miR-19 in blood plasma reflects lung cancer occurrence but is not specifically associated with radon exposure

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Abstract. Radon is one of the most powerful carcinogens, particularly in terms of lung cancer onset and development. miRNAs may be considered not only as markers of the ongoing tumorigenesis but also as a hallmark of exposure to radiation, including radon and its progeny. Therefore, the purpose of the present study was to estimate the value of plasma miR-19b-3p level as the prospective marker of the response to radon exposure in lung cancer pathogenesis. A total of 136 subjects were examined, including 49 radon-exposed patients with lung cancer, 37 patients with lung cancer without radon exposure and 50 age/sex matched healthy controls. Total RNA from blood samples was extracted and used to detect miR-19b-3p expression via reverse transcription quantitative-polymerase chain reaction. The 2−ΔΔCq method was used to quantify the amount of relative miRNA. The plasma level of p53 protein was determined using a Human p53 ELISA kit. Plasma miR-19b-3p level was significantly higher in the patients with lung cancer groups, compared with the healthy control group (P<0.0001). No other statistically significant differences were determined in the expression level of plasma miR-19b-3p between patients diagnosed with lung cancer exposed to radon and not exposed to radon. The expression level of free circulating miR-19b-3p was higher in the group of non-smoking patients with lung cancer, compared with smokers with lung cancer. The miR-19b-3p was 1.4-fold higher in non-smokers than in smokers (P<0.05). No association between plasma levels of p53 protein and miR-19b-3p freely circulating in patients with lung cancer was observed. No other statistically significant differences were determined in the plasma p53 protein level between patients diagnosed with lung cancer exposed and not exposed to radon.

These results indicated that detection of miR-19b-3p levels in plasma potentially could be exploited as a noninvasive method for the lung cancer diagnostics. However, this miRNA is not suitable as the precise marker for radon impact.

Introduction

Lung cancer causes almost 1.3 million mortalities/year globally (1). Cigarette smoke and radon exposure are the major risk factors for lung cancer (2). Primary prevention is fundamental in lung cancer, which is a tumor characterized by a long latency period (3). Circulating miRNAs may be proposed as biomarkers for diagnosis, prognosis or monitoring curative effects in various cancer types, including lung cancer (4). In cells, miRNAs are present as short (18-22 nucleotides), non-coding molecules involved in post-transcriptional gene regulation, mRNA degradation and/or translation inhibition (5). Aberrant expression of miRNAs has been established in diverse tumor types, including hepatocellular carcinoma (6), breast (7), stomach (8), prostate (9) and lung cancer (10).

A numbers of studies have demonstrated that increased expression of miR-19 confers negative prognosis for patients with lung cancer (11-13). According to these studies, the miR-19 family is responsible for epithelial-mesenchymal transformation of cells, which, in turn, further facilitates the processes of cell migration and invasion.

Recent data indicated that miRNAs are engaged in the regulation of cellular processes induced by radiation and, consequently, miRNAs can potentially be used as biomarkers to assess the degree of exposure to radiation in humans (14). There is evidence that the expression profile of a number of miRNAs, including miR19b-3p in human bronchial epithelium BEAS2B cells, have been altered upon exposure to radon (15).

A key role in maintaining the genome stability pertains to p53, including response to damage caused by radiation (16). TP53 mutation was identified in the development of tumors of a number of localizations, including lung cancer (17). Specific ‘hotspot’ mutations in cancer-relevant genes have been described in radon-induced lung cancer (16,18-20).

Different studies have indicated that TP53 can directly suppress the expression of oncogenic miRNAs, and as a transcription factor, can activate the expression of oncosuppressor

Key words: miRNA, lung cancer, biomarker, radon, p53
miRNAs (21-23). However, the latest data also indicated the presence of miRNA-mediated post-transcriptional regulation of TP53 in mammalian cells (24). Fan et al (24) have demonstrated that TP53 is the target of miR-19b and the over-expression of miR-19b in cancer cells leads to a decrease in the level of the p53 protein and its targets, Bax and p21 proteins.

