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Mutation and expression of the *C-KIT* gene on population of Kazakhstan

Abstract: Lung cancer is still a leading cause of death from malignant tumors worldwide. Due to late diagnosis, results of treatment remain unsatisfactory. In Kazakhstan about 3,669 new cases of lung cancer are detected every year, with a 5-year survival rate for 2016 equal to 48.0%. The aim of the current work is to study the expression of C-KIT protein and identify mutations in patients with lung cancer living in Almaty region. Literature data showed that anomalous expression of the corresponding gene and presence of mutations lead to a number of malignant neoplasms. Creation of drugs targeting C-KIT protein, promotes the development of clinical diagnosis and treatment of cancer. Blood samples and biopsy material obtained from patients diagnosed with lung cancer, treated at the Almaty Oncology Center and residing in Almaty region served the objects of the study. Written informed consent was obtained from all patients. Analysis of restriction fragment length polymorphism and immunohistochemical analysis followed the polymerase chain reaction. For immunohistochemical analysis tissue fixed in formalin and embedded in paraffin blocks was used. The presence of mutations in codon 557 was revealed by the genetic analysis of 11 exon of *C-KIT* gene. RFLP analysis and sequencing showed mutations in the codon. Immunohistochemical analysis revealed overexpression of *C-KIT* gene in four (9.09%) patients, 14 (31.82%) patients had moderate expression, 24 (54.54%) patients had weak expression and two (4.55%) patients had no expression. In total, 18 (40.91%) patients had a positive response to immunohistochemical analysis. This suggests that the disorders occur in the cells of tumor tissue and are not inherited. In fact, in most cases the occurrence of lung cancer can be associated with smoking.
Key words: *C-KIT* gene, mutation, expression, immunohistochemical analysis.

Introduction

Cancer remains the leading cause of death worldwide, despite impressive advances in the early diagnosis and treatment of patients with malignant neoplasms. Incidence rates in Kazakhstan are highest among CIS countries [1]; with 36 813 cases detected in 2016 [2]. Lung cancer is one of the most common types of cancer and the leading cause of death from cancer worldwide [3]. This type of cancer is the most common in the world, and in 2016, it amounted to 9.9% of the total number of new cases of cancer in Kazakhstan. In 2016, about 3,635 new cases of lung cancer were detected in Kazakhstan, with five-year survival equal to 48.0%, and 2,498 death cases [2]. High mortality from lung cancer is believed to be caused by aggressiveness, invasive and metastatic disease potential, as well as difficulties in detecting it in the early stages. Therefore, the search for pathogenetic approaches in the treatment of cancer remains an urgent problem today. The lack of significant achievements in the fight against cancer is largely due to an insufficient level of diagnosis. For the vast majority of people, the cancer is still diagnosed in the late stages of development (73.5%), in which the possibilities of modern treatment methods cannot be fully realized, as patients may have resistance to chemotherapy and targeted drugs. Ten years ago, therapeutic strategies for treating lung cancer were based on a histological type of classification. However, in recent years, one of the most interesting advances in the treatment of lung cancer has been the

understanding of genetic changes in cells. Achievements in genomic sequencing and identification of molecular markers over the past decade have clearly demonstrated that cancer is a heterogeneous disease [4; 5].

The studies using some modern methods of molecular biology have allowed us to fundamentally change our ideas about the characteristics of the various forms of the disease that arise and, consequently, the treatment tactics and its predicted course. Developments in genotyping have changed the clinical practice of treating lung cancer and have shown that genetic changes in the *EGFR*, *ALK*, *ROS1*, *HER2*, *KIT* and *BRAF* genes are powerful predictive biomarkers in the treatment of lung cancer [6-10].

In this regard, the purpose of the current research was to study the expression of C-KIT protein and identify mutations in patients with lung cancer living in Almaty region.

Materials and methods

Patients. Surgical specimens were obtained during the surgery from patients diagnosed with a lung cancer. All patients underwent a survey, which contained information on clinical data, gender, age and bad habits. Patients signed a voluntary informed consent before participating in the study. The study protocol was approved by the local ethics committee of the medical faculty of the Higher School of Public Health at Al-Farabi Kazakh National University (Almaty, Kazakhstan) – protocol No. IRB – A024 from September 22, 2017.

DNA preparation. Genomic DNA was isolated and purified from formalin-fixed paraffin-embedded tissues using the GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, USA). Purified DNA samples were aliquoted and stored in a freezer at -20˚C.

