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HUMAN GENETICS

Investigation of the Role of microRNA Associated with the VHL-HIFα-Dependent Pathway in Patients with Clear Cell Renal Cell Carcinoma

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Abstract—Renal cell carcinoma (RCC) is a common renal neoplasia of various morphological types, among which clear cell RCC is the most common. About 70% of sporadic cases of clear cell renal cell carcinoma are accompanied by inactivation of the von Hippel–Lindau gene (*VHL*). It is known that *VHL* is a target for several miRNAs—small noncoding RNAs that carry out post-transcriptional regulation of genes. The purpose of this study was to analyze the expression levels of miRNAs which target the *VHL* gene and also to analyze the association of genotypes and alleles of the *rs1642742* polymorphic locus of the *VHL* gene located at the miRNA binding site with the risk of developing clear cell renal cell carcinoma (ccRCC). As a result of the analysis, no statistically significant changes in the expression level were found, although miR-21 and miR-224 showed a tendency to increase expression in the tumor compared with normal kidney tissue ($p = 0.0597$ and $p = 0.0846$, respectively). Also, in a comparative analysis of the frequencies of genotypes and alleles of the polymorphic locus *rs1642742* of the *VHL* gene, an association of the *rs1642742*GG* genotype was found with the risk of developing ccRCC in a group of people over 55 years old ($p = 0.0381$; OR = 1.84; 95% CI (1.03–3.31)). Undoubtedly, further study of miRNAs on large groups of samples is necessary, which will make it possible both to identify new molecular markers of the risk of developing the disease and to form panels of prognostic markers.

Keywords: renal cell carcinoma, miRNA, *VHL*, gene expression
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INTRODUCTION

Renal cancer (RC) is a heterogeneous group of malignant tumors, the vast majority of which are renal cell carcinomas of various morphological types. More than 300000 new cases of RC are registered annually in the world. Among the genitourinary tumors, renal cancer takes third place and is first in mortality [1]. In 2016, in Russia, kidney tumors were primarily diagnosed in 23908 patients, and the increase in the incidence rate from 2006 to 2016 was 43.43% [2]. In the pathogenesis of renal cell carcinomas, particular attention is paid to a number of tumor suppressor genes involved in the suppression of oncogenes activity. It is known that a change in the activity of these genes can lead to the development and progression of the tumor. About 70% of sporadic cases of clear cell renal cell carcinoma are accompanied by inactivation of the von Hippel–Lindau gene (*VHL*) located on the short arm of chromosome 3 in 3p25 position (2011) [3]. The main function of *VHL* that inhibits tumor development is the mediation of degradation by the hypoxia-induced factor (HIF), which stimulates the expression of many target genes with oncogenic functions [4], such as *VEGF* (vascular endothelial growth factor) and *PDGFB* (platelet growth factor), as well as mitogenic factors, such as TGFα (transforming growth factor) [5]. Previously, we searched for changes in the nucleotide sequence of the gene *VHL*, as a result of which several mutations, resulting in destabilization of the pVHL domains, and altered protein synthesis were found [6].

To date, RC is detected mainly using various visualization methods: ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), etc. Renal cancer is characterized by an asymptomatic course; therefore, when a patient deals with discomfort and complaints, it is usually already locally advanced cancer, spread beyond the renal capsule, into the renal or

lower vena cava. In this regard, there is a clear need for new molecular markers that will make it possible to perform an early diagnosis of renal cell carcinoma (RCC), to carry out more effective risk assessments, to select patients for more aggressive treatment methods, and to select molecules that will serve as new targets for drugs. These molecular markers can be microRNAs—small single-stranded noncoding RNAs that participate in post-transcriptional regulation of genes and are promising diagnostic markers of the risk of developing many oncological diseases, including renal cancer [7]. It is known that for each miRNA there can be several target mRNAs, and vice versa, one coding gene can be the target of a number of different miRNAs. Analysis of miRNA expression profiles can be used for molecular classification of cancer by analogy with DNA microarrays and mRNA profiles [8]. In some oncourological diseases, specific microRNA expression profiles have been found that allow for early diagnosis and prognosis of the disease and prediction of the response to therapy [9]. In connection with the foregoing, the goal of this study was to analyze the levels of miRNA expression targeting the gene *VHL* and also analyze the association of genotypes and alleles of the polymorphic locus *rs1642742* of *VHL* gene located at the miRNA binding site with a risk of developing clear cell renal cell carcinoma (ccRCC)

