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Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) Contributes to Tamoxifen Resistance in Estrogen-Positive Breast Cancer Patients

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Abstract—Crosstalk between the estrogen receptors and the receptor tyrosine kinases, including vascular endothelial growth factor receptor type II (VEGFR2), is a key mechanism in breast cancer resistance to antiestrogen therapy with tamoxifen. A high level of VEGFR2 expression in a tumor serves as a marker of tamoxifen resistance. The tamoxifen efficacy prognostic value of functional polymorphisms in the *VEGFR2/KDR* gene has not been established. Using qRT-PCR, we detected the rs2071559 and the rs2305948 variants and the levels of *KDR* gene expression in 122 breast tumor tissue samples from cohorts of patients with progression (distant metastases or relapse) and patients with no progression during tamoxifen therapy. The expression levels of VEGFR2 protein were analyzed by immunohistochemistry. The frequency of heterozygous and mutant genotypes of the rs2305948 SNP was significantly higher in patients without progression than in the cohort with progression. *KDR* rs2305948 was associated with high survival rates in breast cancer patients. A correlation between the mRNA of the *ESR1* and *KDR* genes in patients without progression was detected. The results indicate the prognostic value of rs2305948 and its potential contribution to the tumor phenotype sensitive to tamoxifen.

Keywords: vascular endothelial growth factor receptor type II (VEGFR2), single nucleotide polymorphisms, gene expression, estrogen receptor, tamoxifen resistance, breast cancer

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INTRODUCTION

Antiestrogen drugs are one of the main components of the complex therapy for estrogen-positive breast cancer (BC). However, despite the wide arsenal of antiestrogens, tamoxifen, a partial estrogen receptor antagonist (ERα), remains the standard of endocrine therapy in both advanced and early breast cancer [1]. The use of tamoxifen leads to a decrease in the risk of recurrence and an increase in life expectancy, however, in 20–30% of breast cancer patients, the antiestrogenic effect of tamoxifen is not realized [2].

The mechanisms of resistance to tamoxifen are associated with dysregulation of integral signaling systems (PI3K/Akt, Ras/MAPK, etc.) that control the processes of protein synthesis, metabolism, and cell growth [3, 4]. One of the most important effectors of these intracellular cascades are proteins of the vascular endothelial growth factor (VEGF) family, the biological action of which is determined by interaction with specific tyrosine kinases—the type I VEGF receptors (VEGFR1/Flt-1) and type II VEGF receptors(VEGFR2/Flk-1, KDR), with VEGFR2 activation being the most important for intracellular signal transduction [5]. It has been shown that the growth of estrogen-dependent breast cancer cell lines is mediated by the autocrine action of VEGF, causing VEGFR2 activation and subsequent p38 stimulation [6, 7]. In vitro studies indicate that estradiol is able to induce VEGF secretion, while tamoxifen, on the contrary, inhibits the production of VEGF and VEGFR2 in MCF-7 cell cultures, canceling the angiogenic effect of estradiol [8]. Clinical evidence has been obtained for the prognostic role of VEGFR2 as a marker of resistance to endocrine therapy. It has been established that a high level of VEGFR2 in a

Abbreviations: BC, breast cancer; ERα, estrogen receptor; VEGF, vascular endothelial growth factor; VEGFR1/Flt-1, vascular endothelial growth factor receptor type I; VEGFR2/Flk-1, KDR, vascular endothelial growth factor receptor type II; SNP, single nucleotide polymorphism.

Table 1. Sequences of primers and TaqMan probes for genotyping and gene expression analysis

Locus/gene	Primers, nucleotide sequences, 5' → 3'	Probes, nucleotide sequences, 5' → 3'	T_{ann}, °C
KDR rs2071559	AATCTGGTTGCTCTTAATCAGAAA CACTTCAAACTTGGAGCCG	FAM-TGCCCAGTTCGCCAGCATT ROX-CTTGCCCAGTTCGCCAACATT	60
KDR rs2305948	CTGTTCTTCTTGGTCATCAGC TCTGGGAGTGAGATGAAGAAA	FAM-TGAGCACCTTAACTATAGATGGTATAACC ROX-TGAGCACCTTAACTATAGATGGTGTAAC	61
KDR	ACTCTCTCTGCCTACCTCACCT TACTGACTGATTCCTGCTGTGTT	FAM-TGTATGGAGGAGGAGGAAGTATGTG	60
ESR1	CAGGGTGGCAGAGAAAGATT GTAGCGAGTCTCCTTGGCA	FAM-TGACAAGGGAAGTATGGCTATGGA	60

tumor is associated with low rates of relapse-free survival in breast cancer patients who received tamoxifen as adjuvant therapy [9, 10].

