Association of angiogenesis and inflammation-related gene functional polymorphisms with early-stage breast cancer prognosis

ERIKA KOROBEINIKOVA[1], RASA UGENSKIENE[2,3], RUTA INSODAITE[3], VIKTORAS RUDZIANSKAS[1], EVELINA JASELSKE[1], LINA POSKIENE[4] and ELONA JUOZAITYTE[1]

[1]Oncology Institute, Lithuanian University of Health Sciences; [2]Oncology Research Laboratory, Oncology Institute, Lithuanian University of Health Sciences, LT-50009 Kaunas; [3]Biology System and Genetic Research Institute, Lithuanian University of Health Sciences, LT-50161 Kaunas; [4]Department of Pathological Anatomy, Medical Academy, Lithuanian University of Health Sciences, LT-50009 Kaunas, Lithuania

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Abstract. Genetic variations in inflammation- and angiogenesis-related genes may alter the coded protein level and impact the pathogenesis of breast cancer (BC). The present study investigated the association of functional single nucleotide polymorphisms (SNPs) in the *VEGFA*, *IL-1β*, *IL-1α* and *IL-6* genes with the early-stage BC phenotype and survival. Genomic DNA and clinical data were collected for 202 adult Eastern European (Lithuanian) women with primary I-II stage BC. Genotyping of the SNPs was performed using TaqMan SNP genotyping assays. Nine *VEGFA*, *IL-1β*, *IL-1α* and *IL-6* polymorphisms were analysed. The *VEGFA* and *IL-6* haplotypes were inferred using Phase software. Patients were prospectively followed-up for recurrence, occurrence of metastasis and mortality until April 30, 2019. All studied genotypes were in Hardy-Weinberg equilibrium and had the same distribution as the 1,000 Genomes project Phase 3 dataset for European population. Significant associations of the studied SNPs with clinicopathologic variables were observed between *IL-1α* rs1800587 C allele and larger primary tumour size; *IL-6* rs1800797 A allele, rs1800797 GA genotype, rs1800795 C allele, *IL-6* (rs1800797-re1800795) AC diplotype and hormonal receptor-positive disease; *IL-6* rs1800797 A allele and HER2 negative status. In univariate Cox survival analysis, *IL-1α* rs1800587 CC and *IL-6* rs1800797 GG genotype carriers exhibited worse disease-free survival (DFS), metastasis-free survival (MFS) and overall survival (OS). The *IL-6* rs1800795 GG genotype was associated with worse OS. *IL-6* (rs1800797, rs1800795) GG/GG diplotype carriers had shorter MFS and OS. Multivariate Cox survival analysis revealed that the *IL-1α* rs1800587 CC genotype was an independent negative prognostic factor for DFS, MFS and OS, and the IL6 GG/GG diplotype was an independent negative prognostic factor for MFS and OS. According to the present study, functional SNPs in the *IL-1α* and *IL-6* genes may contribute to the identification of patients at higher risk of BC recurrence, development of metastases and worse OS among early-stage patients with BC.

Correspondence to: Dr Erika Korobeinikova, Oncology Institute, Lithuanian University of Health Sciences, Eiveniu str. 2, LT-50009 Kaunas, Lithuania
E-mail: erika.korobeinikova@lsmuni.lt

Abbreviations: BC, breast cancer; SNP, single nucleotide polymorphism; VEGFA, vascular endothelial growth factor A; IL-6, interleukin-6; IL-1α, interleukin-1α; IL-1β, interleukin-1β; A, adenine; G, guanine; T, thymine; C, cytosine; DFS, disease-free survival; MFS, metastasis-free survival; OS, overall survival; HR, hazard ratio; OR, odds ratio; CI, confidence interval; HER2, human epidermal growth factor receptor 2; GWAS, genome-wide association study

Key words: breast cancer, prognosis, single nucleotide polymorphisms, inflammation, angiogenesis, *VEGFA, IL-6, IL-1α, IL-1β*

Introduction

Breast cancer (BC) is one of the most commonly diagnosed cancers and the leading cause of cancer death among women worldwide (1). Improved diagnostic capabilities has led to an increased rate of BC identified at an early stage (2). However, despite early diagnosis and treatment, the rate of recurrence and metastasis following radical treatment remains disappointingly high, and survival varies considerably between patients with closely matching tumour characteristics.

