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Associated risks of lung cancer with radon emanation

Lung cancer occupies an exceptional position. It is a rare example of a malignant disease with firmly established and delineated etiological factors. In fact, in most cases, the occurrence of the lung cancer can be associated with smoking; Moreover, this tumor can be connected with other carcinogenic agents in the inhaled air such as radon gas. Radon is the second leading cause of lung cancer. The aim of our work was to study the C-KIT protein expression by immunohistochemical method and identification of germ-line mutations in lung cancer patients who live in Almaty, where the radon level exceeds its norm. The methods chosen to carry out this study were a polymerase chain reaction (PCR) with a subsequent analysis of restriction fragment length polymorphism (RFLP) and immunohistochimical analysis (IHC). The object of research were blood samples and biopsies obtained from lung cancer patients who are receiving treatment at the Almaty Oncology Center and living in Almaty. Our studies didn't reveal any mutations in the C-KIT gene, but they showed that 34.09% of lung cancer patients have overexpression in a tumor tissue. It testifies to the fact that disorders occurred in the cells of the tumor tissue, that are not inherited. Based on our observations, we arrived at conclusion that a high radon emanation has caused disorder in the C-KIT gene, which led to its overexpression.

Key words: C-KIT, Expression, radon, IHC, lung cancer.

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Өкпе ісігінің радон эманациясымен түйіндесуі

Өкпе ісігі онкологияда айрықша орын алады – бұл қатаң орнатылған және этиологиялық факторлармен сызылған қатерлі аурудың сирек мысалы болып табылады. Расында, көптеген басыңқы жағдайларда өкпе ісігінің пайда болуын темекі шегумен байланыстыруға болады, сонымен қатар берілген жаңа түзілімді дем алатын ауада кездесетін радонды газ сияқты басқа да канцорагенді агенттермен ұқсастырылған болу мүмкін. Радон өкпе ісігінің дамуына алып келетін екінші себебі болып табылады. Біздің жұмыстың мақсаты радон қалыпты деңгейден жоғары жерде, Алматы қаласында тұратын, иммуногистохимия әдісімен C-KIT ақуызының экспрессиясын және өкпе ісігі бар аурулардағы germ-line мутациясының көрінуін зерттеу. Зерттеу әдістері ретінде полимеразды тізбекті реакциясы (ПТР) рестрикционды фрагменттер ұзындықтарының полиморфизмінін кезекті анализімен және иммуногистохимиялық анализ (IHC). Зерттеу объектілері ретінде Алматы қаласында тұратын және Алматы онкологиялық орталығында ем алатын өкпе ісігі диагнозы бар пациенттерден алынған қан үлгілері және биопсиялық материал болды. Біздің зерттеулерде C-KIT генінде мутация анықталған жоқ, бірақ 34,09% өкпе ісігі бар аурулардың ісік ұлпасында жоғары экспрессия бар екенін көрсетті. Яғни, бұзылу ісік ұлпа клеткаларында болатынын және тұқым қуаламайтынын айтуға болады. Біздің бақылауларымыздың негізінде радонның жоғары эманациясы C-KIT геніндегі бұзылуды тудырған болатын, бұл оның жоғары экспрессиясына әкеліп соқтырады деген нәтижеге келдік.

Түйін сөздер: C-KIT, экспрессия, мутация, радон, иммуногистохимия, өкпе ісігі.

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Сопряженность риска рака легкого с эманацией радона

Рак легкого занимает в онкологии исключительную позицию – это редкий пример злокачественного заболевания с, казалось бы, твердо установленными и очерченными этиологическими факторами. Действительно, в подавляющем большинстве случаев возникновение рака легкого можно связать с курением; кроме того, данное новообразование может быть ассоциировано с другими канцерогенными агентами, находящимися во вдыхаемом воздухе такими как радоновый газ. Радон является второй причиной развития рака легкого. Целью нашей работы было изучение экспрессии белка C-KIT иммуногистохимическим методом и выявление germ-line мутаций у больных раком легких, которые проживают в городе Алматы, где уровень радона превышает нормы. В качестве методов исследования были выбраны полимеразной цепной реакции (ПЦР) с последующим анализом полиморфизма длин рестрикционных фрагментов (ПДРФ) и иммуногистохимический анализ (IHC). Объектом исследования служили образцы крови и биопсийный материал полученные от пациентов с диагнозом рак легких, находящихся на лечении в Алматинском онкологическом центре и проживающих в городе Алматы. Наши исследования не выявили мутаций в гене C-KIT, но было показано, что 34,09% больных раком легких имеют избыточную экспрессию в опухолевой ткани. Что говорит о том, что нарушения произошли в клетках опухолевой ткани и не наследуются. На основании наших наблюдений, мы пришли к выводу, что высокая эманация радона вызвала нарушение в гене C-KIT, что привело к ее повышенной экспрессии.

