CLINICAL-DIAGNOSTIC STUDIES

EXPRESSION OF AhR-REGULATED miRNAs IN NON-SMALL CELL LUNG CANCER IN SMOKERS AND NEVER SMOKERS

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Smoking is a risk factor for non-small cell lung cancer (NSCLC). The most common subtypes of NSCLC are lung adenocarcinoma (LAC) and squamous cell carcinoma (SCC). The cigarette smoke contains aryl hydrocarbon receptor (AhR) ligands, such as benzo(a)pyrene (BaP). By activating the AhR, BaP can change the expression of many genes, including miRNA-encoding genes. In this study, we have evaluated the expression of few miRNAs potentially regulated by AhR (miR-21, -342, -93, -181a, -146a), as well as *CYP1A1*, a known AhR target gene, in lung tumor samples from smoking (n=40) and non-smoking (n=30) patients with LAC and from smoking patients with SCC (n=40). We have also collected macroscopically normal lung tissue >5 cm from the tumor margin. We compared the obtained data on the miRNA expression in tumors with data from The Cancer Genome Atlas (TCGA). We found that in 76.7% of non-smoking LAC patients, *CYP1A1* mRNA was not detected in tumor and normal lung tissues, while in smoking patients, *CYP1A1* expression was detected in tumors in almost half of the cases (47.5% for SCC and 42.5% for LAC). The expression profile of AhR-regulated miRNAs differed between LAC and SCC and depended on the smoking status. In LAC patients, the expression of oncogenic miRNA-21 and miRNA-93 in tumors was higher than in normal lung tissue from the same patients. However, in SCC patients from our sample, the levels of these miRNAs in tumor and non-transformed lung tissue did not differ significantly. The results of our studies and TCGA data indicate that the expression levels of miRNA-181a and miRNA-146a in LAC are associated with smoking: expression of these miRNAs was significantly lower in tumors of smokers. It is possible that their expression is regulated by AhR and AhRR (AhR repressor), and inhibition of AhR by AhRR leads to a decrease in miRNA expression in tumors of smoking patients. Overall, these results confirm that smoking has an effect on the miRNA expression profile. This should be taken into account when searching for new diagnostic and therapeutic targets for NSCLC.

Key words: aryl hydrocarbon receptor; benzo(a)pyrene; non-small cell lung cancer; microRNA

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INTRODUCTION

Arylhydrocarbon receptor (AhR) is a ligand-activated transcription factor belonging to the bHLH/PAS family of proteins involved in regulation of many physiological processes [1]. AhR was originally identified as a receptor binding the environmental toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related toxic halogenated aromatic industrial compounds [2]. The results of numerous studies have shown that this receptor binds structurally diverse ligands of both foreign and endogenous nature [3]. Moreover, the biological effect of such interactions depends on the specific receptor-binding ligands [4]. Signaling pathways regulated by AhR are activated in various tumors; they influence the main stages of oncogenesis — initiation, promotion, progression, and metastasis [5, 6]. The role of AhR signaling pathways in lung carcinogenesis attracts much attention due to the significant human exposure to cigarette smoke and other environmental pollutants, which are potential AhR ligands [7]. Smoking is the most important risk factor for the development of non-small cell lung cancer (NSCLC), especially squamous cell carcinoma (SCC): about 90–95% of cases are associated with tobacco smoking. There are 510 polycyclic aromatic hydrocarbons (PAHs) reported in cigarette smoke, including 16 PAHs that have been classified by the International Agency for Research on Cancer as carcinogenic to humans [8]. One such carcinogenic compound is benzo(a)pyrene (BaP), which can activate AhR. According to a study conducted on Canadian cigarette brands in 1992, the BaP content in the smoke from a single cigarette could vary from 3.36 ng to 28.39 ng [9]. Therefore, it can be expected that smoking and concomitant exposure to BaP in humans leads to changes in the expression of many AhR target genes in lung tissues.