Inflammation and angiogenesis are the main drivers of cancer. These processes are tightly interconnected in the sense that many pro-inflammatory proteins possess proangiogenic properties and vice versa. The formation of new blood vessels in malignant tumours ensures the supply of nutrients and oxygen, hence promoting the spread of tumour cells (3). Microvessel density is a pivotal risk factor for metastasis and a predictor of poor BC prognosis (4).

The vascular endothelial growth factor A (VEGFA, or VEGF) is the best-known proangiogenic molecule. VEGFA

mediates the growth of new blood vessels by binding to the endothelial cell surface receptors. It promotes endothelial cell proliferation, migration and the formation of tubular structures (5). Another mechanism of tumour neovascularization is the so-called inflammatory angiogenesis. Such pro-inflammatory cytokines as interleukin-1β (IL-1β), interlukin-1α (IL-1α) and interleukin-6 (IL-6) through a variety of signalling pathways promote endothelial cell migration and proliferation, contributing to tumour angiogenesis that facilitates the survival of cancer cells (6,7). Apart from stimulating angiogenesis, VEGFA, IL-1β1, IL-1α and IL-6 are also involved in inflammatory processes. These proteins may prevent apoptosis and promote cancer cell proliferation, differentiation, migration and metastasis.

The association between the above-mentioned proteins and BC prognosis was demonstrated by several authors. The elevated serum level of VEGFA in metastatic BC patients is linked to worse progression-free survival (PFS) and overall survival (OS) (8). Another cytokine, IL-6, induces epithelial-mesenchymal phenotype and therapeutic resistance in BC cells (9). Higher circulating levels of IL6 were observed in more advanced stages of the disease (10). It was also found that high tumour co-expression of the VEGF and IL-6 family cytokines significantly lowers the human epidermal growth factor receptor 2 (HER2) negative BC survival (11). Moreover, higher expression of pro-inflammatory IL-1β cytokine is correlated with higher BC stage and significantly worse survival (12). In addition, IL-1α acts as a pro-inflammatory molecule itself and also promotes the activity of IL-1β, resulting in an increased growth of BC cells and tumour progression (13).

Common polymorphisms in proinflammatory and proangiogenic cytokine genes may influence their coded protein production and play a role in the course of BC. The polymorphisms with proved functional activity are *VEGFA* (rs699947, rs833061, rs25648, rs1005230), *IL-1β* (rs1143634, rs16944), *IL-1α* (rs1800587) and *IL-6* (rs1800795, rs1800797) (14-21). Candidate gene studies as well as moderate-sized genome-wide association studies (GWAS) highlight the important role of these polymorphisms in BC risk and aggressiveness (22-32), although substantial heterogeneity across studies exists. The currently available results are inconsistent in terms of different ethnicity and cancer stage; therefore, it is necessary to further investigate the role of *VEGFA*, *IL-1β*, *IL-1α* and *IL-6* gene polymorphisms in breast carcinogenesis and cancer progression.

This paper describes a cohort study that aimed to examine the contribution of *VEGFA*, *IL-1β*, *IL-1α* and *IL-6* gene polymorphisms to the clinicopathologic features and survival in a homogeneous group of Eastern European (specifically, Lithuanian) early-stage BC patients.

Materials and methods

Patients. The study consisted of 202 adult Lithuanian women with primary I-II stage BC. All patients were treated in the Hospital of Lithuanian University of Health Sciences Kaunas Clinics. The exclusion criteria were other malignancies, significant comorbidities and/or incomplete medical documentation. Surgery and adjuvant therapy were chosen by the clinicians, based on pathomorphologic characteristics and validated prognostic factors. The patients were followed until 30 April, 2019 (censoring date).

Candidate polymorphisms. The genes and polymorphisms known to modulate inflammation and angiogenesis were selected. The selection criteria included: i) functional single nucleotide polymorphisms (SNPs) in the *VEGFA*, *IL-1β*, *IL-1α* and *IL-6* genes predicting alterations in the protein level; ii) SNP relevant to outcomes in other settings; and iii) SNP with a minor allele frequency greater than 15% in the study population. We selected nine SNPs: The *VEGFA* gene rs699947, rs833061, rs25648, and rs1005230; the *IL-1β* gene rs1143634 and rs16944; the *IL-1α* gene rs1800587; and the *IL-6* gene rs1800795 and rs1800797.