MATERIALS AND METHODS

Analysis of miRNA expression was carried out on 30 paired DNA samples isolated from tumor tissue of the kidney and adjacent normal renal parenchyma of unrelated patients with ccRCC living in the Republic of Bashkortostan. All those examined were patients at the clinic of the Bashkir State Medical University of Ufa. The sampling of tissues and venous blood was carried out by employees of the Department of Urology. The study was approved by the bioethics committee of the Institute of Biochemistry and Genetics. The study group included patients with the initial stages of the disease (stages I–II of the malignant process, according to the TNM classification). The age of patients at the time of diagnosis ranged from 37 to 79 years. Analysis of association of genotypes and alleles of *rs1642742* of gene *VHL* with a risk of developing ccRCC was performed in 450 patients with clear cell renal cancer and 490 individuals from the control group. The control group according to age, sex, and territory of residence corresponded to the group of patients. All biological materials were obtained with the informed consent of patients.

Isolation of total RNA and miRNA was performed using the Direct-zol™ RNA MiniPrep kit (Zymo Research) using Zymo-Spin™ II C Column spin columns. DNA was isolated from venous blood by phenol-chloroform extraction. To determine the expression level, real-time quantitative PCR was performed in triplicate for each sample using the TaqMan MicroRNA Assays kit (Applied Biosystems) and the CFX96™ real-time PCR product detection system (BioRad). The method $2^{-\Delta Ct}$ was used to quantify gene expression; it is based on the fact that the difference in the value of the "threshold cycle" (ΔCt) between the target gene and the control gene is proportional to the level of relative expression of the target gene. As an endogenous control, small nuclear RNA U6 was used, characterized by constant expression in different tissues and cells.

Identification of genotypes of polymorphic locus *rs1642742* of microRNA binding site in the gene *VHL* was carried out using the TaqMan allele discrimination method. Allelic discrimination analysis was performed using the CFX96 Touch™ Real-Time PCR Detection System.

Statistical Data Processing

The MS Office Excel 2003 software package (Microsoft) and GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA) were used as a calculation tool. In pairwise comparison of the frequencies of genotypes and alleles in groups of patients and healthy individuals, the χ^2 criterion was used for 2 × 2 contingency tables with Yates correction for continuity. For all the analysis methods used, the differences were considered statistically significant at $p < 0.05$. The hypothesis of the significance of independent factors was tested on the basis of the coefficient *t*-statistics and significance level (*p*-value) for coefficient *t*. The exponential individual regression coefficient was interpreted as the odds ratio (OR) for the logistic model with the calculation of the 95% confidence interval (95% CI).

RESULTS

On the basis of published data, the following miRNAs were included in the study: miRNA-17, miRNA-21, miRNA-92a, miRNA-106a, miRNA-106b, and miRNA-224. A study of their expression profile was carried out on 30 paired samples of tumor and normal tissue of the kidney of patients with clear cell renal cell carcinoma. Clinical and pathological characteristics included in the study of patients are presented in Table 1. As a result of statistical processing of the obtained data, no significant differences in the levels of miRNA expression between the tumor tissue of the kidney and normal renal parenchyma were found (Fig. 1).

The frequency distribution of genotypes and alleles of the polymorphic locus *rs1642742* of gene *VHL* was studied in a group of patients with ccRCC ($N = 450$) and in a control group ($N = 490$). The characteristics of the studied groups are presented in Table 2. In a comparative analysis of the frequencies of genotypes and alleles of the polymorphic locus *rs1642742* of gene *VHL*, it was shown that the genotype *rs1642742*AA* ($p = 0.0165$; OR = 0.5978; 95% CI (0.3915–0.9213))

Table 1. Clinical and pathological characteristics of patients with ccRCC

Characteristic	Values
Age (years), range; average	37–79; 58
Sex	*n* (%)
men	18 (60.0)
women	12 (40.0)
Tumor size, cm	2.4–6.5
Furman gradation	*n* (%)
1–2	27 (90.0)
3–4	3 (3.0)

was found in patients with ccRCC at the age of 55 years and older significantly less than in the control group of the same age category, while the genotype *rs1642742*GG* was more common in patients, being a marker of an increased risk of developing ccRCC (Table 3).

Next, we performed an analysis of the association of genotypes and alleles of the polymorphic variant *rs1642742* with overall survival in patients with clear cell renal cancer. Follow-up information was available for 394 of 450 patients with ccRCC (87.3%). One patient died of postoperative complications within 30 days at the beginning of the study period, and this case was excluded from the analysis. The mean follow-up time for the remaining 393 patients with ccRCC was 29.6 months (range 1.0–60.0 months; 95% CI: 27.6–30.2 months). Forty-eight patients (12.2%) died of ccRCC during follow-up, 319 patients (81.2%) remained alive, and 26 (6.6%) died of other causes or were lost for follow-up. Survival curves for *rs1642742* were constructed and compared using dominant and recessive models (Fig. 2, Table 4). Statistically significant differences in survival of patients with ccRCC between carriers of genotypes *rs1642742*AA* and *rs1642742*GG* were not found.