Using genome-wide analysis, numerous AhR binding sites have been identified in human DNA. Recently performed comparison of genome-wide binding profiles of AhR and AhRR (AhR repressor) in human breast cancer MCF-7 cells treated with TCDD [10] resulted in identification of 3915 binding sites for AhR and 2811 binding sites for AhRR; 974 binding sites (35%) were common for both factors. However, functionality of the identified sites remains an issue for further research.

Besides protein coding genes AhR can regulate expression of microRNAs (miRs, miRNAs) — small RNAs 18–22 nucleotides in length; dysregulation of their expression can contribute to carcinogenesis [11]. Previously, using *in silico* methods, we searched for AHRE elements (AhR binding sites) in the genomes of rats, mice, and humans and found at least 154 putative miRNA promoters containing AHRE sequences in the human genome [12]. However, the role of miRNAs regulated by AhR in NSCLC and the relationships between the expression profile of such miRNAs and the smoking status have not been fully studied. Thus, the aim of this study was to determine the expression of AhR-regulated miRNAs in tumor samples from smoking and non-smoking patients with NSCLC, to confirm the association of their expression profile with smoking and to identify new potential therapeutic targets in lung cancer. We have also performed an *in silico* study based on TCGA data on the expression of these miRNAs in malignant lung tumors.

MATERIALS AND METHODS

Tissue Samples

A biocollection of 110 pairs of samples of tumors and non-transformed human lung tissues was collected at the Novosibirsk Regional Oncology Center in the 3rd Oncology (thoracic) Department. Lung tissue samples were obtained during thoracoscopic lung resection from male patients who did not undergo neoadjuvant chemotherapy. Macroscopically unchanged (conditionally normal) lung tissue was taken at a distance of at least 5 cm from the tumor margin. This method of selecting control tissue is widely used in scientific work and makes it possible to identify genes with altered expression profile, which are associated with carcinogenesis [13–15]. A total of 40 pairs of samples were collected from smoking patients with SCC and lung adenocarcinoma (LAC) and 30 pairs of samples from never-smoking patients with LAC. Samples of macroscopically normal and tumor lung tissue were placed in RNA stabilization solution and stored at -20°C.

Isolation of miRNAs

miRNAs were extracted from samples by thermo-induced lysis with guanidine isothiocyanate according to a previously published protocol [16].

Real-Time RT-PCR

Relative miRNA expression levels were measured using real-time reverse transcription-PCR. The reverse transcription reaction was carried out using stem-loop primers [17] and a commercial kit RT-M-MuLV-RH (BiolabMix, Russia). Real-time PCR was performed using the BioMaster UDG HS-qPCR (2×) reaction mixture (BiolabMix). The CFX96™ detection system (Bio-Rad Laboratories, USA) was used to detect PCR products. Small nuclear RNAs U44 and U48 were used to normalize the data. Primers for reverse transcription are given in Table 1. The oligonucleotides given in Table 2 were used for PCR.

Each sample was analyzed in triplicate. The fold change of each miRNA was calculated using the threshold cycle method ($2^{-\Delta\Delta Ct}$).

RNA Isolation, cDNA Synthesis, and Real-Time PCR

RNA was isolated using the TRIzol™ reagent (Invitrogen, USA) according to the manufacturer's recommendations. RNA integrity was monitored by agarose gel electrophoresis. Concentration and purity were determined spectrophotometrically at 260 nm and 280 nm using an Agilent-8453 spectrophotometer (Agilent Technologies, USA). Reverse transcription was performed using the OT-M-MuLV-RH kit (BiolabMix) according to the manufacturer's recommendations. 1 μg of RNA was taken per reaction. The resultant cDNA was used to determine the levels

Table 1. Primer sequences for reverse transcription of miRNA

miRNA	Primer sequences
U48	5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACGGTCAG-3′
U44	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCAGTT-3′
miR-21	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAACATC-3′
miR-342	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACGGGTG-3′
miR-181a	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTCACCG-3′
miR-146a	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGCCTA-3′
miR-93	5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCTGC-3′