Assay methods. Peripheral blood samples from the study population were collected in 2009-2017. For genomic DNA extraction from peripheral blood leukocytes, a commercially available DNA extraction kit (i.e., Thermo Fisher Scientific Baltics, Lithuania) was used. The DNA was stored at -20˚C prior to usage.

Genotyping of the selected polymorphisms was performed at the Dr. K. Janusauskas Laboratory of Genetics of the Institute of Biology Systems and Genetic Research of Lithuanian University of Health Sciences Kaunas Clinics. The SNPs of the target genes were estimated by using TaqMan SNP Genotyping Assays (C_8311602_10, C_1647381_10, C_791476_10, C_8311612_10, C_1839697_20, C_1839695_20, C_9546517_10, C_1839943_10, C_1839943_10; Applied Biosystems; Thermo Fisher Scientific, Inc.). The polymerase chain reaction was performed in a reaction volume of 25 μl containing template DNA (2 ng), 2X TaqMan Universal Master Mix II, no UNG (Applied Biosystems; Thermo Fisher Scientific, Inc.)-12,5 μl, 20X TaqMan SNP Genotyping Assay stock (initial stock of 40X or 80X was diluted to get 20X working stock)-1,25 μl. The final volume of 25 μl was adjusted by adding nuclease free ddH_2O. Finally, the 2 μl of DNA was added from each sample. For negative control, nuclease free ddH_2O was used instead of patient DNA, while for positive control, the DNA of the known genotype was used. Each sample genotyping was repeated twice for accuracy.

The Applied Biosystems 7900HT Real-Time Polymerase Chain Reaction System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for SNP detection. The cycling program started from heating up to 95˚C for 10 min followed by 40 cycles (at 95˚C for 15 sec and at 60˚C for 1 min). Finally, allelic discrimination was done by using the SDS 2.3 software provided by Applied Biosystems; Thermo Fisher Scientific, Inc.

Study design. A prospective cohort study was conducted at the Hospital of Lithuanian University of Health Sciences. For the case selection, information on primarily BC patients was retrieved from the hospital's Pathology Department. The patients who fulfilled the inclusion criteria and signed the informed consent document (approved by the Kaunas Regional Ethics Committee for Biomedical research; Protocol number BE-2-10) were enrolled in the study and their peripheral blood samples were obtained. The characteristics of clinical and pathological features and the course of the disease were

obtained for all study subjects. The date of histological BC verification was time zero in the survival analysis. The endpoints of interest were disease-free survival (DFS), metastasis-free survival (MFS) and OS. We checked for associations of SNPs with the known BC prognostic factors and survival endpoints. The guidelines for the reporting of tumour marker prognostic studies were applied while conducting the study (33,34).

Statistical analysis. The allele frequency distributions of the investigated SNPs were compared with the European population data from the 1000 Genomes project phase 3 database (35). For each SNP a Hardy-Weinberg equilibrium was assessed by using Pearson's chi square and Fisher's exact tests. The Haploview v4.1 software was used to check for the linkage disequilibrium between SNPs (36). The *VEGFA* and *IL-6* haplotypes were inferred from the tested SNPs by Bayesian methods as implemented in the Phase software (v2.1; Department of Statistics, University of Washington, Seattle, WA, USA) (37,38). The SNPs were analysed under genotype, allelic and haplotype (for *IL-6* and *VEGFA* SNPs) models. The associations of polymorphisms with clinicopathologic variables were evaluated by Pearson's Chi-square or Fisher's exact test. The Bonferroni-corrected α level was used in the association analysis for multiple comparisons. Moreover, the Cox proportional hazards model was used to estimate the prognostic factors for DFS, MFS and OS. In addition, multivariate analysis was used to determine the interdependency of genotypes and other known prognostic factors, such as age, tumour differentiation grade, tumour size, lymph node status, oestrogen receptor status, progesterone receptor status and human epidermal growth factor receptor 2 (HER2) status. Hazard ratios (HR) and their 95% confidence intervals (CI) were recorded for each tested marker. Finally, the Kaplan-Meier analysis with the log-rank test was applied to compare the survival of the patients with different genotypes. Statistical significance was set at 5% ($P<0.05$). Statistical analysis was performed using SPSS for Windows v20.0 (Released 2011; IBM Corp).