DISCUSSION

Clear cell renal cell carcinoma is the most common subtype of renal cancer. The study of molecular markers of ccRCC is extremely important for early diagnosis and timely and correct treatment of patients.

In this work, we analyzed the expression of a number of miRNAs whose immediate target is the gene *VHL*. As a result of the analysis, no statistically significant changes in the expression level were found, although for miRNA-21 and miRNA-224 there was a tendency toward increased expression in the tumor compared to normal kidney tissue ($p = 0.0597$ and $p = 0.0846$, respectively). It is known that miRNA-21 plays a key role in tumor development by regulating various molecular mechanisms, such as cell proliferation, metastasis, angiogenesis, and antiapoptosis [10, 11]. It is known that miRNA-21 levels are elevated in various malignant neoplasms, such as cancer of the breast, lung, prostate, stomach, colon, pancreas [12], and kidney [13]. MicroRNA-21, together with miRNA-210 and miRNA-155, can reduce proapoptotic signaling in response to a hypoxic environment and have stably increased expression in various types of human tumors [14]. Besides *VHL*, other target genes involved in the pathogenesis of renal cancer are known for miRNA-21. Thus, in a study by H. Liu et al. [12], two miRNA-21 target genes were identified—*SLC12A1* and *TCF21*. Gene *SLC12A1* is a kidney-specific sodium-potassium chloride transporter; it plays a key role in the resorption of sodium chloride. In a study by S. Schrödter et al., a significant decrease in expression of *SLC12A1* was shown in kidney tumor tissue compared to normal tissue. In addition, measuring the expression of this

Table 2. Clinical, pathological, and demographic characteristics of groups of ccRCC patients and control

Characteristic	Patients ($n = 450$)	Control ($n = 490$)	*p*-value	χ^2
Age, years (mean ± SD)	56.74 ± 0.61	56.46 ± 0.45	0.736	0.935
Sex				
men	250 (55.6)	275 (56.1)	0.914	0.0124
women	200 (44.6)	215 (43.9)		
TNM stage				
I–II	243 (54.0)			
III–IV	207 (46.0)			
Ethnicity				
Bashkirs	111 (24.7)	119 (24.3)	0.9532	0.004
Tatars	159 (35.3)	221 (45.1)	0.0038	8.894
Russians	180 (40.0)	150 (30.6)	0.0042	8.6674

The fraction is given in parentheses in %.