Table 2. Primer sequences for real-time PCR for evaluation of miRNA expression

miRNA	Primer sequences	
U44	Forward	5′-GCCGCTCTTAATTAGCTCT-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACAGTCAGTT-(BHQ1)-3′
U48	Forward	5′-GAGTGATGATGACCCCAGGTAA-3′
	Reverse	5′-GTGCAGGGTCCGAGGT-3′
	Probe	5′-(R6G)-TTCGCACCAGAGCCAACGGTCAG-(BHQ1)-3′
miR-21	Forward	5′-GCCGCTAGCTTATCAGACT-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACTCAACATC-(BHQ1)-3′
miR-342	Forward	5′-GCCGCTCTCACACAGAAATCG-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACACGGGTGC-(BHQ1)-3′
miR-181a	Forward	5′-GCCGCAACATTCAACGCTGT-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACACTCACCG-(BHQ1)-3′
miR-146a	Forward	5′-GCCGTGAGAACTGAATTCCA-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACACAGCCTA-(BHQ1)-3′
miR-93	Forward	5′-GCCGCCAAAGTGCTGTTCGT-3′
	Reverse	5′-AGTGCAGGGTCCGAGGTA-3′
	Probe	5′-(R6G)-TTCGCACTGGATACGACCTACCTGC-(BHQ1)-3′

of *AHR* and *CYP1A1* mRNAs by real-time PCR using the BioMaster HS-qPCR SYBR Blue (2×) reaction mixture (BiolabMix) on a CFX96™ detection system (Bio-Rad Laboratories). *18S* and *POLR2A* were used as normalization genes. The following specific primers were used:

AHR 5′-GTCGTCTAAGGTGTCTGCTGGA-3′, 5′-CGCAAACAAAGCCAACTGAGGTG-3′;
CYP1A1 5′-GGTCAAGGAGCACTACAAACC-3′, 5′-TGGACATTGGCGTTCTCAT-3′;
POLR2A 5′-GCATGGCAGAGGAGTTTCGGCT-3′, 5′-ATTTCCCCGGGATGCGCAATGG-3′;
18S 5′-CGGCTACCACATCCAAGGAA-3′, 5′-GCTGGAATTACCGCGGCT-3′.

The optimal concentration of each primer was 300 nM.

Each PCR reaction was performed using 0.3 μl of cDNA in a final volume of 20 μl under the following conditions: initial denaturation at 95°C for 5 min, then 40 cycles: denaturation at 95°C for 15 s, annealing at 60°C for 20 s, elongation at 72°C for 30 s with fluorescence reading. PCR specificity was monitored by melting curves. Samples were analyzed in three technical replicates. Relative gene expression levels were assessed using threshold cycle (Ct) values taking into account reaction efficiency (E) for the gene of interest and the normalization gene.

Bioinformatics Analysis

miRNA-21, miRNA-342, and miRNA-93 were selected according to data from a previously performed bioinformatics analysis [12]. In addition, using the Harmonizome database [18], which contains information on the results of ChIP analyses, miRNA-181a and miRNA-146a have been also included in this study.

The information contained in the TCGA database on the expression of the genes *CYP1A1*, *AHRR*, and AHR-regulated miRNAs has been analyzed using the UALCAN resource [19].

Statistical Analysis

Statistical data analysis was performed using the STATISTICA v.12 program. Data are presented as median values. Statistical analysis was performed using the nonparametric Mann-Whitney U test. Differences were considered statistically significant at $p<0.05$.

RESULTS

Expression of CYP1A1 and AhR in Tumor Tissues of Patients with NSCLC

First of all, we performed a comparative analysis of the expression of the AhR target gene, *CYP1A1*, in tumor and conditionally normal tissues of smoking

and non-smoking patients with LAC and smoking patients with SCC. In almost half of the cases (47.5% for SCC and 42.5% for LAC), *CYP1A1* mRNA was detected in the tumor tissue of smoking patients (Table 3). However, in all tumor tissue samples from non-smoking patients with LAC no expression of *CYP1A1* was detected. In 23.3% of non-smoking patients with LAC, the cytochrome gene expression was recorded, but only in conditionally normal tissues. It should be noted that in 30% of smoking patients with LAC, *CYP1A1* expression was also found only in normal tissue.