Variability in VEGFR2 expression can be modulated by polymorphisms in the coding and noncoding regions of the *KDR* gene. The polymorphic loci rs2071559 and rs2305948 are two of the numerous single nucleotide substitutions (SNPs) in the *KDR* gene. These two SNPs have important functional effects on *KDR* gene activity or the expression of its protein [11]. However, the contribution of these genetic variants to the development of a tumor phenotype resistant to tamoxifen is currently not well defined. In addition, because of the established involvement of VEGFR2 in the breast cancer progression, it seems promising to study the relationship between the genetic characteristics of the expression of this tyrosine kinase and the receptor status of the tumor.

The aim of our work was to study the influence of the rs2071559 and rs2305948 polymorphic loci of the *KDR* gene, its transcriptional activity, and the expression of the protein encoded by it in the tumor, on the tamoxifen therapy efficacy in patients with estrogen-positive breast cancer and to assess the prognostic potential of these factors.

EXPERIMENTAL

Patients. We used samples of tumor and adjacent morphologically normal tissue obtained from 122 women who underwent treatment at the Cancer Research Institute of the Tomsk National Research Medical Center for breast cancer in the period from 2002 to 2014. The clinical diagnosis was confirmed by histological examination**.** All patients underwent radical surgery and had received tamoxifen as an adjuvant hormonal therapy (20 mg/1 time per day). The general group of patients was divided into subgroups according to the progression of the disease: 27 patients with distant metastases or relapses made up the tamoxifen-resistant group; 95 patients without progression were included in the tamoxifen sensitive group. The mean time to progression was 28.5 ± 17.8 months. The study groups were matched for age, tumor stage, and the treatment.

Isolation of DNA. DNA from breast cancer samples was isolated using the QIAamp DNA mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and purity of the isolated DNA were determined using a NanoDrop-1000 spectrophotometer ("Thermo Scientific", USA) (25–400 ng/μL, the ratio A_{260}/A_{280} = 2.10–2.35). DNA integrity was assessed using capillary electrophoresis (TapeStation, Agilent Technologies, USA).

Genotyping. Genotyping the *KDR* gene rs2071559 and rs2305948 SNPs was carried out by real-time PCR using hybridization TaqMan probes. Primers and probes were selected using the OligoAnalysisVector NTI software www.ncbi.nlm.nih.gov (Table 1). The reaction conditions, as well as the sequences of primers and TaqMan probes, were described earlier [12]. The fluorescence accumulation curves were analyzed using the Bio-Rad CFX96 Manager 3.1 software (Bio-Rad, USA). The quality of genotyping was controlled by re-amplification of 5% randomly selected samples with 100% reproducibility of results.

Isolation of RNA. RNA was isolated from tumor and adjacent normal tissue samples using the RNeasy Plus mini Kit containing DNase I (Qiagen, Germany). The concentration and quality of the isolated RNA were assessed spectrophotometrically using a NanoDrop-1000 instrument (Thermo Scientific). RNA concentration ranged from 40 to 200 ng/μL, A_{260}/A_{280} = 1.95–2.05.