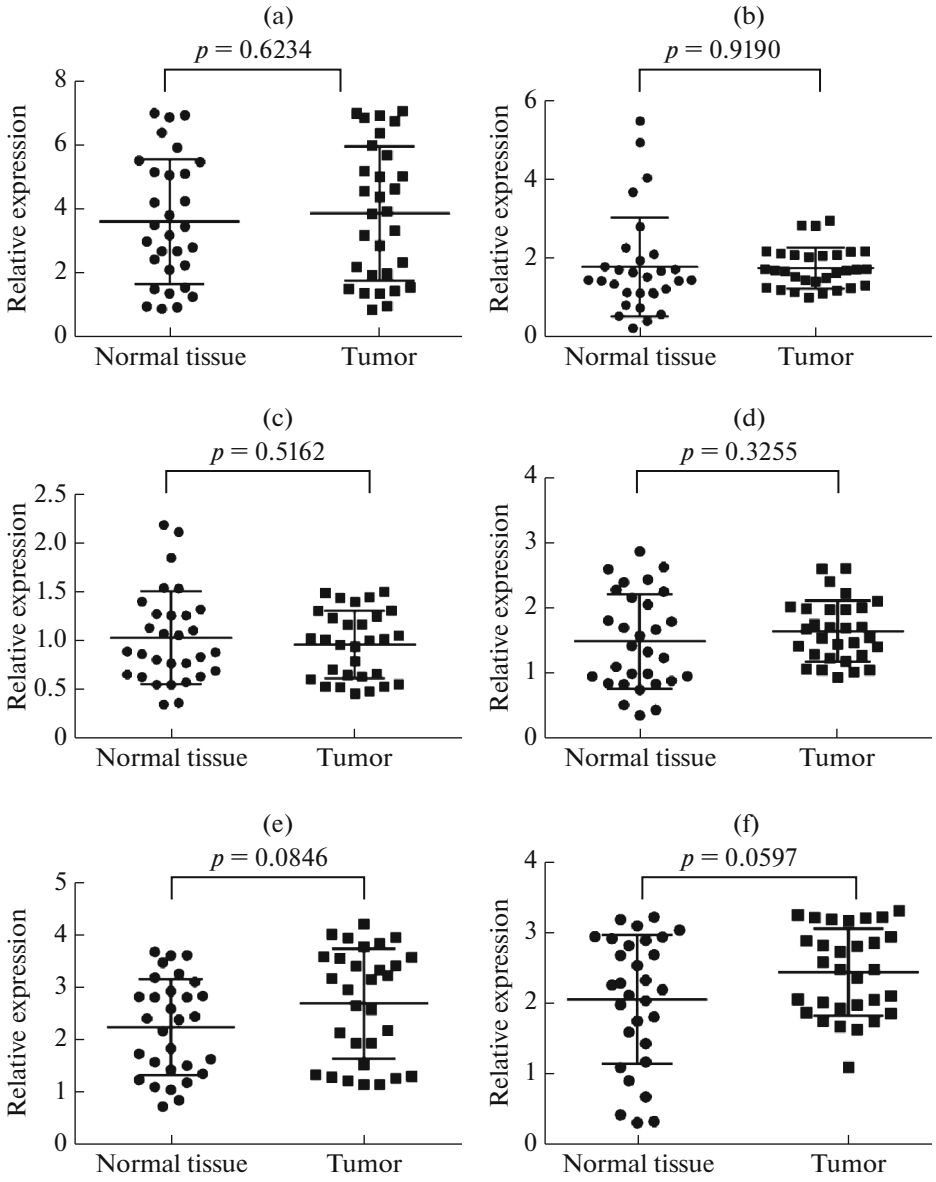

Fig. 1. Analysis of miRNA expression in samples of tumor and normal kidney tissue of patients with clear cell renal cell carcinoma. MicroRNA-17 (a), miRNA-92a (b), miRNA-106a (c), and miRNA-106b (d) did not show significant differences in the expression level between the tumor and normal kidney tissue. MicroRNA-224 (e) and miRNA-21 (f) showed a tendency to increased expression in tumor tissue compared to normal tissue, although the differences did not reach statistical significance. Significance level (*p*-value) was defined using the Student's *t*-test.

gene made it possible to separate benign and malignant samples with 100% specificity and sensitivity, which indicates its high diagnostic value [15].

Gene *TCF21*—transcription factor 21—is described as a tumor suppressor. With clear cell renal cancer, a decrease in its expression in tumor tissue is shown, which correlates with poor survival of patients with ccRCC [16]. In addition, decreased expression of *TCF21* induced by miRNA-21 leads to a decrease in expression of *KISS1*—a member of the metastasis suppressor family—which increases the likelihood of metastases [17].

The expression of miRNA-224 is also altered in various types of malignant neoplasms. Thus, an increase in miRNA-224 expression has been described in colorectal cancer [18], non-small cell lung cancer [19], hepatocellular carcinoma [20], renal cancer [21], and others. Functional analysis by N. Fujii et al. [21] on ccRCC cells showed that miRNA-224 promotes cell viability and invasion and inhibits apoptosis. The target of this miRNA, in addition to *VHL*, is the gene

Table 3. Frequency distribution of genotypes and alleles of polymorphic locus *rs1642742* of gene *VHL* in the group of patients with ccRCC and in the control over 55 years old

Genotype, allele	Patients		Control		χ^2	OR (95% CI)	*p*-value
	n	pi ± Sp (95% CI)	*n*	pi ± Sp (95% CI)			
AA	79	34.5 ± 3.14 (28.36–41.04)	82	46.86 ± 3.77 (39.29–54.53)	5.81	0.59 (0.39–0.92)	0.0165
AG	102	44.54 ± 3.28 (37.99–51.23)	71	40.57 ± 3.71 (33.23–48.24)	0.36	1.15 (0.76–1.76)	0.5748
GG	48	20.96 ± 2.69 (15.88–26.81)	22	12.57 ± 2.51 (8.05–18.41)	4.30	1.84 (1.03–3.31)	0.0381
A	260	56.77 ± 2.31 (52.09–61.36)	235	67.14 ± 2.51 (61.95–72.04)	8.56	0.64 (0.48–0.87)	0.0043
G	198	43.23 ± 2.31 (38.64–47.91)	115	32.86 ± 2.51 (27.96–38.05)	8.56	1.55 (1.15–2.10)	0.0043

Table 4. Associations between genotypes of *rs1642742* of gene *VHL* and overall survival of patients with ccRCC

Inheritance model		Number of patients $N = 393$		Average OS value	OR (95% CI)	log-rank *P*-value
		n, %	deaths			
Recessive	*GG*	95 (24.2)	14	84.6	0.66 (0.17–2.28)	0.4851
	AA + *AG*	298 (75.8)	34	89.3	1.00	
Dominant	*AG* + *GG*	240 (61.1)	30	88.0	0.91 (0.31–2.68)	0.8647
	AA	153 (38.9)	18	89.1	1.00	

OS—overall survival; OR—odds ratio.