Ключевые слова: C-KIT, экспрессия, мутация, радон, иммуногис-

UDK: 573.7; 575.22; 577.346

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ASSOCIATED RISKS OF LUNG CANCER WITH RADON EMANATION

Introduction

In accordance with the current seismic zoning map of Kazakhstan, the territory of Almaty is crossed by 5 tectonic faults. Consequently, there is a continuous emanation of radon from these faults that penetrates the houses and industrial premises. In many countries, radon is the second leading cause of lung cancer. The proportion of all lung cancers caused by radon makes up from 3% to 14% [1]. In this connection, the aim of our study is to investigate topological regularities of soil radon emanation and their effects on cancer incidence in population of the Republic of Kazakhstan. Lung cancer is one of the most closely studied cancer, which is not surprising taking into account a high frequency of this disease and more than modest success of a standard therapy [1-2]. Every year approximately nearly 3669 new cases of lung cancer are detected in Kazakhstan. The five year survival rate for 2014 was 33.2%, and 2738 people died [3].

The results of studies using some modern methods of molecular biology made it possible radically to change our ideas concerning peculiarities of emerging various forms of the disease and, consequently, treatment tactics and its predict course. The development in the field of genotyping changed lung cancer treatment clinical practice and showed gene involvement in the pathogenesis and prognosis such as the: EGFR, KRAS, C-KIT, BRAF, MET, ROS2 et al.[4].

The aim of our work was to study of the protein C-KIT the expression by immunohistochemical method and identification of germ-line mutations in lung cancer patients who live along the tectonic faults. C-KIT proto-oncogene is a transmembrane tyrosine kinase receptor of type III, that has a high homology with platelet-derived growth factor receptor (PDGF) and colony stimulating growth factor (CSF-1 or-fms) [5]. This gene is expressed on hematopoietic stem cells, mast cells, gametocytes, melanocytes, intraepithelial lymphocytes within epithelium of mammary glands interstitial cells of Cajal in the gastrointestinal tract [6]. Receptor activation occurs while binding to the C-KIT ligand, which is considered a stem cell factor. Binding of a ligand leads to receptor dimerization and interior tyrosine kinase activation that leads to that results in signaling

pathway, playing an essential role in cell survival, proliferation and cell differentiation [7]. There are several protein isoforms. Literature data showed that abnormal gene expression and mutations lead to the signaling ways activation and cell proliferation. KIT mutations are associated with a number of malignancies. Drug production, where for the C-KIT is a target, contributes to the development of clinical diagnosis and cancer treatment [8].

C-KIT receptor signaling pathway plays an important role in regulating the synthesis of erythrocytes, proliferation of lymphocyte development and function of mast cells, the formation of melanin and gamete formation. Specific binding of SCF causes homologous dimerization and signal transduction in the future. Subsequently it regulates gene expression, growth, proliferation and differentiation of cells.

The mechanism of C-KIT receptor activation has been studied by Satoru Yuzawa et al. SCF binding to the extracellular domain of C-KIT leads to a receptor dimerization of the two monomers and thus to its activation. As a result of C-KIT receptor activation tyrosine residues autophosphorylation occurs mainly both outside and inside kinase domain at a position of 823 and 900 [9,10]. Autophosphorylation performs two functions: firstly, it increases kinase activity and, secondly, it creates a high affinity Src homolog 2 (SH2) interaction protein or phosphotyrosine binding (PTB) domain [11, 12]. Proteins that interact with the activated receptor can, in its turn phosphorylat and initiate signaling. Besides serine and threonine phosphorylate as well. The importance of the phosphorylation is not clear at present. However, in the case of PKC-dependent phosphorylation of S741 and S746 with the inhibition of the C-KIT tyrosine kinase activity there is negative feedback [13].

The participation of C-KIT receptor in tumor development. The C-KIT role in the formation of tumor is ambiguous. On the one hand, a few types of tumors are associated either with C-KIT activation or through overexpression, expression of its ligand or mutation. On the other hand, there are such tumors as breast cancer, thyroid carcinoma and melanoma, where the malignant progression occurs at the same time at C-KIT expression loss. In fact, the forced of C-KIT expression in a highly metastatic melanoma leads to apoptosis [14].