Results

Sample characteristics. A total of 202 Lithuanian women with early-stage BC were included in the current analysis. The frequency distributions of clinical and tumour biological factors are shown in Table I. For all study participants, primary treatments included surgery (100%), chemotherapy (77%), hormone therapy (71%), trastuzumab (19%) and radiation therapy (97%).

All the patients were genotyped for a panel of nine SNPs: The *VEGFA* gene rs699947, rs833061, rs25648 and rs1005230; the *IL-1β* gene rs1143634 and rs16944; the *IL-1α* gene rs1800587; the *IL-6* gene rs1800795 and rs1800797. The genotypes were found to be in Hardy-Weinberg equilibrium in all the nine SNPs. A strong linkage disequilibrium between four *VEGFA* and two *IL-6* polymorphisms was confirmed (Fig. 1). Our cohort had similar allele distribution to that of the 1000 Genomes project phase 3 for European population. The genotype and allele frequency data is presented in Table II.

Inferential analysis. The data on associations between the analysed polymorphisms and clinicopathologic tumour features is shown in Tables SI-SVI. In the single-locus analysis, the genotype model revealed a significant link between the *IL-6* rs1800797 genotypes and the oestrogen receptor ($P=0.005$) and the progesterone receptor ($P=0.007$) status (Bonferoni adjusted; significant p value <0.008). The allelic model showed that the A allele of this SNP is associated with positive oestrogen receptors (OR 2.23; 95% CI: 1.19-4.08; $P=0.014$). Patients carrying the *IL-6* rs 1800797 A allele were also predisposed to higher rates of HER2 negative BC (OR 2.21; 95% CI: 1.07-4.57; $P=0.042$). Additionally, another *IL-6* polymorphism, rs1800795, in both genotype and allelic models was linked to oestrogen receptor positive BC. Specifically, 72% of patients carrying the *IL-6* rs1800795 C allele had oestrogen receptor positive disease, compared to 58% of non-carriers (OR 1.92; 95% CI: 1.04-3.57; $P=0.04$).

Linkage disequilibrium analysis showed a high degree of disequilibrium between the two *IL-6* SNPs ($r^2=0.89$), meaning that the associations were not independent. As such, haplotype analysis was performed to further explore the relationship between *IL-6* variations and other prognostic factors. Phasing revealed three possible *IL-6* (rs1800797, rs1800795) haplotypes: GG (51.7%), AC (45.5%) and GC (2.8%). It was further demonstrated that the AC haplotype was positively associated with oestrogen receptor positive BC (HR 2.13; 95% CI 1.14-3.98; $P=0.018$; Table SII).

Table I. Frequency data for clinical and tumour biological factors.

Factor	Patients, %
Age at diagnosis, years	
<50 years	65
≥50 years	35
Tumour size, cm	
<2	64
2-5	36
Lymph node status	
Positive	55
Negative	45
Grade	
G1 and G2	78
G3	22
ER status	
ER positive	68
ER negative	32
PR status	
PR positive	60
PR negative	40
HER2 status	
HER2 positive	19
HER2 negative	81

ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

Table II. Allele and genotype frequencies of analysed single nucleotide polymorphisms in the study population and in European population data from the 1000 Genomes Project Phase 3 database.

Gene	Polymorphism	Study allele and genotype frequencies (1000 Genomes Project Phase 3 database allele frequencies)				
VEGFA	rs699947	A, 0.53 (0.50)	C, 0.47 (0.50)	AA, 0.28	CA, 0.50	CC, 0.22
	rs833061	T, 0.47 (0.50)	C, 0.53 (0.50)	TT, 0.21	TC, 0.51	CC, 0.28
	rs25648	C, 0.81 (0.83)	T, 0.19 (0.17)	TT, 0.03	CT, 0.32	CC, 0.65
	rs1005230	T, 0.52 (0.50)	C, 0.48 (0.50)	TT, 0.28	CT, 0.49	CC, 0.23
IL-6	rs1800795	C, 0.47 (0.42)	G, 0.53 (0.58)	CC, 0.24	GC, 0.47	GG, 0.29
	rs1800797	A, 0.46 (0.41)	G, 0.54 (0.59)	AA, 0.23	GA, 0.47	GG, 0.30
IL-1β	rs1143634	G, 0.73 (0.75)	A, 0.27 (0.25)	GG, 0.52	GA, 0.42	AA, 0.06
	rs16944	G, 0.66 (0.65)	A, 0.34 (0.35)	GG, 0.43	GA, 0.47	AA, 0.10
IL-1α	rs1800587	C, 0.69 (0.71)	T, 0.31 (0.29)	CC, 0.48	CT, 0.43	TT, 0.09

VEGFA, vascular endothelial growth factor A; IL, interleukin.