Nevertheless, despite the copious amounts of evidence supporting the association between p53 and miRNA in lung cancer, the pathogenic mechanisms underlying this process remain largely unclear particularly concerning the epigenetic modulation. This is particularly true for radon-induced lung cancer (25,26).

The aim of the present study was to determine the alterations in free circulating miR-19b and the level of p53 protein in the plasma of patients with lung cancer exposed to high doses of radon.

Materials and methods

Study design and population. A total of 136 subjects was examined, including: i) 49 radon-exposed patients with lung cancer (RLC); ii) 37 patients with lung cancer without radon exposure (LC); and iii) 50 healthy controls (C). The radon-exposed patients with lung cancer comprised 39 males and 10 females with a mean age of 67.8±1.78 years. The group of lung cancer patients without radon exposure consisted of 31 males and 6 females with a mean age of 57.42±2.34 years. Healthy control was represented by 39 males and 11 females with a mean age of 60.7±1.96 years.

All patients with primary lung cancer were diagnosed between April 2015 and September 2016 at the Astana Oncology Center (Astana, Kazakhstan) and at the Akмолa Region Oncology Hospital (Kokshetau, Kazakhstan). All cases were newly diagnosed, previously untreated and histologically confirmed. Clinical stage was classified according to the sixth edition of the Tumor-Node-Metastasis classification of the International Union Against Cancer (27). Controls were matched for sex, age and smoking status to patients with lung cancer, according to characteristics of subjects which are reported in Table I. At recruitment, each participant was interviewed to obtain detailed information on age, life history of tobacco use and occupational activities.

All information regarding participants was rendered anonymous following data and blood sample collection. Informed consent was obtained from each study participant prior to interview and blood collection. The present study was approved by the Ethical Committee of the Semey State Medical University (Semey, Kazakhstan; approval no. 2).

RNA extraction from blood. A 10 ml sample of whole peripheral blood was collected from each subject into EDTA containing tubes. Blood was centrifuged at 3,000 x g for 10 min at room temperature and supernatant stored in aliquots at -80°C. Total RNA from 200 µl plasma was isolated using the MiRCURY™ RNA Isolation kit-Biofluids (cat no. 300112; Exiqon A/S, Vedbaek, Denmark), in accordance with the manufacturer's protocol. The amount and purity of extracted RNA were evaluated using a fiber optic spectrophotometer (Nanodrop™ ND-1000; Nanodrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the manufacturer's protocols, and the 230/260 (<0.50) and 260/280 (>1.85) absorbance ratios were calculated. The RNA structural integrity was evaluated by capillary electrophoresis using a RNA bioanalyzer (Bioanalyzer Agilent 2100; Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with a RNA oligonucleotide chip (RNA 6000 Nano Ladder Chip; Agilent Technologies, Inc.). The miRNA amounts were accurately standardized among blood serum samples for microarray and quantitative polymerase chain reaction (qPCR) analyses using the Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific, Inc.).

miR-19 analysis by qPCR. The expression levels of miR-19b-3p was determined by evaluating the level of fluorescence emitted by SYBR® Green tracer (cat no. 203403; Exiqon A/S, Denmark). SYBR-Green fluorescent tracer was used to identify PCR amplicons whose identity was checked by melting curve analysis according to previously published procedures (28). MiRCURY LNA™ UNIVERSAL RT microRNA PCR kit, including miR-19b-3p-specific primers (cat no. 204450; Exiqon A/S, Vedbaek, Denmark) and primers for the reference gene RNU6B (cat no. 203907; Exiqon A/S) for reverse transcription (RT)-qPCR, was used to amplify miRNAs. cDNAs were prepared using Universal synthesis kit (cat no. 203301; Exiqon A/S) according to the manufacturer's instructions. PCR amplification was performed in the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Each reaction was carried out using 4 µl of cDNA, 1 µl of PCR primer mix and 5 µl SYBR-Green PCR Master mix (cat nos. 204450 and 203403 respectively; Exiqon A/S, Denmark) in a final 10 µl volume. The RT-qPCR was performed at 95°C for 10 min for one cycle, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec. The specificity of PCR products was evaluated by melting curve analysis. Gene expression was normalized to RNU6B. All reactions were carried out in triplicate, and the 2 ΔΔCq method (ΔCq=CTmir−CTEndogenous) was used to quantify the relative miRNA amount (29).