Next we have analyzed whether the level of AhR expression is associated with the presence of *CYP1A1* expression in the tumor tissue of patients. Significant results were obtained for SCC: AhR expression was higher in tumor tissues in which *CYP1A1* expression was detected (Figure 1).

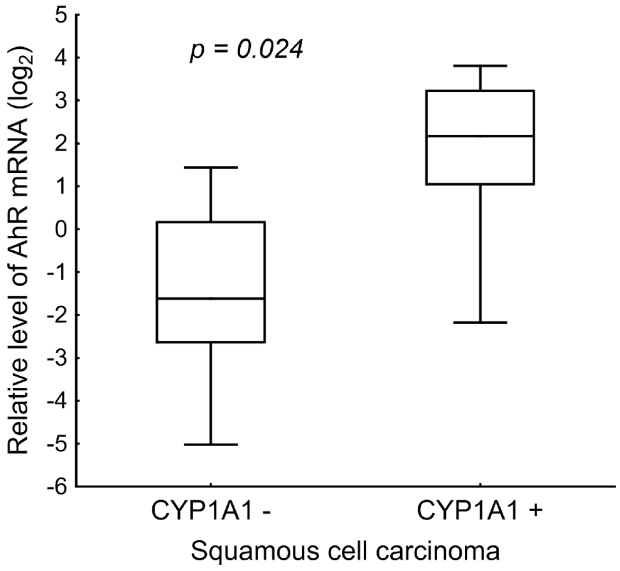

Figure 1. The dependence of AhR mRNA levels in tumors of patients with SCC on the expression status of *CYP1A1*. The upper and lower limits of the boxes represent the 75th and 25th percentiles. The horizontal line is the median of changes in the AhR mRNA level in tumor samples relative to paired conditionally normal (adjacent) tissue from the same patients. The whiskers represent a range of values. CYP1A1- – there is no expression of *CYP1A1* in the tumor; CYP1A1+ – expression of *CYP1A1* in the tumor is registered.

Expression of AhR-Regulated miRNAs in Tumors of NSCLC Patients

In the collected lung cancer samples and conditionally normal lung tissues, we also determined the expression profile of several miRNAs potentially regulated by AhR (miRNA-21, -342, -93, -181a, and -146a). In patients with SCC, the expression levels of miRNA-342 and miRNA-181a were significantly differed in tumor tissue as compared to conditionally normal tissue: in tumor tissue the levels of these miRNAs were about 3 times lower (when comparing median values).

As in the cases of SCC, in smoking patients with LAC, the levels of miRNA-342 and miRNA-181a in tumor tissue were lower as compared to the level of these miRNAs in conditionally normal lung tissue. In addition, the expression of miRNA-21 and miRNA-93 was higher in the tumor tissues of such patients (Table 4). The expression of miRNA-21 and miRNA-93 was also higher in tumor tissues of non-smoking patients with LAC, but the levels of miRNA-342 and miRNA-181a in tumor tissues of non-smoking patients did not differ from their levels in conditionally normal lung tissues of these patients (Table 5).

We also analyzed TCGA data on the levels of these miRNAs in NSCLC tissues. As well as our set of samples, set of samples from TCGA showed a increase in the levels of miRNA-21 and miRNA-93 (5.6- and 2.8-fold respectively) in LAC (n=447) as compared to normal lung tissue (n=44), respectively (Table 6). According to TCGA, the levels of these miRNAs were also increased in SCC by 2.4 and 2.1 times, respectively (n=336). In our set of samples, we observed only a trend towards increased levels of these miRNAs in SCC.