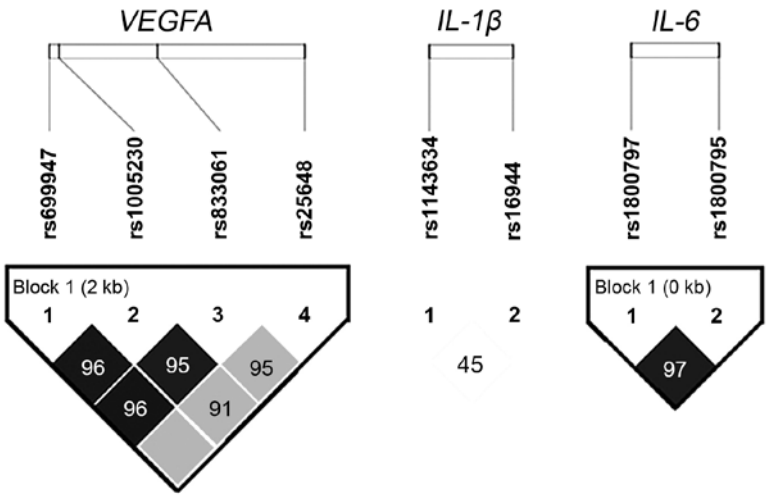

Figure 1. Linkage disequilibrium data for *VEGFA*, *IL-1β* and *IL-6* single nucleotide polymorophisms. Numerical values are given of D' values, whereas the colors are given to encode r2 (dark grey encodes high r2). VEGFA, vascular endothelial growth factor A; IL, interleukin.

By analysing the associations between the *IL-1α* SNP and clinicopathologic factors (Table SIII), we found a link between the *IL-1α* rs1800587 SNP C allele and larger primary tumour size (comparing tumours sized <2 vs. 2-5 cm) (OR 4.91; 95% CI: 1.09-22.01; P=0.022).

Other analysed polymorphisms of *IL-1β* and *VEGFA* revealed no associations with the evaluated prognostic factors in neither genotype nor allelic models (Tables SIV-SVI). Due to the strong linkage disequilibrium, four *VEGFA* polymorphisms were also analysed in the haplotype model. Three main haplotypes were identified: CCTC (45.5%), ATCC (33.4%) and ATCT (17.6%), as well as several rare variants. None of the *VEGFA* haplotypes was associated with clinicopathologic prognostic variables.

Survival analysis. In the mean follow-up time of 67 months (range 28-202), progression of the disease was observed for 33 patients. Of those who progressed, 28 had distant metastases. Twenty-two patients with progressive disease died, all due to cancer. The Kaplan-Meier survival analysis showed that the *IL-1α* rs1800587 SNP is associated with early-stage breast cancer DFS, MFS and OS. In particular, patients homozygous for the C allele (CC) had worse survival rates than patients homozygous and heterozygous for the A allele (CT and TT) (Fig. 2A-C).

The univariate Cox regression analysis (presented in Tables III-V) revealed that *L-1α* rs1800587 CC genotype carriers had 2.48 times higher risk of disease recurrence (95% CI: 1.19-5.11; P=0.014), 3.12 times higher risk of metastasis (95% CI: 1.37-7.10; P=0.007) and 2.63 times higher risk of death (95% CI: 1.07-6.46; P=0.035) than the carriers of CT and TT genotypes (Table III).

By analysing the SNPs of the *IL-6* gene, we found that the rs1800797 GG genotype was a negative prognostic factor for DFS and MFS. Furthermore, *IL-6* rs1800797 GG and *IL-6* rs1800795 GG genotype carriers displayed a shorter OS (Table IV). In a haplotype model, the patients who inherited the GG/GG diplotype (AC haplotype non-carriers) had a higher

Table III. Cox's univariate model for *IL-1β* and *IL-1α* SNPs.