Exposure assessment. Measurement of radon activity was conducted in accordance with the Rapid Measurement Method of radon and thoron (30) using a Canary 222 Digital Electronic Radon Gas Monitor (LR-03) radiometer (Corentium AS, Oslo, Norway). The measurement of radon concentration was carried out at the homes of recruited patients in rooms previously unventilated for at least 24 h. In each room, radon in the air was measured for seven days and the average value was used for further calculations.

The annual effective dose (H) was calculated according to the formula (30): H (mSv/y)=C x F x O x T x D; where C stands for the average radon concentration Bq/m³, F is the occupancy factor for indoor that is set as 0.4, O is the occupancy factor taken as 0.8, T is time in h in a year (8,760 h/y) and D is the dose conversion factor, 1.4x10⁻⁶ Sv/Bq/m³.

Determination of p53 protein in plasma. The plasma level of p53 protein was determined using an ELISA kit (Human p53 ELISA kit; cat no. ab46067; Abcam, Cambridge, UK). The samples were added to 96-microtiter wells pre-coated with the p53 monoclonal antibody from the kit and incubated at room
temperature for 2 h. Samples and biotinylated p53 monoclonal antibody were incubated for 1 h at room temperature. Following washing three times with 200X Wash buffer from the kit, the enzyme Streptavidin-HRP (also from the Human p53 ELISA kit), which binds the biotinylated antibody, was added and incubated for 30 min with washes performed three times with 200X Wash buffer from the kit according to the protocol of the manufacturer. Chromogen TMB (3,3',5,5'-tetramethylbenzidine) substrate solution was added inducing a colored reaction product. The absorbance at 450 nm of each well was determined using a microplate reader (Asys Expert microplate reader; Biochrom, Ltd., Cambridge, UK). All samples were measured in triplicate.

**Determination of cotinine in plasma.** In order to verify smoking status of the individuals, blood plasma cotinine was utilized as a marker. The cotinine level was determined using an ELISA kit (Cotinine ELISA kit; cat no. KA0930; Abnova, Taipei, Taiwan) performed according to the manufacturer's protocols. Briefly, samples and cotinine enzyme conjugate were added to the wells coated with anti-cotinine antibody and horse peroxidase colorimetric reaction performed. Unbound cotinine and cotinine enzyme conjugate were washed three times with 300 µl 1X Wash Buffer, also from the Cotinine ELISA kit. The intensity of color (absorbance wavelength 450 nm) was inversely proportional to the concentration of cotinine in the samples. A standard curve was prepared associating color intensity with the concentration of the cotinine.

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). All data are presented as mean ± SD. Differences between groups were assessed using the Tukey’s post hoc test after one-way analysis. P<0.05 was considered to indicate a statistically significant difference; "P<0.05; ""P<0.01; """"P<0.001; and """"""P<0.0001. χ² was used to calculate the statistical significance in Table I.

**Results**

**Effective annual radon exposure dose and decay product inhalation.** The overall exposure to radon evaluated at the homes of monitored subjects is reported in Table II. The table represents the home addresses of the patients with lung cancer living on the territory with a level above the permissible concentration, where radon levels were measured, and also demonstrated a level of equivalent equilibrium volume activity of radon (EEVA) and effective exposure dose for the population.