Using TCGA, it is possible to evaluate separately the levels of miRNA-181a-1 and miRNA-181a-2. The sequences of these miRNAs are identical, so we have measured the total amount of miRNA-181a using PCR, but only miRNA-181a-1 is predicted to be AhR-regulated. According to our data and the TCGA data, miRNA-181a expression decreased in SCC. However, all other our data on miRNAs levels compared with their levels in normal tissues were not consistent with the TCGA data, which could be due to the different source of normal tissues in our study and in TCGA.

Table 3. CYP1A1 mRNA abundance in tissues of patients with SCC and LAC

Tissue samples from patients	*CYP1A1* expression		
	Lack of expression in normal and tumor tissues	Expression*	
		In normal tissue	In tumor
sSCC (% of patients)	52.5	12.5	47.5
nsLAC (% patients)	76.7	23.3	0.0
sLAC (% of patients)	27.5	62.5	42.5

CsSCC – smoking patients with SCC; sLAC – smoking patients with LAC; nsLAC – non-smoking patients with LAC.
* The expression was considered as positive at Ct from 22 to 37.

Table 4. Relative levels of the studied miRNAs in the tumor samples of smoking patients with LAC (n=40)

Relative levels of miRNA* and *p*-value									
miR-21	*p*	miR-342	*p*	miR-93	*p*	miR-181a	*p*	miR-146a	*p*
2.04 (0.17–14.00)	0.042	0.46 (0.05–4.68)	<0.001	1.60 (0.23–7.81)	0.041	0.68 (0.09–5.32)	0.024	1.14 (0.09–33.17)	0.581

Here and in Table 5: * data represent medians and the range of changes in the level of miRNA in tumor samples versus paired conditionally normal (adjacent) tissue.

Table 5. Relative levels of the studied miRNAs in the tumor samples of non-smoking patients with LAC (n=30)

Relative levels of miRNA* and *p*-value									
miR-21	*p*	miR-342	*p*	miR-93	*p*	miR-181a	*p*	miR-146a	*p*
4.06 (1.95–8.38)	<0.001	0.93 (0.12–4.47)	1.000	2.84 (0.33–4.31)	0.049	0.86 (0.40–2.20)	0.650	1.22 (0.28–6.56)	0.290

Table 6. Data on altered miRNA levels in the studied clinical samples of NSCLC and in samples from TCGA (relative to normal tissues)

miRNA	Changes in the miRNA levels in NSCLC samples as compared to the normal lung tissue				
	Disease	LAC		SCC	
	Smoking status	Smokers	Non-smokers	Smokers	Non-smokers
miRNA-21	Our data	↑	↑	–	No data
	TCGA data	↑	↑	↑	↑
miRNA-342	Our data	↓	–	↓	No data
	TCGA data	–	–	↑	–
miRNA-181a-1	Our data	↓	–	↓	No data
	TCGA data	–	–	↓	↓
miRNA-181a-2	Our data	↓	–	↓	No data
	TCGA data	–	↑	↓	↓
miRNA-93	Our data	↑	↑	–	No data
	TCGA data	↑	↑	↑	↑
miRNA-146a	Our data	–	–	–	No data
	TCGA data	–	↑	↑	↑

↑ — significant increase in the level of miRNA in tumor tissue as compared to its level in normal tissue; ↓ — significant decrease in the level of miRNA in tumor tissue; – — no changes in miRNA expression.

Analysis of the Association between the Levels of AhR-Regulated miRNAs in NSCLC Tumors and Smoking Status

Smoking can lead to changes in the miRNA expression profile not only in tumor tissue, but also in normal lung tissue. Therefore, to confirm the presence of an association between the expression profile of AhR target miRNAs and smoking, we have compared miRNA levels normalized to the RNA levels of the reference genes in tumor tissues of smoking and non-smoking patients from our set of samples, and also analyzed similar data from TCGA.

