs; in addition, the expression of the genes and miRNAs were compared with the pathomorphological features of the tumor and the clinical features of patients. Analysis of the RQ values showed downregulation of *RARβ, FHIT* and miRNA-34a and increased expression of miRNA-141, miRNA-143 and miRNA-217 in all BALf samples (P > 0.05). No correlation was found between the expression of the selected genes and corresponding miRNAs, history of smoking, cancer stage, age and sex of the patients. The presence of the selected genes and miRNAs in BALf material does not seem to have diagnostic potential in patients with SCC; however, the results should be verified on a larger group of patients.

Keywords miRNA · FHIT · RARbeta · Lung cancer · Squamous cell lung cancer

Introduction

Among all deaths due to malignant neoplasia, lung cancer is a common cause, with a 5-year survival rate of around 15%.

Many studies indicate that lung cancer has a strong genetic basis, with the term "genetic landscape of lung cancer" being used increasingly often. NSCLC development is known to be associated with molecular alterations in more than 20 critical genes. These genes belong to the tumor suppressor gene (TSGs) family and changes in their regions of the chromosome are typically regarded as the beginning of carcinogenesis. Loss of gene expression requires the loss of function of both alleles of a gene. While loss of heterozygosity (LOH) is one of the most frequent causes of single allele dysfunction, inactivation of the second allele typically requires the occurrence of mutations or epigenetic alterations such as gene promoter methylation or miRNA regulation [1].

The present study examines the activity of *FHIT* and *RARβ*: two genes whose protein products play an important role in the carcinogenesis of many cancers, including lung cancer.

The fragile histidine triad (*FHIT*) and retinoic acid receptor beta (*RARβ*) genes are located on the short arm of third chromosome (3p). This area is known as a "hot spot mutation site" as it is a common location for molecular abnormalities in lung cancerogenesis. *FHIT* encodes an enzyme involved in purine metabolism, which plays a role in apoptosis and the suppression of tumor metastasis. *FHIT* expression is reduced during the early stage of lung cancer, and

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hence the gene has been proposed as a marker for predicting NSCLC outcome [2]. This downregulation of *FHIT* has also been correlated with rapid progression, especially in lung SCC cells [3]. The *RARβ* gene encodes a receptor which binds retinoic acid, the biologically- active form of vitamin A. The protein mediates cellular signaling in embryogenesis, cell growth and differentiation. [4].

Lung cancer has been found to have its own miRNA profile [5]. During cancerogenesis, miRNAs related to cancer are aberrantly expressed and act as tumor suppressors or oncogenes (TSGs). The particular miRNAs that regulate these TSGs play a significant role in the development and progression of human malignancies, including NSCLC [6], and their activity can provide an insight into the mechanisms behind the regulation of *FHIT* and *RARβ* gene expression.

miRNA-34a regulates tumor cell apoptosis, and one very promising approach in NSCLC therapy involves the restoration of its correct function [7]. miRNA-141 is known as a predictive marker in NSCLC as it regulates sensitivity to cisplatin [8]. Apart from its prognostic value, miRNA-141 overexpression correlates with the more advanced stage of NSCLC and has potential as a diagnostic molecular biomarker. There are only a few studies concerning roles of miRNA-143 and miRNA-217 in NSCLC. Depending on the tissue cell type, miRNA-217 can act as an oncogene or a TSG [9].

The miRNA profile is valuable during diagnostic process in the subclassification of NSCLC [10]. However, no study has so far evaluated the use of miRNA analysis for discriminating between subtypes of lung carcinoma in bronchial alveolar lavage fluid (BALf).

The aim of the study was to determine how selected miRNAs regulate the expression of their associated TSGs (*FHIT, RARβ*) in BALf obtained from patients with SCC of the lung. It also examines the relationship between genotype and the clinical and pathomorphological features of the tumor.

Patients and methods

Clinical characterization of the patients

The investigations were carried in accordance with the 1975 Declaration of Helsinki, revised in 2013.

The study protocol was approved by the Ethical Committee of the Medical University of Lodz, Poland, agreement no. RNN/89/15/KE. All participating patients were fully informed. Written informed consent was obtained from each patient.

Relevant clinical and pathological characteristics of the patients with NSCLC included in the study are summarized in Table 1.

Table 1 Clinical and pathological features of the studied SCC group

Analyzed variables	SCC patients (%)	
	Sample (n=25)	Control (n=25)
Gender		
Man	12 (48)	15 (60)
Woman	13 (52)	10 (40)
Mean age (range), years		
Man	66 (54–73)	58 (32–83)
Woman	69 (53–82)	55 (26–83)
AJCC*		
AJCC IA/IB	1 (4)	
AJCC IIA/IIB	7 (28)	
AJCC III A/IIIB	10 (40)	
pTNM*		
T1	1 (4)	
T2	7 (28)	
T3-4	17 (68)	
Cigarette addiction		
Active smokers	10 (40)	0 (0)
Former smokers	15 (60)	5 (20)
Non-smokers	0 (0)	20 (80)
Packyears (PYs)		
do 40 PYs	10 (40)	5 (100)
≥ 40 PYs	15 (60)	0 (0)

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

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

The control group included 25 healthy non-smokers; all had been admitted to the hospital for routine diagnostic due to chronic cough of unknown origin or the presence of undefined changes on a chest X-ray. If the diagnostic process did not show any abnormalities, the patients were defined as healthy subjects.