Mutations destroying the function of tyrosine kinases, thereby leading to the development of cancer were known in the early 1980s. Activating mutations in this gene are associated with gastrointestinal stromal tumor of the stomach, testicular seminoma, mastocytosis, melanoma and acute myeloid leukemia.

Mutations often occur in membrane-proximal immunoglobulin-like domain (D5, exon 8 and 9), close to the membrane domain (exon 11), and a tyrosine kinase domain (exon 17). Mutations are deletions, point mutations and insertions duplication that can lead to C-KIT receptor activation. According to the latest date, mutations in 11 and 17 exons can reduce self-inhibition that leads to a sustained C-KIT receptor activation [15].

In gastrointestinal stromal tumors (GIST), C-KIT mutations can be found approximately in 85% of tumors and these mutations C-KIT receptor activate, that leads to tumor growth. C-KIT mutations in GIST are often located in exon 11, which encodes a C-KIT juxtamembrane region. This region is associated in a wild-type C-KIT kinase domain, where the inhibition of tyrosine kinase activity occurs. Mutations in this region lead to release of suppression and activation of tyrosine kinases. The less common mutations are in exon 9 (encoding an extracellular portion of C-KIT) and exon 17 (encoding the kinase domain). It should be noted that duplication of stromal gastrointestinal tumors (GIST) fragments Ala503 ~ Tyr502 and Ala502 ~ Phe506 can be found [16].

Deletions or insertions in exon 8 (either absent or replaced Asp419) have been found in acute myeloid leukemia (AML). Almost all the mutations activating proto-oncogene occur on the surface of the D5-D5 and these mutations can increase the affinity of neighboring D5-D5 domains [17]. Moreover, paracrine or autocrine C-KIT receptor activation may plays an essential role in many other human malignancies, such as ovarian cancer, small cell lung cancer, etc [18].

Materials and methods

Study population

The objects of research were peripheral blood samples and histological material obtained at surgery from patients diagnosed with lung cancer, who were the inhabitants of Almaty and receiving a treatment at the Almaty Oncology Center. A voluntary informed consents were obtained from all the patients before sampling. A detailed questionnaire including information about sex, age, smoking status, and other personnel data were collected from patients. Clinical investigation data contain the information about histological type of tumor and developmental stage defined by TNM criteria. The study protocol was approved by the Ethics Commit-

tee of the Asfendiyarov Kazakh National Medical University (Almaty, Kazakhstan).

C-KIT gene mutation analysis

The 44 peripheral blood samples were used for identification of mutations in 11 exons of C-KIT gene at lung cancer patients. A polymerase chain reaction (PCR) with a subsequent analysis of restriction fragment length polymorphism (RFLP) was carried out. To perform the PCR-RFLP analysis a genomic DNA have been isolated from peripheral blood lymphocytes using a GeneJet Genomic DNA Purification Kit (Thermo Scientific, USA) in accordance with the protocol suggested by a manufacturer. Then the PCR analysis with specific primers for 11 exons of the C-KIT gene has been carried out. Design of primers for genotyping V560G (f-GATCTATTTTTCCCTTTCTC and r- AGCCCCT-GTTTCATACTGAC), polymorphism was performed using the PrimerQuest Tool.

20-100 ng of target DNA was amplified in total volume of 20 μl of PCR mixture using the «Mastercycler» (Eppendorf, Germany). PCR reactions contained 10 pM of each specific primer, 10 mM of each dNTP, 2 μl of 10xPCR buffer (10 mM KCl, 100 mM Tris HCl, pH 9.0) and 0.5U of Taq-polymerase (Sigma-Aldrich, USA). PCR conditions: denaturation of DNA at 94^0C for two minutes, then: 94^0C – 40 sec, 55^0C – 30 sec, 72^0C – 40 seconds total – 35 cycles, with a final synthesis at 72^0C for 9 minutes. The PCR products were digested at 37^0C for 3 hours with 1-2U of AgsI endonucleases (Thermo Scientific, USA) in total volume of 10 μL reaction mixture.

Tissue Microarrays (TMAs)

44 histological materials obtained from lung cancer patients were used for creating of TMA for a immunohistochemical analysis. The materials were the formalin-fixed tumor tissues soaked in paraffin blocks. Every selected area of a lung tissue was analyzed by a qualified pathologist and tumor histologic types were determined. Pieces of tissue were taken from selected areas by a hollow cylinder of 2 mm diameter and then transferred together into a single paraffin block (Fig.1). Then a 3-4 micron sections, which were placed on glass slides, were obtained using the microtome (RM2255,Leica, Wetzlar, Germany) [19].