The comparison of the levels of the studied miRNAs in LAC of smoking and never-smoking patients has shown that in the tumor tissues of smoking patients the amount of miRNA-181a and miRNA-146a was 2.4 times lower than in the tumor tissues of non-smoking patients (Table 7). According to TCGA, a significant association with smoking in patients with LAC was observed for miRNA-181a-1 and miRNA-181a-2, miRNA-146a (the amount of these miRNAs was lower in the tumor tissues of smokers), miRNA-93 (the amount of miRNAs was higher in the tumor tissues of smokers). For SCC, TCGA also revealed a trend to an increase in the level of miRNA-93 and a decrease in the level of miRNA-146a in tumor tissues of smokers (as compared to never-smokers), but this relationship was not significant.

DISCUSSION

Results of numerous experimental and epidemiological studies have shown that exposure to environmental toxins such as PAHs has a significant

Table 7. The dependence of tumor miRNA levels in LAC patients on the smoking status

miRNA	Data source	miRNA level in LAC of smoking patients (relative to miRNA level in LAC of non-smoking patients)	miRNA level in SCC of smoking patients (relative to miRNA level in SCC of non-smoking patients)
miRNA-21	Our data	0.75	—
	TCGA data	0.98	1.25
miRNA-342	Our data	1.01	—
	TCGA data	1.04	0.87
miRNA-181a-1	Our data	0.42*	—
	TCGA data	0.87*	1.09
miRNA-181a-2	Our data	0.42*	—
	TCGA data	0.63*	0.90
miRNA-93	Our data	1.12	—
	TCGA data	1.22*	1.36
miRNA-146a	Our data	0.41*	—
	TCGA data	0.67*	0.79

Data represent a median ratio of the miRNA levels in tumor samples from smoking and non-smoking patients. miRNA levels in tumors were normalized to the RNA levels of reference U44 and U48 and were not normalized to miRNA levels in normal tissues. * – $p<0.05$.

impact on the occurrence and progression of lung cancer. One of the main sources of PAHs is smoking [20, 21]. According to IARC data, BaP, a component of cigarette smoke, belongs to group 1 human carcinogens [21]. The toxic effect of BaP is realized via two main mechanisms: genotoxic (formation of DNA adducts) and non-genotoxic or epigenetic [22, 23]. The epigenetic mechanisms include, for example, miRNA-mediated regulation of gene expression at the post-transcriptional level. Although many studies of miRNA expression in malignant tumors, including lung cancer, have shown significant changes in the expression levels of both oncogenic and tumor-suppressive miRNAs, the reasons for such changes are often unknown. It can be speculated that compounds such as BaP may lead to changes in the miRNA expression profile through AhR activation. We have previously found miRNAs containing AHRE sequences (the AhR binding sites) in their promoter regions [12]. In the present study, we have investigated expression of some of these miRNAs (miRNA-21, miRNA-342, and miRNA-93) in lung tumor samples obtained from smoking and non-smoking patients with SCC and LAC. In addition, we have assessed expression of miRNA-181a and miRNA-146a, which are considered as AhR targets in the Harmonizome resource. It should be noted that we were unable to form a group of non-smoking patients with SCC due to their limited number, which complicated statistical analysis.

First of all, to confirm activation of AhR in the lung tissues of smoking patients, we have determined the expression level of the AhR "classical" target gene *CYP1A1*. As a rule, the constitutive expression of this gene in the lungs is low, and this is consistent with our results: in 76.7% of non-smoking LAC patients, *CYP1A1* expression was not detected in both normal and tumor lung tissues. However, among smoking patients, particularly patients with LAC, the proportion of cases, in which *CYP1A1* expression in tissues was absent, decreased. Interestingly, among SCC patients no cases were found when *CYP1A1* expression was detected only in normal lung tissue, while in 30% of smoking patients with LAC *CYP1A1* expression was registered in normal tissue and not detected in tumor tissue. In addition, only in SCC high *AhR* expression corresponded to positive expression of *CYP1A1* in tumor tissues. These results support different mechanisms of AhR-dependent pathogenesis of LAC and SCC.