Bronchoscopy and bronchoalveolar lavage fluid (BALf) collection

Bronchoscopy was performed with a flexible bronchoscope (Pentax, Tokyo, Japan). Patients received topical anesthetic in the nasal passages (using 2% lidocaine) and the oral mucosa (using xylocaine). BAL fluid (BALf) was taken from the medial lobe by administration followed by aspiration 4×50 ml 0.9% NaCl.

RNA extraction, miRNA extraction, real-time PCR (qPCR method)

RNA isolation was performed with the use of a Universal RNA Purification Kit (Eurx, Poland) and mirVana™ miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's protocol.

Evaluation of *FHIT, RARβ* and miRNA extraction

The relative expression of gene was assessed by qPCR reactions using Custom TaqMan® Array Gene Cards and (Applied Biosystems, Carlsbad, CA) and TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The following gene probes were used for the study: catalogue numbers *FHIT*—Hs00179987_m1; *RARß*—Hs00977140_m1 and *ACTB* Hs99999903_m1) and microRNA probes: catalogue numbers hsa-miR-34a 000426, hsa-miR-141 002145, hsa-miR-143 002249 and hsa-miR-217 002337. An Applied Biosystems 7900HT Fast Real- Time PCR System machine was used in the experiment and samples were run in triplicate. All data were analyzed by SEQUENCE DETECTION SYSTEM 2.0 (Applied Biosystems) using TaqMan probes for the genes *FHIT, RARβ* and *ACTB* used as an endogenous control. Relative expression was calculated according to the Ct method $2^{-\Delta\Delta Ct}$, with the adjustment to the β-actvb adjusted to the level of expression of RNU6B RNA (endogenous cin kyexpression level and in relation to the expression level of calibrator (Human Lung Total RNA Ambion®), for which RQ value was equal to 1. The expression levels (RQ values) of the studied miRNA were calculated using the $2^{-\Delta\Delta Ct}$ method, adjusted to the level of expression of RNU6B RNA (endogenous control) and the calibrator (Human Lung Total RNA Ambion®), which was assigned an RQ value of 1.

Statistical analysis

Statistical analysis was performed using Statistica for Windows 10.0 software (StatSoft, Cracow, Poland) (v.10). Normally-distributed variables were summarized as means with standard deviations, while non-normally distributed or ordinal variables were presented as medians and 25–75 percentile ranges. Normality was evaluated using the Shapiro–Wilk test. Outcome variables, due to their ordinal nature, were compared using non-parametric tests: the Mann–Whitney U-test was used for two-group comparisons, Spearman's rank correlation test for correlation assessment, or the Kruskal–Wallis test for multiple group comparisons. The results of relative expression analysis

Table 2 *FHIT* and *RARβ* expression levels (mean RQ values) in SCC subtype and paired macroscopically-unchanged tissues

Gene	Sample	N	Mean RQ	*P* value
FHIT	Cancer	25	0.75	0.62
	Control	25	1.01	
RARβ	Cancer	25	0.61	0.76
	Control	25	0.98	

Table 3 miRNA expression levels in BALf (mean RQ values) and paired macroscopically-unchanged tissues from the same patient, according to SCC subtype

Gene	Sample	n	Mean RQ	P-value
miRNA-141	Cancer (SCC)	25	1.56	0.21
	Control	25	0.97	
miRNA-143	Cancer (SCC)	25	1.23	0.64
	Control	25	0.95	
miRNA-217	Cancer (SCC)	25	1.75	0.48
	Control	25	1.02	
miRNA-34	Cancer (SCC)	25	0.67	0.66
	Control	25	1.03	

(RQ value) are presented as median values. Statistical significance was determined at $P < 0.05$.

Results

Evaluation of *FHIT* and *RARβ* gene expression in SCC patients versus controls

FHIT and *RARβ* expression was elevated in comparison to the control group ($P > 0.05$, U-Mann–Whitney test). Data presented in Table 2.

No statistically significant relationships were found between *FHIT* and *RARβ* expression and the following parameters: smoking history (the length of addiction in years, or tobacco intake in PYs), grade of SCC (according to TNM and AJCC), patient age or gender ($P > 0.05$, Kruskal–Wallis test, Mann–Whitney U-test).
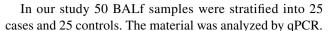
Relative expression of the studied miRNAs in SCC patients versus controls

miRNA-34 expression was lower in the patient group than in the control group ($P > 0.05$, U-Mann–Whitney test). However, miRNA-141, miRNA-143 and miRNA-217 expression was increased in all BALf samples ($P > 0.05$, Mann–Whitney U-test). Data presented in Table 3.