Gene expression analysis of *KDR* and *ESR1*. cDNA for determining the level of gene expression was synthesized from the obtained RNA samples using the RevertAid™ kit (Fermentas, USA) according to the instructions for the kit. Real-time PCR was performed using specific primers and TaqMan probes (Table 1) on a CFX96 (Bio-Rad). The reaction mixture contained 250 μM dNTP (Sibenzyme, Russia), 300 nM forward and reverse primers, 200 nM probe, 2.5 mM $MgCl_2$, 1× SE buffer (67 mM Tris-HCl pH 8.8 at 25°C, 16.6 mM $(NH_4)_2SO_4$, 0.01% Tween-20),

Table 2. Distribution of genotypes and alleles of the *KDR* gene in patient groups

SNP_ID	Genotype	Genotype frequency			Allele	Allele frequency		
		TAM-S[a], *n*, %	TAM-R[b], *n*, %	*p*		TAM-S[a], *n*, %	TAM-R[b], *n*, %	*p*
KDR rs2071559	TT	31(32.6)	64(67.4)		T	101(53.2)	89(46.8)	
	TC + CC	12(24.4)	15(55.6)	0.264	C	34(63.0)	20(37.0)	0.201
KDR rs2305948	GG	77(83.7)	15(16.3)		G	166(90.2)	18(9.8)	
	GA + AA	26(100.0)	0(0.0)	0.040	A	52(100.0)	0(0.0)	0.015

[a] Tamoxifen sensitive group. [b] Tamoxifen-resistant group.

2.5 units/reaction HotStart Taq polymerase (Sibenzyme) and 50 ng cDNA. The reaction was carried as follows: preliminary denaturation (94°C, 10 min); then 40 cycles—94°C, 10 s and 60°C, 20 s. Relative *KDR* and *ESR1* gene expression was determined using the $2^{-\Delta\Delta CT}$ method [13] using the *GAPDH* gene as a reference.

Immunohistochemical study. Expression of steroid hormone receptors as well as *KDR* gene protein products were analyzed on paraffin sections of breast tumor tissue using the streptavidin-biotin method and the Dako LSAB2 HRP imaging system. We used mouse antibodies to ERα from Dako (clone 1D5, RTU) and progesterone receptor (PR) (clone PgR 636, RTU), antibodies to VEGFR2 from Novus Biologicals (clone 1B6, 1 : 100). The results of immunohistochemical analysis were assessed semi-quantitatively using a light microscope at a magnification of ×400, taking into account the percentage of positively stained cells and the intensity of their staining in ten fields of view of each section. Moderate (2+) or strong (3+) cytoplasmic and/or membrane staining in more than 10% of tumor cells was considered as VEGFR2-positive, negative staining (0) or weak (1+) expression in <10% of cells—as VEGFR2-negative.

Statistical processing. Statistical analysis was performed using SPSS 21.0 software (IBM SPSS Statistics, Armonk, NY, USA) and GraphPad Prism 8.0.1. The allele and genotype frequencies of the studied SNPs were calculated and tested for deviations from the Hardy–Weinberg equilibrium. Differences in mRNA *KDR* and *ESR1* gene expression between groups of patients was assessed using the Mann-Whitney U-test. The significance of differences between the proportion of VEGFR2-expressing cells in the studied groups was determined by the χ^2 test. Correlation relationships were assessed using Spearman's nonparametric test. Metastatic-free survival rates were calculated using the Kaplan–Meier method. All tests were two-tailed, differences were considered statistically significant when $p < 0.05$.

RESULTS

Frequency of Polymorphic Variants of the KDR Gene in Patients with Estrogen-Positive Breast Cancer Depending on the Tamoxifen Treatment Efficacy

Results of the analysis of two polymorphic gene loci *KDR* in patients with progression and without breast cancer progression while taking tamoxifen are presented in Table 2. The distribution of genotype frequencies at the rs2071559 locus in both groups were consistent with Hardy–Weinberg equilibrium ($p = 0.531$ and $p = 0.629$, respectively). The frequency distribution of genotypes of polymorphic locus rs2305948, corresponding to the Hardy–Weinberg equilibrium, was observed only in the group of patients without breast cancer progression ($p = 0.902$). The absence of heterozygotes and homozygotes for the mutant allele rs2305948 did not allow us to determine the significance of deviations from the Hardy–Weinberg equilibrium in the group with tumor progression. Comparative analysis of the frequencies of genotypes and alleles of the rs2071559 locus of the *KDR* gene did not reveal significant differences between the patients of the study groups. However, in patients without progression, mutant alleles and genotypes of the rs2305948 locus were more common than in patients with progression of breast cancer during tamoxifen therapy ($p = 0.015$ and $p = 0.040$; Table 2).