Immunohistochemical analysis of the C-KIT gene

For immunohistochemical analysis of patients using tumor tissue. The slides were deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Antigen retrieval was performed by heating tissue sections in ethylenediaminetetraacetic acid (EDTA) buffer (1 mM, pH 9.0) in a pressure cooker for one minute. Endogenous peroxidase was blocked with hydrogen peroxide (3%) for 10 minutes and Tris-buffered saline plus Tween 20 (0.05%, pH 7.4) was used for all washes and diluents Immunohistochemical staining was performed using a 1:500 dilution of the polyclonal antibody CD117 (DAKO, Glostrup, Denmark), was added to the slides and incubated at 4°C overnight, followed by the second antibody. The slides were briefly counterstained with hematoxylin. Preparations were analyzed using NanoZoomer-XR Digital slide scanner C12000 (Hamamatsu Photonics, Japan) software NDP.scan 2.5.

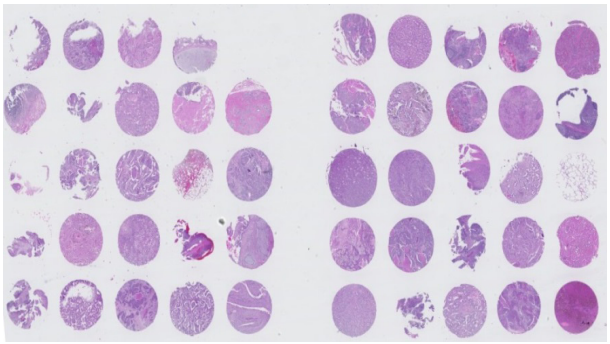


Figure 1 – Tissue microarray, slid with cores of 2 mm in diameter

Results of research and discussions

Lung cancer patients cohort

44 patients suffering from lung cancer participating in this study were received their treatment from January 2013 to February 2016. Moreover, all operations were performed by the same surgeon. The collecting of questionnaires and voluntary informed consents was done before the operation and fallowing sampling the biomaterials. By the time of conducting analysis, 42 patients were still alive, two died during a postoperative period. The main characteristics of investigated patients are shown in Table 1.

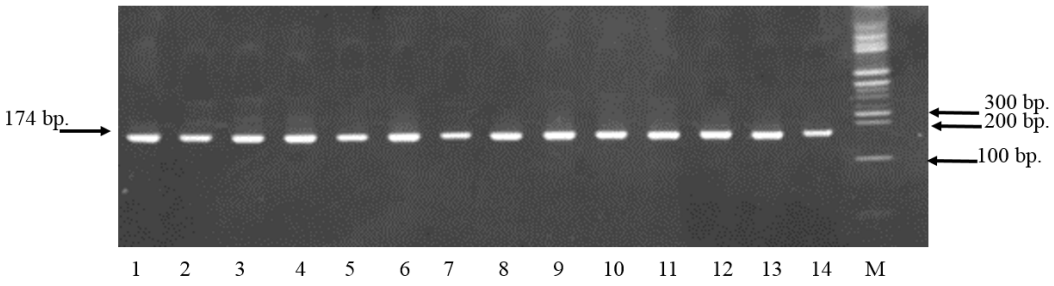
Among 44 patients there was 70.5% male and 29.5% female, that corresponds to the literature data on a more frequent cases of this type cancer in men. It is also known that smoking is a major risk factor for the disease. In our group there were 95.5% smokers. According to a histological type all cancer patients were distributed in the following way: a squamous cell of lung cancer – 63.6%, adenocarcinoma – 34.1%, a small cell lung cancer – 2.3%. All tumors were staged using TNM criteria: stage

I – 10 cases (22.73%); stage II – 15 cases (34.09%); stage III – 15 cases (34.09%) and stage IV – 4 cases (9.09%).

PCR products were digested with AgsI, 10x Buffer Tango (*Thermo Fisher Scientific, USA*). To visualize the restriction products was carried out by electrophoresis in 15% polyacrylamide gel. PCR products in size 174 bp in case of presence of mutations must have a restriction site for AgsI endonuclease, which leads to formation two fragments of 50 bp and 124 bp. The absence mutations does not lead to hydrolysis of the PCR product (Fig.2).

