ORIGINAL RESEARCH

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FREQUENCY OF HETEROZYGOUS CARRIAGE OF MUTATIONS IN THE GENES OF NOTCH SIGNAL PATH IN PATIENTS WITH CLEAR CELL KIDNEY CANCER AND IN THE POPULATIONS OF THE VOLGA-URAL REGION

Aim of the study: to determine the frequency of mutations in the Notch signaling pathway (*DLL4* (rs35748882), *HEY2* (rs61737181), *JAG1* (rs1801140, rs1801139, rs45575136), *NOTCH1* (rs61751542), *NOTCH2* (rs3795666), *NOTCH4* (rs8192576, rs8192579, rs8192585) identified earlier as a result of exome sequencing in an expanded group of patients with clear cell renal cell carcinoma.

Materials and methods. The study included 238 paired samples of tumor and normal kidney tissue from patients with clear cell renal cell carcinoma. Detection of nucleotide sequence alterations of genes was performed using PCR followed by RFLP analysis. Restriction enzymes were selected using the NEBcutter V2.0 Internet resource.

Results. On average, the frequency of detected changes in the group of clear cell renal cell carcinoma patients was higher than the general population values. The highest frequency was found for rs8192579 and rs8192585 NOTCH4 gene. Clinical and pathological characteristics of the tumors in which mutations were identified, were heterogeneous and included patients with both early and late stages.

Conclusions. The results obtained in this study may indicate the contribution Notch signaling pathway gene alterations to the pathogenesis of clear cell renal cell carcinoma, as well as the possibility of their use in creating a molecular markers panel for the diagnosis and prognosis of the course of the disease.

Introduction. Renal cancer (RC) is a heterogeneous group of malignant tumors, the overwhelming majority of which are renal cell carcinomas of various morphological types. More than 300 thousand new cases of RC are registered in the world every year (Mohammadian et al., 2017). Due to the asymptomatic course of renal cancer, the disease is often detected in the late stages, aggravated by metastasis. In this regard, there is a clear need for an in-depth molecular genetic study of the pathogenesis of renal cancer, which will make it possible to identify new molecular markers for the early diagnosis of RC, to carry out more effective risk assessments, to select patients for more aggressive treatment methods, and to select molecules that will serve as new drug targets. The main molecular genetic event in the development of renal cancer is the change in the activity of the von Hippel-Lindau tumor suppressor gene (*VHL*), which accompanies about 70% of cases of sporadic renal cancer. Along with the VHL gene, a number of genes involved in different molecular pathways are involved in the pathogenesis of renal cancer. Transmembrane receptors of the Notch family carry out regulatory actions, affecting proliferation, apoptosis, differentiation, angiogenesis, metastasis, and other cellular processes that induce the onset and development of malignant tumors. Signaling through Notch receptors protects cells from a variety of apoptotic stimuli [10]. The four Notch receptors (Notch-1, -2, -3, and -4) are single-pass heterodimeric transmembrane proteins that are activated by binding to one of five ligands, Delta-like 1/3/4 or Jagged 1/2, expressed on adjacent cells [11]. Several studies have shown that the Notch signaling pathway plays an important role in the formation of mammalian kidneys. It has been shown that inhibition of Notch signaling in mice leads to a decrease in the epithelial compartment in the developing kidney with degradation of the proximal tubules [13].

It is known that abnormal activation of the Notch signaling pathway genes is observed in various types of tumors. It is believed that the Notch and Wnt pathways can be used as therapeutic targets. In contrast to *VHL*, mutations in Wnt and Notch pathway members in renal cell carcinoma are rare [5]. However, *VHL* and other important molecular genetic factors responsible for the development of kidney cancer activate the Wnt and Notch pathways. For example, loss of *VHL* stabilizes β-catenin via *JADE1*. B-catenin activation, in turn, is associated with advanced renal cancer and lower patient survival. At the same time, it was shown that Notch pathway signaling does not depend on the activity of *VHL*, *HIF1α*, *HIF2α* and the level of cell oxygenation. [8].

Previously, we performed exome analysis in patients with clear cell renal cell carcinoma, as a result of which the most pathogenic changes in the nucleotide sequence were found in the genes of the Notch pathway. The purpose of this work was to determine the frequency of mutations in the Notch signaling pathway (*DLL4* (rs35748882), *HEY2* (rs61737181), *JAG1* (rs1801140, rs1801139, rs45575136), *NOTCH1* (rs61751542), *NOTCH2* (rs3795666), *NOTCH4* (rs8192576, rs8192579, rs8192585) identified earlier as a result of exome sequencing in an expanded group of patients with clear cell renal cell carcinoma.

Materials and methods. The study included 238 paired samples of tumor

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and normal kidney tissue from patients with clear cell renal cell carcinoma. All examined were patients of the clinic of the Bashkir State Medical University in Ufa. The collection of tissue samples was carried out by the staff of the Department of Urology. The study was approved by the Bioethical Committee of the Institute of Biochemistry and Genetics. In the study group, 56.7% of patients had early stages of the disease (stages I-II of the malignant process according to the TNM classification) and 43.3% of patients had late stages (III-IV stages of the malignant process according to the TNM classification). The age of the patients ranged from 37 to 89 years.