Analysis of mRNA Expression of the KDR and ESR1 Genes in Patients of the Study Groups

Gene expression analysis showed that in patients with a favorable response to tamoxifen, the *ESR1* mRNA relative expression was higher than in patients with breast cancer progression (12.21 ± 3.22 and 0.85 ± 0.32, respectively, $p = 0.009$; Fig. 1a). A similar trend was noted in *KDR* gene transcriptional activity (the relative of mRNA expression was 1.85 ± 0.56 in the group without breast cancer progression and 0.30 ± 0.11 in the group with progression), but no statistically significant differences were found (Fig. 1b).

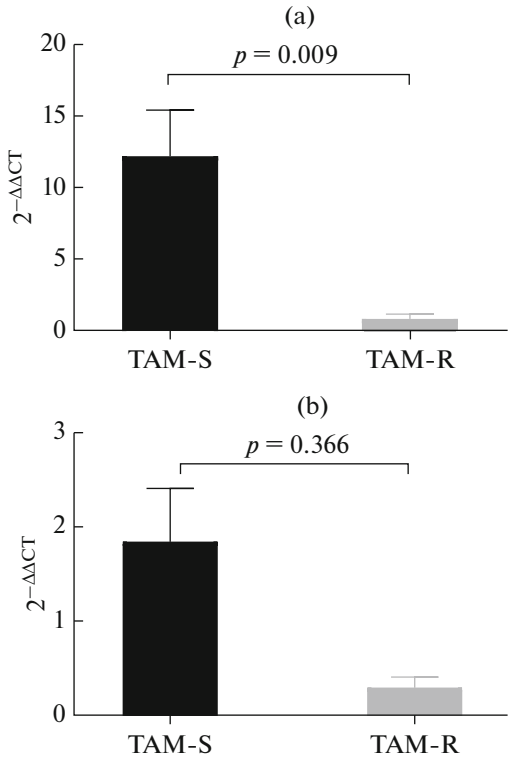

Fig. 1. *ESR1* (a) and *KDR* (b) gene mRNA expression level in breast cancer samples from patients of the TAM-S, sensitive to tamoxifen, and TAM-R, resistant to tamoxifen, groups. Results are presented as $M \pm SE$, where M is mean value and SE is standard error of the mean.

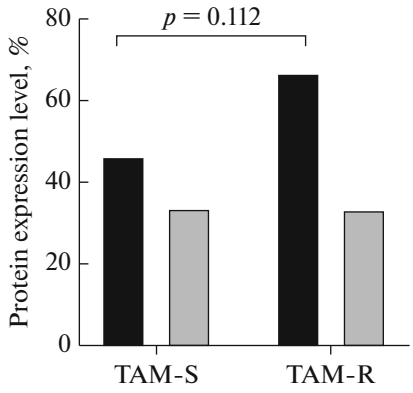

Fig. 2. VEGFR2 level in patients of the TAM-S group, which is sensitive to tamoxifen, and TAM-R, which is resistant to tamoxifen. ■ VEGFR2 positive; ▩ VEGFR2 negative.

Analysis of the relationship between gene expression profiles in the group of patients sensitive to tamoxifen showed a significant correlation ($r = 0.458$; $p = 0.003$), in contrast to the group of patients resistant to therapy, in which there was no such correlation ($r = 0.657$; $p = 0.156$).

Analysis of VEGFR2 Expression in Patients of the Study Groups

Immunohistochemical imaging of the protein product of the *KDR* gene showed that VEGFR2 is more often expressed in tumors resistant to tamoxifen (68.8%), while VEGFR2-negative expression is more often observed in tumors sensitive to tamoxifen (53.8%). However, the differences found did not reach statistical significance ($p = 0.112$; Fig. 2).