PCR and RLFP analysis. Specific primers (forward 5'-GATCTATTTTTCCCTTTCTC-3' and reverse 5'-AGCCCCTGTTTCATACTGAC-3') were designed using the PrimerQuest Tool. Each PCR reaction mixture contained forward and reverse primers (10 pmol each), 25 ng of genomic DNA, 10 µL of Mmix (2x, Thermo Fisher Scientific, USA) and nuclease-free water. PCR was performed in 0.2 mL microtubes on Mastercycler^{R} Nexus thermal cycler (Thermocycler Eppendorf™, USA). PCR conditions consisted of initial denaturation step at 95°C for 4 min, then 35 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 45 sec, and final extension step at 72°C for 5 min.

The PCR products were analyzed using electrophoresis on 1% agarose gel. The correspondence of the obtained fragments was evaluated using the DNA Ladder Gene Ruler 100 bp marker (Thermo Fisher Scientific, USA). Resrtriction endonucleases were selected using WatCut online software for SNP-RFLP analysis. The PCR products were digested with restriction endonucleases SsiI and FokI. 5 µL of the PCR product and 10U of endonuclease were used in the reaction carried out at 3˚C for 3 h. Restricted DNA was analyzed on 8% polyacrylamide gel. The polyacrylamide gel was stained with ethidium bromide. DNA Ladder Gene Ruler 100 bp (Thermo Fisher Scientific, USA) was used for detection of the restriction fragments length.

Tissue microarrays. Before creating a Tissue Microarrays (TMAs) slide, all tumor tissues were classified according to the international classification of stages of malignant neoplasms – TNM [11].

As a material for the creation of TMAs slides and immunohistochemical analysis, 44 histological materials obtained from patients with lung cancer fixed in formalin and embedded in paraffin blocks were used. Each selected area of lung tissue was analyzed by a qualified pathologist to determine histological types of tumors. From selected sites, selected tissue was carefully taken with a hollow cylinder (2 mm in diameter) and transferred into one common paraffin block. Then, a 3-4 micron thick section was cut from this block using a microtome (RM2255, Leica, Wetzlar, Germany) and placed on a glass slide [12] (Figure 1).

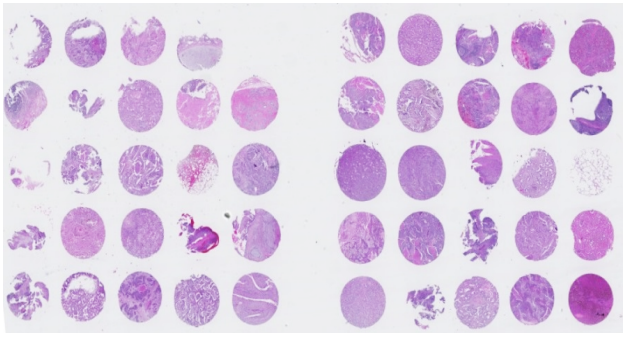

Figure 1 – Tissue Microarrays, slid with cores of 2 mm in diameter

Figure 1 shows the location of all TMAs samples on a glass slide. All 44 samples were divided into 5 rows of 9 samples (5th row – 8 samples).

Immunohistochemistry. Immunohistochemical staining was performed on slides using TMAs. For staining polyclonal antibodies CD117 (DAKO, Glos-

trup, Denmark) were used at dilution of 1:500. The preparations were analyzed using a NanoZoomer-XR C12000 digital slide scanner (Hamamatsu Photonics, Japan) with NDP.scan 2.5 software. The evaluation of immunohistological staining was distributed as follows: 0, no stained cells or weak staining intensity, with less than 10% of tumor cells; 1+, pale or weak color intensity, with more than 10% of tumor cells; 2+, moderate staining intensity; and 3+, strong, granular staining intensity; 2+ and 3+ were identified as positive, and 0 and 1+ as negative [13].

Results and discussion

The patients with lung cancer included in this study were treated at the Oncology center from January, 2013 to February, 2016. Questionnaires and voluntary informed consent were collected prior to the collection of the biomaterial. The main characteristics of the patients are shown in Table 1.

As can be seen from the Table 1, among 44 patients, 70.45% were men and 29.55% women, which corresponds to the data on more frequent cases of this type of cancer in men. Tumors were classified according to TNM criteria, and patients were divided by the following stages of cancer: 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%). According to the histological type, the distribution was as follows: squamous cell lung cancer – 63.6%, adenocarcinoma – 34.1%, small cell lung cancer – 2.3%.