According to the generally accepted opinion in the world scientific community, the distribution of EEVA parameters obeys the lognormal law (30); therefore, the median value was used as the average value for calculating the exposure power of radon and its decay products. The characteristics of the data array and the parameters of the lognormal distribution are depicted in Table III. Thus, the average effective annual dose for patients diagnosed with lung cancer living in the areas with a high level of EEVA was 4.55 mSv/y. Participants in the study selected for control groups (C and LC) lived in areas with a low level of EEVA. These areas were determined according to the results reported in a previous study (15). The EEVA data used to calculate the average effective annual dose for participants in the control group study (C and LC) are depicted in Table IV. Thus, the average effective annual dose for study participants living in areas with a low level of EEVA was 0.88 mSv/y.

**miR-19b-3p expression level in the plasma of patients with lung cancer with and without radon exposure.** The total circulating miRNA was isolated from blood plasma using the protocol aforementioned and divided to three groups: Patients diagnosed with lung cancer living in areas with a radon concentration below 200 Bq/m³ for ≥5 years; patients diagnosed with lung cancer who had been exposed to high doses of radon for 5 years; and a control group consisting of healthy individuals, who did not have pulmonary pathology and lived in a EEVA area with <200 Bq/m³. The expression levels of miRNA-19b-3p
in all three groups were depicted in Fig. 1. miR-19b-3p expression levels in patients of the RLC group were 6.5 times higher (P<0.0001) than in the C group. miR -19 expression levels in the LC group was 6.9 times increased (P<0.0001), compared with those detected in the C group. Comparative analysis of the relative miR‑19b‑3p expression level in the RLC group vs. the LC group indicated that the miR‑19b‑3p expression profile in both groups did not differ significantly.

miR-19b-3p expression level in the plasma associated with smoking habits. The association of miR-19b-3p expression with smoking status was examined. The results of the present study demonstrated that miR-19b-3p expression levels do not differ in patients with lung cancer with or without radon exposure, and these groups were combined into one ‘patients with lung cancer’ group. This group was divided into current smokers and non-smokers regardless of radon exposure. Cotinine levels <10 ng/ml was considered as an indicator of the absence of current smoking.

The ‘Smokers’ group consisted of 34 people, and the ‘Non-smokers’ group 33 people. Former smokers (subjects having stopped smoking <6 months ago) have been excluded from any of these groups (19 subjects). Furthermore, healthy control group (50 subjects) have been not included to this particular analysis due to, according to the previous experiments, miR‑19b‑3p expression level was significantly lower in the control group than in two groups with lung cancer diagnosis, that is, these groups are incompatible. As it can be observed from Fig. 2, the level of free circulating miR-19b-3p is higher in plasma in the group of non-smoking patients with lung cancer, compared with smokers. The mean plasma miR-19b-3p expression level was 6.45±0.4 in the ‘Non-smokers’ group in comparison with the ‘Smokers’ group, where the mean miR-19b-3p expression level was 4.74±0.5, miR-19 being 1.4-fold higher in non-smokers than in smokers (P<0.05).
p53 protein in plasma of patients with lung cancer as associated with radon exposure. The results of the p53 protein measurement in plasma are given in Table V representing minimum, maximum, median and mean values. p53 protein levels of the LC were determined in a range from 5.17-36.81 U/ml, with a median value of 15.24 U/ml and the mean of 17.92±1.53 U/ml. RLC demonstrated the highest plasma p53 in the present study. In this group, the plasma levels of p53 had a range from 0.1-26.94 U/ml, median value of 13.0 U/ml and mean of 18.76±3.31 U/ml. The p53 protein has not been detected in plasma in 16% of the samples from C group. In 84% of samples from group C, the plasma levels of p53 were determined to range from 1.96-39.64 U/ml, with a median of 6.49 U/ml and mean value of 8.646±1.25 U/ml. The p53 plasma levels in the LC group were 2 times higher than in those of healthy subjects (P<0.05; Fig. 3). The p53 protein level was also 2 times higher in radon-exposed patients with lung cancer than in healthy volunteers (P<0.01). There were no significant differences in p53 protein concentrations between LC and RLC (P=0.265). However, there was no association between the level of miR-19b-3p and the plasma p53 level in patients with lung cancer, as indicated via regression analysis (r=0.06; P=0.24).