Correlation analysis did not reveal a statistically significant relationship between the level of mRNA expression and the proportion (%) of VEGFR2-stained cells ($r = 0.219$; $p = 0.262$ in the tamoxifen sensitive group; $r = 0.500$; $p = 0.667$ in the tamoxifen-resistant group).

Relationship between the Studied Markers and Metastatic-Free Survival Rates in Patients with Estrogen-Positive Breast Cancer

According to the comparative analysis of the dependence of metastatic survival of patients on the carriage of polymorphic gene loci *KDR*, the best indicators of positive outcomes of long-term treatment are observed in the group of carriers of heterozygous and mutant genotypes in comparison to carriers of the wild variant rs2305948 (log-rank $p = 0.024$; Fig. 3a). The rs2071559 polymorphic locus of the *KDR* gene was not associated with survival rates (log-rank $p = 0.824$; Fig. 3b).

The relative mRNA level of the *ESR1* and *KDR* genes, as well as the expression of VEGFR2 in the tumor did not have a significant effect on the metastatic survival rates of patients (data not shown).

DISCUSSION

The present study made it possible to study the role of VEGFR2 tyrosine kinase in the development of resistance to tamoxifen in patients with estrogen-positive breast cancer, taking into account the contribution of its genetic variants, transcriptional activity, and protein profile. We showed an associative relationship between the carriage of heterozygous and mutant variants of the rs2305948 locus of the *KDR* gene in breast cancer patients with a favorable response to tamoxifen. The presence of these genetic variants is highly correlated with a better prognosis for survival. In addition, a correlation between the level of gene expression of *ESR1* and *KDR* was found in patients with successful hormonal therapy.

It should be noted that the studies regarding the effect of rs2305948 polymorphism on tamoxifen efficacy and prognosis are very limited. Most of the them are analyzed the risk significance of this genetic variant; however, the data obtained do not confirm its influence on breast cancer risk [14–16]. Försti et al. [14] revealed a tendency for the association of the mutant genotype SNP rs2305948 with high rates of

overall survival in breast cancer patients; other authors did not find evidence of its prognostic value [17]. Previously, we found that overall survival rates were significantly higher in carriers of heterozygous and mutant variants of the rs2305948 locus in the group of estrogen-positive breast cancer patients who received tamoxifen [18]. In the present study, the prognostic significance of these genotypes is also shown in relation to metastatic-free survival.

The rs2305948 polymorphism is localized in exon 7 of the *KDR* gene, which encodes the extracellular part of the receptor containing seven (D1–D7) Ig-like domains. Structural and functional studies have shown that domains D2–D3 are critical for ligand binding with high affinity [19, 20]. Substitution of valine (V), a Cβ-residue, with a larger Cγ-hydrophobic residue, isoleucine (I) at position 297 of the protein, leads to a change in the conformation of the β-sheet connecting loop in the Ig-like domain of D3, preventing its dimerization [21, 22]. Thus, rs2305948 can affect the activity of *trans*-autophosphorylation and intracellular signal transduction [11, 23, 24]. It is believed that by decreasing the efficiency of VEGFR2 binding to the ligand, this mutation can determine the low activity of VEGFR2-mediated proliferative signaling pathways in the tumor, contributing to a more effective therapeutic response and a favorable prognosis.

The functional variants of the *KDR* gene studied in this work were previously selected to assess their predictive potential in patients with estrogen-negative breast cancer who received preoperative chemotherapy. Interestingly, the wild genotypes of the rs2071559 locus of the *KDR* are markers of the preoperative treatment efficacy with the capecitabine, while in carriers of wild genotypes of the polymorphic variant rs2305948, only a tendency to an increase of pathologic complete response was revealed [12]. It is likely that the contribution of the VEGF system to the mechanisms of progression and development of drug resistance of estrogen-positive and estrogen-negative tumors is different. Thus, in estrogen-negative breast cancer, VEGFR2-mediated stimulation is one of the main regulatory mechanisms for the formation of resistance of tumor cells to the action of cytostatic drugs. A decrease in the activation of proliferative VEGFR2-dependent effects may be due to the modulating influence of other intracellular pathways, which have both pro- and antitumor action [25, 26]. In experiments in vitro the ability of transforming growth factor-β to directly suppress transcription *VEGFR2* in endothelial cells has been shown [27]. In the estrogen-positive tumors, ERα signaling is the key pathway that controls hormonal resistance, through which VEGFR2 expression is regulated [28, 29]. We found a correlation between expression levels of *ESR1* and *KDR* in patients with successful hormonal therapy, this may indicate the possibility of coreceptor interactions of these genes to provide the antitumor effect of tamoxifen [30]. It is worth noting that we have not found a direct correlation between *KDR* gene expression and its protein product in the tumors of such patients. This discrepancy is probably associated with various regulatory mechanisms that affect the protein level, namely, the disruption of translation, synthesis and degradation of mRNA, and post-translational modification of protein products.