Table 1 – Patients' clinical characteristics

Number of patients	Median age, y	Histology, cases			Sex, cases		Smoking, cases	
		Adenocarcinoma	Squamous	Small cell lung cancer	M	F	Smoker	Never smoker
44	62±7 (46-76)	15	28	1	31	13	42	2



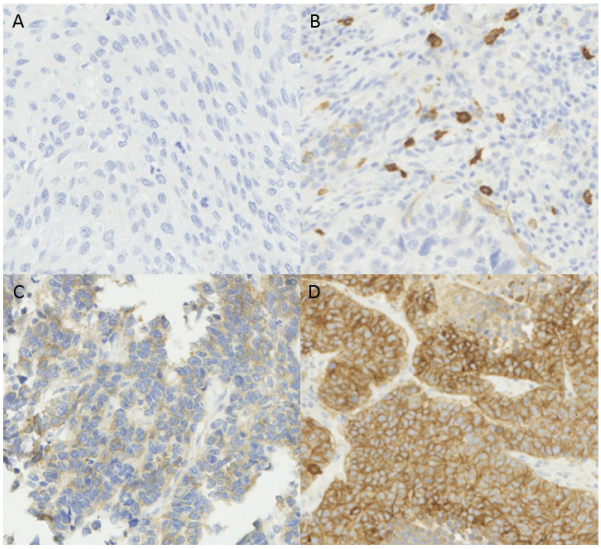
M – molecular weight marker 25bp.;
1-14 DNA samples from lung cancer patients without mutations in exon 21.

Figure 2 – The restriction products Electrophoregram amplificate C-KIT gene for mutations in exon 11 V560G

The Immunohistochemical staining was carried out on slides using the TMA. The assessment of an immunohistological staining was as follows: (0) no stained cells, (1+) faint or weak color intensity, less than 10% of tumor cells; (2+) moderate intensity staining, more than 10% of tumor cells; and (3+), strong, granular staining intensity. 2+ and 3+ have been identified as positive, and 0 and 1+ as negative ones (Fig. 3) [20].

The conducted immunohistochemical analysis revealed that 3 (6,82%) patients had the EGFR overexpression gene, 12 (27,27%) patients had a moderate expression (more than 10%), 22 (50 %) patients showed a weak expression 1+ (less than 10 %) and 7 (15.91 %) patients had no expression at all. On the whole 15 (34,09 %) patients demonstrated a positive response to the immunohistochemical analysis (Table 2**)**.

There are a lot of genetic mutations associated with the occurrence of cancer, EGFR mutation in NSCLC patients, the KRAS in colorectal cancer and lung cancer, and C-KIT mutations in patients with GIST in particular [21]. C-KIT tyrosine kinase can be activated independently on the ligand, through some specific mutations in the oncogene. The studies conducted in Japan and Europe have shown that lung cancer patients have mutations in the C-KIT oncogene [22]. Sekido et al. studied 15 SCLC cell lines as well as 13 primary tumors of lung cancer specimens, and reported that there is a mutation in the C-KIT gene, which occurred in the transmembrane domain at codon 541 with a frequency of 6.7% (1/15) and 7.7% (1/13) in the primary tumor samples, accordingly [23]. In our studies we conducted a genetic analysis of 11 exons for the presence of mutations at codon V560G. The RFLP analysis showed no mutations in codon under investigation. This may be due to the fact that DNA was isolated from the blood cells, rather than that from tumor tissue. That testifies to the fact that disorders occurred in the cells of the tumor tissue, and they are not inherited. Based on our observations, we arrived at conclusion that a high radon emanation has caused disorder in the C-KIT gene, which led to its overexpression.



A – negative tissue sample, B – a sample of tissue with low expression – 1+,
C – a sample of tissue with moderate expression 2+,
D – a tissue sample with high expression – 3+.

Figure 3 – Immunohistochemical analysis of histological material of lung cancer patients

Table 2 – The results of the immunohistochemical analysis

Number of analyzed patients	The number of patients not having the EGFR gene expression (0)	The number of patients with low expression of the EGFR gene (1+)	The number of patients with moderate expression of the EGFR gene (2+)	The number of patients with high expression of the EGFR gene (3+)
44 (100%)	7 (15.91 %)	22 (50%)	12 (27,27%)	3 (6,82%)

The study has been carried out according to the GF4/2554 «The study of radon oncology danger for population by measuring the vertical, horizontal and temporal topology of emanation and its accumulation in biological objects» project. Project Manager Biyasheva Z.M.

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