Discussion

Recent studies have reported that non-coding RNA (ncRNA) may serve a critical role in regulating different cancer types (31,32). These ncRNAs are divided into housekeeping ncRNAs and regulatory ncRNAs (33). The regulatory ncRNAs have emerged as critical regulators of gene expression in normal and disease states (34). This heterogeneous group depending on their size can be divided into IncRNAs (≥200 nucleotides) and small ncRNAs (<200 nucleotides) (35). miRNAs represent the most studied small ncRNAs. These molecules predominantly bind to 3’-UTRs of mRNAs to mediate post-transcriptional gene silencing (36). A number of studies have identified aberrant miRNA expression in different types of cancer (35). Circulating miRNAs, passively leaked or actively transported outside cells, can be stably detected in blood and may be proposed as biomarkers for diagnosis, prognosis or monitoring curative effect in various cancer types, including lung cancer (37). Since miRNAs are involved in the development and progression of lung cancer, there is a particular interest in miRNAs not only as novel biomarkers but also as potential tools for treatment (38).

In the present study, changes in the level of miR-19b-3p expression were analyzed, which serve a significant role in the development of a number of cancer types (39,40), as well as in metastasis (41) and tumor response to drug therapy (42). A miRNA panel including miR-19b-3p in peripheral plasma was identified and proposed as a biomarker in diagnosis of adenocarcinoma lung cancer as reported by Zhou et al (43). Furthermore, obtained results are consistent with the previous data of Li et al (41), who discovered that miR-19b expression was significantly higher in lung adenocarcinoma cell lines.
circulating freely in the blood plasma was analyzed. The level of miRNA downregulated by mainstream cigarette smoke has opposite effects in the lungs and blood, with the lungs downregulating miRNAs and miRNAs being upregulated in blood (52). This situation is associated with the inhibition of DICER processing induced by cigarette smoke exposure, which causes a blockage in miRNA maturation and release from cells into the blood stream of miRNA precursor in the absence of their mature counterpart (53).

miRNAs downregulated by mainstream cigarette smoke are miR-19b and miR-292, which are involved in the regulation of DICER, a key molecule in miRNA biogenesis, as demonstrated by Balansky et al (54). However, in the present study there are a number of limitations: First, the level of miRNA circulating freely in the blood plasma was analyzed. The level of free circulating miRNAs can differ significantly from the level of the same miRNAs in the tissues due to different types of mammalian tissues varying in the profile of miRNAs expressed in them (55). An example of this case is let-7 miRNA, which serves a significant role in the embryonic development of the lung, and the expression of this miRNA remains at a high level in the postembryonic period (56). As Mendell (57) depicted, the miR-17-92 miRNA cluster, which includes miR-19, serves a crucial role in the embryonic development of the heart, lungs and immune system. Furthermore, the loss of the function of the miR-17-92 cluster leads to a decrease in the size of embryos and the immediate postnatal mortality of all animals (57). This is possibly due to pronounced lung hypoplasia and interventricular septum defects in the hearts of mice devoid of miR-17-92 (57).

The observations aforementioned are consistent with an earlier demonstration that miR-17-92 miRNA clusters usually have a high level of expression in the embryonic lung, and later its levels decrease as the mice matures (58). Furthermore, the transgenic expression of these miRNAs specifically in the pulmonary epithelium leads to severe developmental defects with increased proliferation and inhibition of epithelial cell differentiation (58). Despite the fact that exposure to tobacco smoke experiments in animal models lead to a decrease in body weight and have in general negative effect on cell proliferation and survival, an opposite pattern has been observed in target tissues, including lungs distinguished by hyperplasia of pulmonary tissue (54). Considering the aforementioned, it appears necessary to investigate the tissue-specific miR-19b-3p profile in the lungs of smokers and non-smokers to improve the understanding of the role of this miRNA in the development of lung cancer induced by smoking. The present study has demonstrated that the level of miR-19b-3p circulating in the blood plasma significantly decreases in smokers with lung cancer, and this is consistent with previous data, indicating the negative effect of tobacco smoke on proliferative activity in the body as a whole (59,60).