Relative expression of the studied miRNAs in SCC patients according to clinical and pathological classification

Statistically significant relationships were found between miRNA expression and smoking history (the length of addiction in years, or tobacco intake in PYs), grade of SCC (according to TNM and AJCC), patient age and gender (P > 0.05, Kruskal–Wallis test, Mann–Whitney U-test).

Correlations between genes and miRNA expression values

No correlations were found between the expression of the analyzed miRNAs and selected genes (P > 0.05, Spearman's rank test).

Discussion

The development of many cancers, including lung cancer, is commonly associated with a loss of function of tumor suppressor genes. Greater understanding of the genetic background of cancer has allowed the creation of personalized targeted therapy. In the case of lung cancer, such treatment is proposed to patients with adenocarcinoma and squamous cell lung cancer whose histopathology material reveals the presence of targeted mutations [11]. Targeted treatment allows many serious complications of classical chemotherapy to be avoided, and significantly improves survival in advanced stages of lung cancer. Hence there is a great need for studies focused on the molecular basis of the development and progression of this aggressive cancer, particularly less invasive ones intended to identify early diagnostic markers in material such as BALf.

Although routine lung cancer diagnosis consists of radiological and microscopic examinations, the final diagnosis of lung cancer is typically based on the analysis of material taken from the lung tumor itself, such as lung tumor tissue, a cytology probe obtained by biopsy, bronchial brushings or BALf. Few studies have examined the molecular alterations in BALf taken from patients with advanced lung cancer. This lack of literature be associated with the difficulty faced in collecting such material in the advanced stage of lung cancer: during the present study, the procedure frequently appeared to be poorly tolerated by the patients, and some probes could not be used for genetic analysis due poor material recovery after fluid injection and low cell amount. Additionally, differences in methodology prevent straightforward comparisons with other findings.

In our study 50 BALf samples were stratified into 25 cases and 25 controls. The material was analyzed by qPCR.

It was found that *FHIT* and *RARß* expression levels were decreased; similarly, previous studies also indicate that these genes were downregulated in NSCLC tissue compared to normal samples [12]; however, the present study used BALf as source material. Although the present study only examined SCC patients, previous studies have also found *FHIT* assessments to display differentiating potential, with the least expression observed in cases of SCC [13].

The present study may have limitations associated with the choice of population group; for example, some studies on the diagnostic role of *FHIT* have been performed in Asian populations with a high prevalence of adenocarcinoma. As such, their results undermine the diagnostic value of *FHIT* in Caucasian population [14]. The present study concerns only the Caucasian population, where the SCC subtype was the most common. The presence of such differences between populations complicates any comparison between and may cause the discrepancies in the results of our work.

By gaining a better understanding of the role played by miRNAs in the course of disease, it will be possible to develop new diagnostic biomarkers and individualized therapeutic tools. Of the miRNAs used in the present study, miRNA-34a and miRNA-141 are known to regulate the expression of *RARß* while miRNA-143 and miR-217 influence that of *FHIT*. These mechanisms play an important role in the development of a range cancers and offer promise as potential diagnostic and prognostic markers in some types of lung cancer [15].

Our results indicate higher expression of miRNAs all SCC BALf samples apart from that of miRNA-34a, which was decreased.

Previous studies have also identified overexpression of miRNA-141 and miRNA-217 in tumor tissue [8]. Interestingly, upregulation of miRNA-217 was associated with an advanced status of NSCLC. Contrary to our present findings, miRNA-143 expression has been found to be downregulated in NSCLC [5, 16–18]. These discrepancies might be explained by differences between populations used for the studies and the fact that the present study was restricted only to the SCC histologic subtype.

Although no correlations were found between alterations in the expression of the studied miRNAs with regard to particular stages of SCC in the present study, Kim et al. (2015) propose the use of miRNA-143 for early detection NSCLC [19]. Other studies also describe panels of miRNAs that were significantly up-regulated in patients with lung cancer in BALf samples, confirmed its potential as a diagnostic biomarker in lung cancer [20].

The considerable variation between the studies described above confirm that the methods used for molecular and genetic analysis are not well standardized, particularly on

unusual biological material, such as BALf. Hence, it must be remembered when comparing studies that a "methodological bias" may well exist that can significantly affect the quality of the results.

Conclusions

The levels of the studied miRNAs appear to have no direct influence on the regulation of *RARβ* and *FHIT* expression in the BALf of SCC patients.

Therefore, miRNAs level and genes expression in BALf do not seem to be suitable diagnostic biomarkers in SCC; however, these tests should be repeated on a larger study group. Further molecular studies are needed, especially on less invasive material, to determine their clinical significance in the development of lung cancer.

Compliance with ethical standards

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