The mutation frequency was also studied in a group of healthy individuals from the population control of Bashkir, Russian and Tatar ethnicity (50 individuals in each ethnic group).

Isolation of genomic DNA from paired samples of kidney tumor tissue and adjacent normal renal parenchyma was carried out by phenol-chloroform extraction. The frequency of mutations was determined in the genes of the Notch pathway identified by exome sequencing. The most pathogenic variants were selected for analysis using six in silico programs (SIFT, PolyPhen-2, LRT, Mutation Assessor, Mutation-Taster, phyloP, and GERP ++) from dbNSFP v. 3.0a. Detection of changes in the nucleotide sequence of genes performed using PCR followed by RFLP analysis. Restriction enzymes were selected using the NEBcutter V2.0 Internet resource [12].

Results and discussion. We analyzed 10 gene loci of the Notch signaling pathway in tumor and normal kidney tissue in 238 patients with clear cell renal cell carcinoma: (*DLL4* (rs35748882), *HEY2* (rs61737181), *JAG1* (rs1801140, rs1801139, rs45575136), *NOTCH1* (rs61751542), *NOTCH2* (rs3795666), *NOTCH4* (rs8192576, rs8192579, rs8192585). The frequencies of Notch pathway gene mutations in the study group of patients are shown in Table 1.

All mutations studied here were found in a heterozygous state. On average, the frequency of detected changes in the group of patients with clear cell renal cell carcinoma was higher than the general population values. Clinical and pathological characteristics of the tumors in which mutations were identified, were heterogeneous and included patients with both early and late stages. There were also no significant differences in mutation frequencies with the sex and age of patients. No changes were found in the population control.

The highest frequency of occurrence among the studied loci was demonstrated by changes in the NOTCH4 gene (rs8192579 and rs8192585). The NOTCH4 protein is a single-pass transmembrane receptor containing extracellular (NECD) and intracellular domains (NICD). The extracellular domain NECD contains repeats similar to Epidermal Growth Factor 29 (EGF) and serves to bind ligands and calcium. When bound to ligands (including Jag 1 and 2, Delta-like 1, 3, and 4), NOTCH proteolysis occurs, which releases the intracellular NICD domains from the cell membrane. The NICD then moves to the nucleus. NICD, in turn, activates the expression of a group of downstream genes such as *Hes* and *Hay*. Notch signaling is an important pathway involving cell proliferation, differentiation, and apoptosis [1]. It has been shown that *NOTCH4* plays an important role in the regulation of breast growth and development. Moreover, abnormal expression of *NOTCH4* can inhibit the differentiation of breast stem cells, and mutations in this gene are associated with increased proliferation of epithelial cells [9]. Increased expression of *NOTCH4* is also noted in tumor tissues in liver and colorectal cancer [7], as well as in prostate cancer cell lines [8]. On the other hand, mutations in the *NOTCH4* gene were found to be associated with an increase in the overall survival of patients with gastric cancer who received immunotherapy with PD-1/PD-L1 inhibitors [2].

It is known that the activity of the *NOTCH4* gene is associated with proliferation, invasion, and migration of renal cancer cells, as well as with the size of the tumor and the level of its differentiation [6]. In addition, a recent study has demonstrated that mutations of the Notch signaling pathway genes can influence the effectiveness of immunotherapy in clear cell renal cell carcinoma [3].

Conclusions. The results obtained in this study may indicate the contribution of the studied changes in the nucleotide sequence of the Notch signaling pathway genes to the pathogenesis of clear cell renal cell carcinoma, as well as the possibility of their use in creating a molecular markers panel for the diagnosis and prognosis of the course of the disease. However, further studies are required in larger patient populations.

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Frequency of nucleotide sequence alterations of Notch signaling pathway genes in patients with clear cell renal cell carcinoma

Gene	Type of substitution	Frequency, n (%)	Average frequency in the general population according to Ensembl DB - 1000 Genomes Project Phase 3 (%)*
DLL4 c.C1239T (rs35748882)	synonymous	0/238 (0)	1.0
HEY2 c.G588C (rs61737181)	synonymous	1/238 (0.42)	5.0
JAG1 c.A2214C (rs1801140)	synonymous	2/238 (0.81)	1.0
JAG1 c.C1578T (rs1801139)	synonymous	5/238 (2.10)	1.0
JAG1 c.C924T (rs45575136)	synonymous	1/238 (0.42)	3.0
NOTCH1 c.C4129T (rs61751542)	nonsynonymous	5/238 (2.10)	9.0
NOTCH2 c.C6421T (rs3795666)	synonymous	0/238 (0)	5.0
NOTCH4 c.T4828C (rs8192576)	synonymous	3/238 (1.26)	5.0
NOTCH4 c.A5427G (rs8192579)	synonymous	7/238 (2.94)	8.0
NOTCH4 c.C731T (rs8192585)	nonsynonymous	8/238 (3.36)	2.0

* No changes were found in the populations of Russians, Tatars and Bashkirs from the Volga-Ural region of Russia.

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