				PFS		MFS		OS	
Reference SNP ID	Model	Genotype/allele/ haplotype	Patients n	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (CI)	P-value	Multivariate hazard ratio (95% CI)	P-value
IL-1β rs1143634	Genotype	AA	12	1	0.043	1	0.062	1	0.271
		GA	85	0.443 (0.094-2.100)	0.305	0.945 (0.116-7.688)	0.360	0.773 (0.093-6.421)	0.811
		GG	105	1.243 (0.292-5.291)	0.768	2.555 (0.343-19.048)	0.958	1.667 (0.220-12.630)	0.621
	Allelic	A allele carriers	97	1		1		1	
		A allele non carriers	105	2.490 (0.944-5.246)	0.056	2.686 (0.932-6.102)	0.058	2.087 (0.851-5.122)	0.108
		G allele carriers	190	1		1		1	
		G allele non carriers	12	1.171 (0.279-4.913)	0.829	0.563 (0.076-4.148)	0.573	0.799 (0.107-5.939)	0.826
IL-1-β rs16944	Genotype	AA	20	1	0.335	1	0.347	1	0.715
		GA	95	1.657 (0.387-7.096)	0.496	1.386 (0.320-6.005)	0.663	1.183 (0.264-5.287)	0.826
		GG	87	0.974 (0.216-4.396)	0.973	0.763 (0.165-3.533)	0.729	0.814 (0.173-3.834)	0.794
	Allelic	A allele carriers	115	1		1		1	
		A allele non carriers	87	0.623 (0.302-1.294)	0.201	0.573 (0.259-1.267)	0.169	0.706 (0.296-1.684)	0.432
		G allele carriers	182	1		1		1	
		G allele non carriers	20	0.754 (0.180-3.155)	0.70	0.926 (0.220-3.905)	0.917	0.999 (0.233-4.277)	0.99
IL-1α rs1800587	Genotype	TT	18	1	0.049	1	0.025	1	0.108
		CT	87	0.805 (0.174-3.729)	0.782	1.292 (0.159-10.506)	0.811	1.015 (0.122-8.428)	0.989
		CC	97	2.067 (0.486-8.794)	0.326	3.891 (0.521-29.034)	0.185	2.664 (0.352-20.187)	0.343
	Allelic	T allele carriers	105	1		1		1	
		T allele non carriers	97	2.480 (1.199-5.114)	0.014[a]	3.122 (1.372-7.104)	0.007[a]	2.632 (1.072-6.461)	0.035[a]
		C allele carriers	184	1		1		1	
		C allele non carriers	18	0.703 (0.168-2.940)	0.630	0.392 (0.053-2.888)	0.358	0.551 (0.074-4.096)	0.560

[a]Significant. Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype and allelic model. IL, interleukin; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival; SNP, single nucleotide polymorphism.

Table IV. Cox's univariate model for *IL-6* SNPs.

				PFS		MFS		OS	
Reference SNP ID	Model	Genotype/allele/ haplotype	Patients, n	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value
IL-6 rs1800795	Genotype	CC	48	1	0.239	1	0.160	1	0.101
		GC	95	1.441 (0.518-4.010)	0.484	0.983 (0.336-2.881)	0.972	1.216 (0.321-4.560)	0.779
		GG	49	2.257 (0.811-6.277)	0.119	2.053 (0.736-5.761)	0.174	2.842 (0.798-10.193)	0.109
	Allelic	C allele carriers	143	1		1		1	
		C allele non carriers	49	1.750 (0.874-3.502)	0.114	2.074 (0.983-4.375)	0.055	2.484 (1.075-5.737)	0.033[a]
		G allele carriers	144	1		1		1	
		G allele non carriers	48	0.568 (0.219-1.473)	0.245	0.716 (0.272-1.888)	0.499	0.552 (0.163-1.866)	0.339
IL-6 rs1800797	Genotype	AA	46	1	0.170	1	0.136	1	0.117
		GA	95	1.724 (0.621-4.787)	0.296	1.173 (0.406-3.441)	0.771	1.536 (0.418-5.783)	0.528
		GG	61	2.596 (0.934-7.215)	0.067	2.361 (0.841-6.627)	0.104	3.174 (0.858-11.355)	0.077
	Allelic	A allele carriers	141	1		1		1	
		A allele non carriers	61	2.370 (1.032-5.464)	0.044[a]	2.154 (1.009-4.476)	0.047[a]	2.373 (1.033-5.461)	0.044[a]
		G allele carriers	156	1		1		1	
		G allele non carriers	46	0.483 (0.186-1.251)	0.134	0.610 (0.232-1.606)	0.317	0.458 (0.135-1.548)	0.209
IL-6 ts1800797- rs1800795	Diplotype	AC/AC	48	1	0.339	1	0.292	1	0.250
		GG/AC	80	1.666 (0.599-4.634)	0.328	1.125 (0.383-3.305)	0.830	1.141 (0.382-5.434)	0.589
		GG/GG	63	2.146 (0.772-5.967)	0.143	1.953 (0.695-5.485)	0.204	2.555 (0.711-9.161)	0.150
	Haplotype	AC haplotype carriers	136	1		1		1	
		AC haplotype non carriers	60	1.809 (0.905-3.617)	0.094	2.142 (1.021-4.514)	0.045[a]	2.455 (1.062-5.661)	0.035[a]
		GG haplotype carriers	149	1		1		1	
		GG haplotype non carriers	48	1.818 (0.701-4.713)	0.219	1.438 (0.546-3.791)	0.462	1.894 (0.560-6.401)	0.304