Table 1 – The characteristics of the lung cancer case and control cohorts

Cohort (persons)	Years of birth (average age)	Sex, persons (%)		Ethnicity, persons (%)		Smoking habit, persons (%)		
		Males	Females	Asians (Kazakhs, Uigur, Koreans)	Russian	Smokers	Non-smokers	Ex-smokers
Case (44)	1937-1967 (62.6±7.7)	31 (70.45)	13 (29.55)	23 (52.27)	21 (47.73)	31 (70.45)	12 (27.27)	1 (2.27)
Control (50)	1934-1968 (61.7±9.2)	32 (64.00)	18 (36.00)	35 (70.00)	15 (30.00)	13 (26.00)	36 (72.00)	1 (2.0)
t_{st}	0.075	0.381	0.533	1.147	1.374	2.348	2.207	0.103
P	*0.952	0.768	0.688	0.456	0.400	0.256	0.271	0.935

Analysis of the restriction products for the presence of the Trp557Arg and Val559Asp mutations was analysed on 8% polyacrylamide gel. When analyzing restriction products for the presence of the Trp557Arg mutation, the normal allele in homozygous state has a single fragment of 258 bp in size. The mutant allele in homozygous state has two fragments 169 bp and 89 bp. Consequently, the heterozygous genotype has 3 fragments of 258 bp, 169 bp and 89 bp. RFLP analysis of 11 exon for the presence of Val559Asp mutation homozygous wild allele has one fragment of 258 bp, a homozygous mutant allele – two fragments of 150 and 108 bp, and the heterozygous allele – three fragments of 258 bp, 150 bp and 108 bp (Figure 2).

Figure 2 shows that sample number seven has three fragments measuring 258 bp, 169 bp and 89 bp, which indicates the heterozygosity of this gene for Trp557Arg mutations.

After RLFP analysis DNA samples were sent for sequencing analysis (9, 11 and 13 exons) to the National Cancer Institute, the National Institutes of Health in Bethesda, Maryland. The sequence analysis also did not reveal genetic abnormalities in the *C-KIT* gene, with the exception of the Trp557Arg mutation in sample number 7.

Figure 3 shows the level of expression of the C-KIT gene, which may be the cause of the development of cancer; immunohistochemical staining is applied.

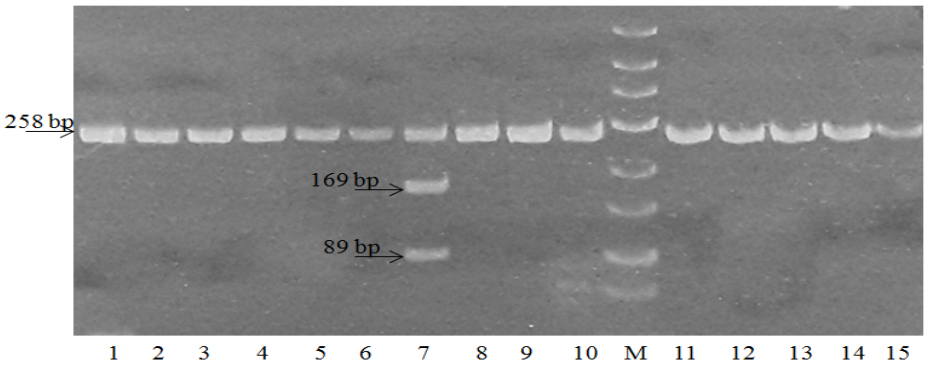

Figure 2 – The electropherogram of the restriction products of the *C-KIT* gene (11 exon, Trp557Arg and Val559Asp). Note: M – molecular weight marker 25bp; 1–6, 8–10 PCR products without mutations Trp557Arg; 7 – PCR products with mutation Trp557Arg; 11–15 PCR products without mutations Val559Asp