DIO1, the product of which catalyzes two types of deiodination reaction, which leads, respectively, to the activation and inactivation of thyroid hormones. Clear cell renal cancer has been shown to be associated with decreased expression of gene *DIO1* [22].

A number of studies indicate the potential association of SNPs at miRNA binding sites with the development of malignant neoplasms and their progression [23–25]. Interest in the study of these polymorphic variants is due to the fact that a change in the nature of the interaction with the microRNA binding site as a result of a single nucleotide substitution can contribute to a change in the expression of target genes involved in the onset and development of tumors. Polymorphic locus *rs1642742* is located in the 3'-untranslated region of the *VHL* gene, both inside and near several microRNA binding sites. In this study, in a comparative analysis of the frequencies of genotypes and alleles of the polymorphic locus *rs1642742* of gene *VHL*, the association of genotype *rs1642742*GG* with a risk of developing ccRCC in a group of people over 55 years of age was found ($p = 0.0381$; OR = 1.84; 95% CI (1.03–3.31)). Moore et al. showed that the polymor-

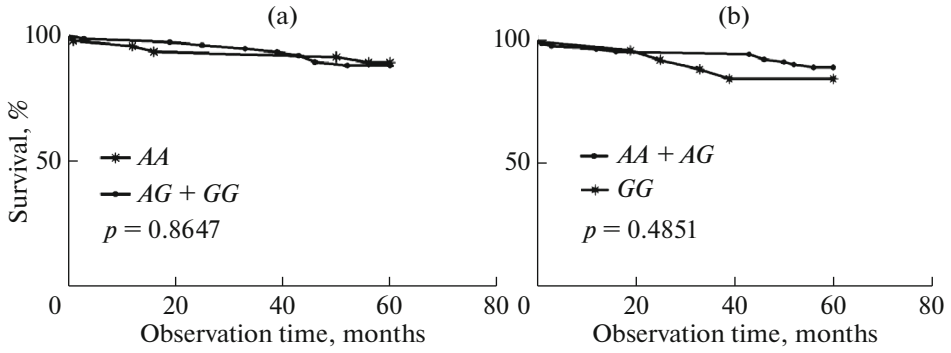

Fig. 2. Survival curves for patients with clear cell RCC: (a) dominant model, (b) recessive model.

phic variant *rs1642742* of *VHL* gene was associated with a risk of *VHL* promotor hypermethylation. Cases of ccRCC with specific haplotypes of polymorphic variants in the *VHL* gene they were more likely to inactivate this gene by promoter hypermethylation than by changing the DNA sequence of tumor tissue. Thus, it can be assumed that the presence of this SNP may represent an example of an inherited tendency to epigenetic variation in kidney tissue [26]. In this study, for individuals who had the *G* allele in *rs1642742*, an increased risk of developing ccRCC at 55 years of age and older was found. Similar results were presented in a study by Wen-Chung Wang et al., where the frequency of allele *G* in *rs1642742* was much higher in advanced renal carcinoma in patients from Taiwan [27].

A recent study examining the relationship between the variability of polymorphic loci of *VHL* gene and the response to ccRCC therapy showed that, with metastatic ccRCC, allele *G* in *rs1642742* is associated with poorer outcomes in patients receiving first-line treatment with tyrosine kinase inhibitors (TKIs). In addition, it was found that the genotype *rs1642742*GG* is a predictor of shorter overall survival, and there was also a tendency to lower survival without signs of disease progression. It is supposed that allele *G* in *rs1642742* disrupts the binding of a number of miRNAs that regulate expression of *VHL* gene [28].

Undoubtedly, further study of small noncoding RNAs on large groups of samples is necessary, which will allow both the identification of new molecular markers of the risk of development of the disease and the formation of prognostic marker panels.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interest.

Statement of compliance with standards of research involving humans as subjects. All procedures performed in a study involving people comply with the ethical standards of the institutional and/or national committee for research ethics and the 1964 Helsinki Declaration and its subsequent changes or comparable ethical standards. Informed consent was obtained from each of the participants in the study.

AUTHOR CONTRIBUTIONS

E.A. Klimentova and I.R. Gilyazova equally contributed to this study.

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