Among numerous AhR target genes, miRNAs encoding genes are of particular interest. In our study, we have confirmed that the expression profile of AhR-regulated miRNA-21, -342, -93, -181a, and -146a differs in dependence on tumor type (LAC or SCC) and the smoking status. For example, in smoking patients with SCC and LAC, the expression of miRNA-342 and miRNA-181a was lower in tumor tissues than in normal lung tissues from the same patients. These results do not coincide with the TCGA data; however, the statistically significant decrease in the expression of miRNA-342 and miRNA-181a in tumor tissue samples is consistent with the recent results of other researchers who also showed a decrease in the levels of these miRNAs in NSCLC [24, 25]. Overall, the differences between our data and the TCGA data may be due to different sources of normal tissue. For example, in TCGA, tissues from healthy individuals with unknown smoking status have been use as the normal tissue. In our study, miRNA levels in tumors were normalized to miRNA levels in normal lung tissues from the same patients.

For LAC patients, both smokers and non-smokers, we have found a significant increase in the expression of oncogenic miRNA-21 and miRNA-93 in tumor tissues as compared to conditionally normal tissues; this is also consistent with previously obtained data [26, 27] and the TCGA data. We did not find a significant increase in the expression of these miRNAs in SCC, but observed a trend towards an increase in their levels. In the TCGA sample set, miRNA-21 and miRNA-93 levels were significantly increased in SCC, which could be a consequence of the larger number of samples (336 tumor samples and 44 normal tissue samples) and a different source of normal tissue.

However, expression of the miRNAs we studied may differ in normal lung tissues of smoking and non-smoking patients. Therefore, we also analyzed the difference in the levels of the studied miRNAs in tumor tissues of smoking and non-smoking patients with LAC, without normalization to conditionally normal patient tissue. We observed a decrease in the amount of miRNA-181a and miRNA-146a in tumors of smoking patients as compared to their amount in tumors of non-smokers. The decrease in the expression of these miRNAs in samples from smoking patients that we observed could be associated with an increase in the amount of AhRR, the AhR repressor. For example, according to the TCGA data, AHRR expression is significantly higher in tumor tissues of smoking patients with SCC or LAC (compared to non-smoking patients). In addition, it is known that smoking leads to demethylation of cg05575921 in the *AHRR* gene enhancer, which increases its expression [28].

In the set of samples from TCGA, a significant decrease in miRNA-181a and miRNA-146a expression was also found in tumor tissues of smoking patients with LAC, while miRNA-93 levels were significantly higher (as compared to never-smoking patients). In SCC patients, no significant dependence of the expression of all studied miRNAs on the smoking status was found in TCGA; however, a trend towards an increase in the expression of miRNA-93 and a decrease in the expression of miRNA-146a was detected in smoking patients as compared to never smokers. It is possible that the lack of a significant association with smoking in SCC patients from the TCGA set is due to the insufficient number of SCC samples from non-smoking patients. For example, the number of smoking and never-smoking patients with LAC in TCGA is 104 and 66, respectively, while the number of smoking and never-smoking patients with SCC is 105 and 11.

Our results confirm that smoking affects the miRNA expression profile even in macroscopically normal lung tissue. For example, in smoking patients, miRNA-342 expression was lower in tumors than in normal lung tissues. However, comparison of the level of this miRNA in tumors of smoking and non-smoking patients revealed no significant difference, thus suggesting an increase in the expression of AhR-regulated miRNA-342 in conditionally normal lung tissue of smoking patients. There was also no difference in the amount of miRNA-146a in tumor and normal lung tissues of patients. However, it has been shown that the expression of miRNA-146a is significantly lower in the tumor tissue of smoking patients than in lung tumor tissue of never-smokers.

In general, we can clearly conclude that expression of at least two AhR-regulated miRNAs, miRNA-181a and miRNA-146a, in LAC depends on smoking.