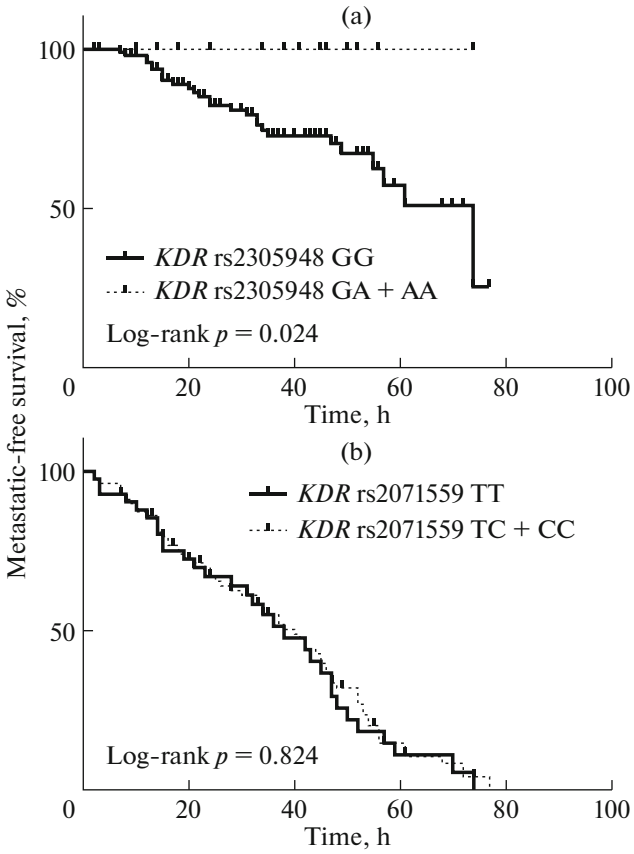

Fig. 3. Metastatic-free survival rates in breast cancer patients who are carriers of the polymorphic loci rs2305948 (a) or rs2071559 (b) of the *KDR* gene.

It is known that changes in the level of *ESR1* gene expression along with the ERα status in the tumor, correlate with the response to tamoxifen therapy in patients with estrogen-positive breast cancer, and a high level of *ESR1* transcriptional activity is associated with sensitivity to tamoxifen [31], and a low level, on the contrary, with resistance to such antiestrogen therapy [32]. In our study, a high level of mRNA of the gene *ESR1* was observed in tumors of patients who did not have disease progression after tamoxifen therapy, which is consistent with the results of the above studies.

Thus, our data indicate that the effect of hormonal therapy with tamoxifen to depends on rs2305948 mutation in the *KDR* gene. The high frequency of heterozygous and mutant genotypes of this polymorphic locus in patients with successful hormonal therapy, as well as their association with a long life expectancy

without breast cancer progression, allows us to consider these genetic markers as factors that determine a favorable prognosis in this disease. Coordinated expression of *ESR1* and *KDR* may indicate a low metastatic potential of the tumor and its sensitivity to tamoxifen.

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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. The study was carried out in compliance with the principles of voluntariness and confidentiality in accordance with the "Fundamentals of the legislation of the Russian Federation on the protection of public health" (Decree of the President of the Russian Federation of 24.12.93 no. 2288) on the basis of the permission of the Biomedical Ethics Committee of the Research Institute of Oncology of the Tomsk NIMC.

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