[a]Significant. Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype, allelic and haplotype models. IL-6, interleukin-6; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival; SNP, single nucleotide polymorphism.

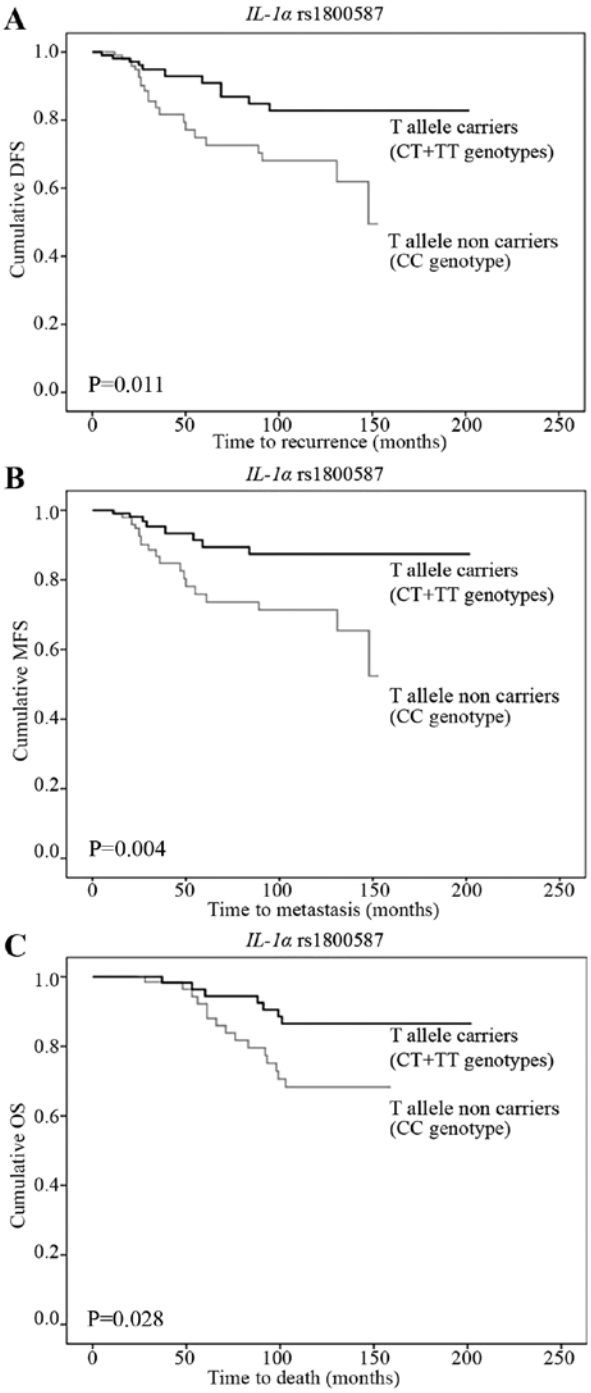

Figure 2. Kaplan-Meier survival curves for *IL-1α* rs1800587 single nucleotide polymorphism. (A) DFS, (B) MFS and (C) OS. IL, interleukin; DFS, disease-free survival; MFS, metastasis-free survival; OS, overall survival.

risk of disease recurrence, metastasis and death (Table IV; Fig. 3A and B).