miR-19 is a key member of the miR-17/92 cluster, which targets several critical apoptosis associated genes, including TP53 (24). Fan et al (24) demonstrated that when miR-19b is transfected into HeLa cells, following 48 h, the p53 protein level is reduced by 50%, while the mRNA level remains unchanged.
Indeed, miR-19b directly targets p53 by base-pairing to its 3'UTR. The TP53 gene was the first tumor-suppressor gene to be identified. The p53 network, which present in all cells of the organism is normally ‘off’ (61). In the absence of damage to the genetic apparatus, the p53 protein is in an inactive state, and when DNA lesions occur, it becomes activated (62). The p53 protein is also activated by stimuli that remind those damages when p53 is active or are a signal regarding the unfavorable state of the cell (stress state) (63). The function of the p53 protein is to remove from the pool of replicating oncogenic cells (62). Consequently, it can be assumed that one of the mechanisms underlying the lung cancer development is a decrease in the level of the p53 protein due to targeting its mRNA by miR-19b-3p and is mediated by this process of prevention of apoptosis in cancer cells.

In the present study, utilizing the Human p53 ELISA kit, it was determined that the level of the p53 protein in plasma of patients with lung cancer exposed to high doses of radon as well as in those individuals living in areas with a permissible concentration of EEVA, compared with the control group consisting of healthy people. The results of the present study have revealed that the level of p53 is significantly higher in both groups of patients diagnosed with lung cancer, while in a number of samples of healthy people it has not been detected at all. This data does not contradict the available data (64). According to a previous study (65), the level of antibodies against p53 in the blood serum increases with any pathology of the lung not only in malignant but even in benign tumors (65).

The present study paid close attention to two aspects. Firstly, whether there was a association between the p53 level and miR-19b-3p circulating freely in the blood plasma; additionally, the second aspect has concerned the issue of the effect of plasma p53 level on radon and its progeny.

The present study revealed no association between the plasma p53 and miR-19b-3p levels freely circulating in the blood plasma in patients with lung cancer. Thus, in both cases, the level of p53 protein and miR-19b-3p has been determined in the blood plasma and not in the lung tissue, so it cannot be concluded that these two components are not involved in a single mechanism underlying the malignant tumor onset in the lung. In addition, no other statistically significant differences have been determined in the plasma p53 protein level between RLC and LC. Also, the results of the present study are consistent with the data of other authors (66,67). In the studies of Schneider et al (67), no appreciably elevated or diminished p53 protein or anti-p53 antibodies values in serum of Former Uranium Miners was determined. Also, there was no association of these indicators with Working Level Months (WLM). Thereby, plasma p53 protein level is ineffective as a biomarker for the detection of lung cancer associated with radon and its progeny.

Collectively, these studies indicated that miR-19b-3p is not a suitable biomarker for radon-induced lung cancer. Despite the available data in the literature that overexpression of miR19b observed in human cancer cells lines can diminish p53 protein levels, the present study did not determine any association between the plasma p53 and the miR-19b-3p levels freely circulating in the blood plasma in patients with lung cancer. Furthermore, any changes in the plasma p53 protein levels depending on the effect of radon were not determined.

However, further research for biomarkers including miRNAs will provide a biomonitoring strategy to face health-risk in population living in radon-polluted areas and professionally exposed workers.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

RB, AI provided the study concept and design. RB, OB, AI and AP wrote and revised the manuscript. AP participated in discussion and made a significant contribution to the interpretation of the results. OB, DZ and AK collected the data, and performed the experiments. AK and DZ performed the RNA extraction from blood samples. OB and DZ performed the determination of p53 protein and cotinine in plasma and qPCR. OB and AK performed the radon exposure assessment. OB performed the statistical analyses. The final version of the manuscripts was read and approved by all authors.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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