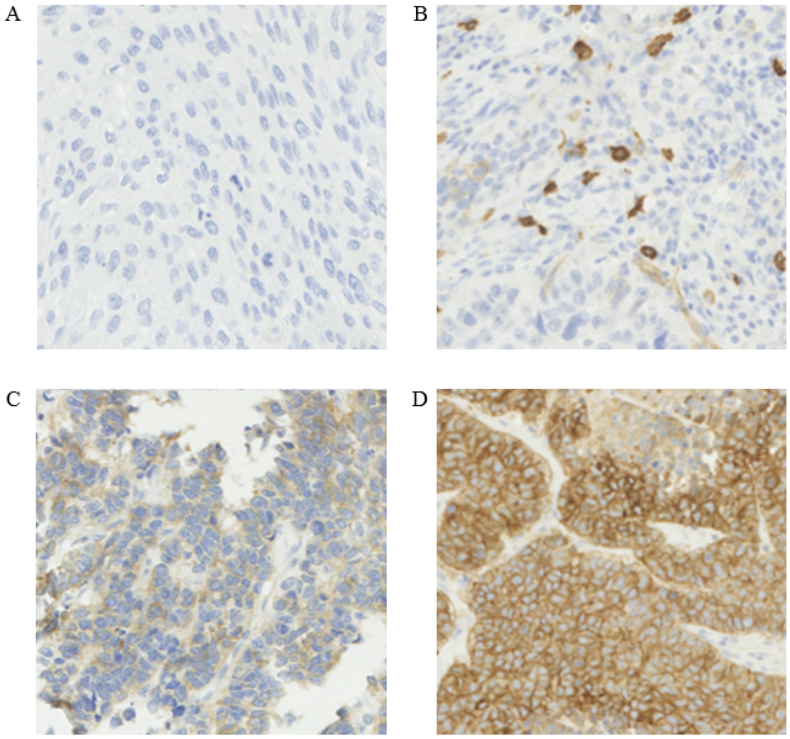

Figure 3 – Immunohistochemical analysis of histological material of lung cancer patients

Figure 3.A shows negative staining, indicating lack of expression; Figure 3.B – a sample of tissue with weak staining shows moderate expression; Figures 3. C and 3.D – the samples of tissue with moderate staining (2+) and strong staining (3+) show a high level of expression of the analyzed gene.

Table 2 shows the results of the immunohistochemical analysis. Immunohistochemical staining revealed 4 patients (9.09%) had overexpression of the *C-KIT* gene, 14 (31.82%) had moderate expression (more than 10%), 24 (54.54 %) showed weak expression of 1+ (less than 10%) and 2 patients (4.55%) did not have expression.

Table 2 – The results of the immunohistochemical analysis

Number of analyzed patients	The number of patients not having the C-KIT gene expression (0)	The number of patients with low expression of the C-KIT gene (1+)	The number of patients with moderate expression of the C-KIT gene (2+)	The number of patients with high expression of the C-KIT gene (3+)
44	2 (4.55 %)	24 (54.54%)	14 (31.82%)	4 (9.09%)

As can be seen from the Table 2, patients having a positive response to immunohistochemical analysis amounted to 40.91%, what could be the cause of the lung cancer. Accordingly, the cause of the lung cancer in these patients may be an increased expression of the *C-KIT* gene.

The *C-KIT* gene is an oncogene that regulates cell division and interacts with cell cycle factors. Solid tumors such as breast cancer, neuroblastoma, colon cancer, gynecological tumors, glioma, and SCLC reported to express *C-KIT* [14]. Autocrine or paracrine activation of the C-CIT receptor using GFR has been postulated for the treatment of lung cancer. The receptor can also be constitutionally activated independent of the ligand by specific mutations of the *C-KIT* gene. The receptor can also be constitutively activated independently on the ligand by specific mutations of the *C-KIT* gene. Expression of C-KIT protein was detected in 79-88% of SCLC cell lines, and during the inactivation of *C-KIT* the tyrosine kinase inhibitor imatinib had an inhibitory effect on SCLC cell growth [15].

Micke et al. showed that *C-KIT* expression is associated with reduced survival [16], however Blackhall et al. in their studies demonstrated *C-KIT* expression in 51% of tumors and concluded that *C-KIT* expression is not a predictor of survival [17]. Studies of the correlation between the level of C-KIT protein synthesis and the rate of the cancer development have led to the conflicting results. In some cases, the correlation was significant, while in other experiments it was absent [18; 19].

However, there is a little information on the mutations in the studied gene and protein expression in non-small cell lung cancer. Antonescu et al. revealed a Trp557Arg mutation in the gastrointestinal stromal tumor [20]. Like other *C-KIT* mutations occurring in exon 11, this mutation is thought to be associated with increased sensitivity to imatinib and other *C-KIT* inhibitors [21].

In our studies, a mutation in the *C-KIT* gene was detected in one patient with a diagnosis of adenocarcinoma and with a stage of the disease – T2aN-0M0G2R0. The results of immunohistochemical analysis showed that this patient has a moderate level of expression of C-KIT protein (2+). However, despite the data obtained, our results showed that for non-small cell lung cancer, the expression of the *C-KIT* gene is not associated with mutations and are rare as in other researchers. Analysis of the expression level showed that 40.91% of patients had increased expression of the *C-KIT* gene, which is a high indicator for the development of cancer. Thus, the relationship between gene expression and mutations was not observed in our studies, as well as the relationship between expression and cancer stages in patients. *C-KIT* gene expression level may be due to gene amplification rather than mutations.

Conclusion

Our results show that the level of C-KIT gene expression is high for patients with lung cancer in Kazakhstan and is much more common than genetic disorders in this gene. Due to the high frequency of expression of this gene (40.91%), it is highly recommended for patients to undergo various forms of lung screening tests.

This will help to find the spectrum of causes of the disease and find the right treatment for the targeted therapy. There was not any relationship between disease stages and survival, despite the high level of *C-KIT* gene expression.

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