CONCLUSIONS

According to the results of our studies, the expression profile of miRNAs potentially regulated by AhR is different in LAC and SCC and depends on the smoking status. The results of our studies and the TCGA data indicate a dependence of the expression of miRNA-181a and miRNA-146a on smoking: expression of these miRNAs is lower in the tumor tissues of smoking patients with LAC thus suggesting their regulation by a pair of transcription factors AhR and AhRR. The expression of oncogenic miRNA-21 and miRNA-93 in tumor tissues was higher than in normal lung tissue in all patients with LAC (in our set of samples and in the set of samples from TCGA). Further study of the regulation of these miRNAs, their role and the role of the genes they regulate in the pathogenesis of NSCLC in smoking and non-smoking patients may contribute to the development of new therapeutic approaches for the treatment of NSCLC.

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COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures were approved by the Bioethical Committee of the Institute of Molecular Biology and Biophysics and comply with the ethical standards of the National Research Ethics Committee and the 1964 Helsinki Declaration

and its subsequent amendments or comparable ethical standards (Protocol No. 3 of March 14, 2017). Informed voluntary consent was obtained from each participant included in the study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ЭКСПРЕССИЯ микроРНК, ПОТЕНЦИАЛЬНО РЕГУЛИРУЕМЫХ AhR, В ТКАНЯХ НЕМЕЛКОКЛЕТОЧНОГО РАКА ЛЁГКОГО У КУРЯЩИХ И НЕКУРЯЩИХ ПАЦИЕНТОВ

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Курение является фактором риска развития немелкоклеточного рака лёгкого (НМКРЛ), наиболее распространённые подтипы которого — аденокарцинома лёгкого (АКЛ) и плоскоклеточный рак лёгкого (ПКРЛ). Сигаретный дым содержит лиганды арилгидрокарбонового рецептора (AhR), например, бензо(а)пирен (BaP), который через активацию AhR может изменять экспрессию многих (в том числе микроРНК-кодирующих) генов. В настоящем исследовании мы оценили экспрессию некоторых из микроРНК, потенциально регулируемых AhR (miRNA-21, -342, -93, -181a, -146a), а также *CYP1A1* — известного гена-мишени AhR. Исследование выполнено в образцах опухолей лёгких курящих и некурящих пациентов с АКЛ и курящих пациентов с ПКРЛ. У этих же пациентов на расстоянии не менее 5 см от границы опухоли были также отобраны макроскопически нормальные ткани лёгкого. Полученные нами результаты для микроРНК мы сравнили с данными из TCGA (The Cancer Genome Atlas). У 76,7% некурящих пациентов с АКЛ в опухолевых или нормальных тканях лёгких мРНК *CYP1A1* не была обнаружена, тогда как среди курящих пациентов почти в половине случаев (47,5% для ПКРЛ и 42,5% для АКЛ) в опухолевых тканях была зарегистрирована экспрессия *CYP1A1*. Мы подтвердили, что профиль экспрессии AhR-регулируемых микроРНК различен в АКЛ и ПКРЛ и зависит от статуса курения: у всех пациентов с АКЛ экспрессия онкогенных miRNA-21 и miRNA-93 в опухолевых тканях была выше, чем в условно-нормальной ткани лёгких от этих же пациентов, однако у пациентов с ПКРЛ из выборки уровни этих микроРНК в опухолевой и условно-нормальной тканях лёгких достоверно не различались. Результаты наших исследований и данные TCGA указывают на зависимость экспрессии miRNA-181a и miRNA-146a у пациентов с АКЛ от курения — экспрессия этих микроРНК значительно ниже в опухолевых тканях курящих пациентов, что может быть связано с регуляцией их экспрессии AhR и AhRR (репрессор AhR). Полученные результаты подтверждают, что курение оказывает влияние на профиль экспрессии микроРНК, что следует учитывать при поиске новых диагностических и терапевтических мишеней при НМКРЛ.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: арил-гидрокарбоновый рецептор; бензо(а)пирен; немелкоклеточный рак лёгкого; микроРНК

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