In a multivariate Cox regression model including age at diagnosis, tumour size, lymph node status, tumour differentiation grade, oestrogen receptor, progesterone receptor and HER2 status, the *IL-1α* 1800587 CC genotype remained a significant predictor of poor DFS, MFS and OS. Furthermore, the *IL-6* GG/GG diplotype (non-carrying the AC haplotype) was an independent negative prognostic factor for MFS and OS (Table VI). Finally, other polymorphisms and *VEGFA* haplotypes were not associated with any of the survival endpoints.

Discussion

In the present study, we investigated the associations between nine functional SNPs in four cytokine genes (i.e., *VEGFA*, *IL-1α*, *IL-1β and IL-6*) and the clinicopathologic profiles and survival rates in a group of Lithuanian women with early-stage BC. We found an association between the IL-6 SNPs and the oestrogen receptor positive, progesterone receptor positive and HER2 negative status, and a link between IL-1α SNP and larger primary tumour size. Furthermore, we confirmed a negative prognostic value of the *IL-6* rs1800797-rs1800795 GG/GG diplotype on MFS and OS and of the *IL-1α* rs1800587 CC genotype on DFS, MFS and OS in a highly homogeneous group of patients.

Several authors have demonstrated that carrying the *IL-6* rs1800795 G or *IL-6* rs1800795 G alleles is associated with higher IL-6 protein production (19-21). Specifically, IL-6 is a cytokine which is considered a prognostic marker as well as a potential therapeutic target for BC patients. This cytokine acts through several pathways, regulating the proliferation, apoptosis and metabolism of BC cells. However, the most important role of IL-6 in breast carcinogenesis is its potential to induce breast metastasis by enhancing angiogenesis and tumour cell migration (39). According to previous studies, *IL-6* rs1800797 and rs1800795 polymorphisms appear to be biologically important; however, the data on their clinical importance is still heterogeneous.

The association of *IL-6* polymorphisms with BC survival was analysed in several studies (Table VII). Snoussi *et al* (32) and DeMichele *et al* (27) analysed patients with non-metastatic stage I-III BC. In both studies the *IL-6* rs1800795 GG genotype was associated with decreased DFS and OS. In DeMichele *et al* (27) the presence of at least one copy of the haplotype rs1800797G-rs1800796G-373(10A/11T)-rs1800795G was associated with worse DFS. Snoussi *et al* (32) also found a significant link between *IL-6* rs1800797 GG polymorphism and decreased DFS and OS. However, in DeMichele *et al* (28), where only locally advanced stage III patients with more than 10 positive lymph nodes were studied, the results were not replicated. Specifically, there was no impact of IL-6 polymorphisms on DFS and OS in the study population. In the same study, only an unplanned sub-analysis showed an association of the rs1800795 GG and rs1800797 GG genotypes with lower DFS but not OS in the oestrogen receptor positive patient subgroup. Our study is the first to confirm the prognostic value of IL-6 polymorphisms (namely, the *IL-6* GG/GG diplotype) on MFS and OS in early-stage BC. Therefore, *IL-6* GG/GG may potentially be used for the selection of patients who need intensified adjuvant BC treatment.

The above-mentioned studies by Snoussi *et al* (32) and DeMichele *et al* (27,28) did not analyse MFS as a survival endpoint. To the best of our knowledge, the only study that evaluated the association between *IL-6* polymorphisms and BC metastasis was a case-control study conducted by Abana *et al* (26). The authors demonstrated the significant link between the *IL-6* rs1800795 GG genotype and the development of BC metastases with an OR of 1.52. However, due to case-control design, we have no data on MFS differences. In our study *IL-6* GG/GG predicted MFS and OS, but not DFS. Taking into account the critical role of IL-6 in the development

Table V. Cox's univariate model for *VEGFA* SNPs.

				PFS		MFS		OS	
Reference SNP ID	Model	Genotype/allele/haplotype	Patients, n	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value
VEGFA rs699947	Genotype	AA	56	1	0.373	1	0.538	1	0.162
		CA	101	1.483 (0.616-3.565)	0.387	1.104 (0.447-2.754)	0.317	1.141 (0.383-3.414)	0.813
		CC	45	2.03 (0.76-5.47)	0.160	1.689 (0.613-4.622)	0.845	2.524 (0.822-7.723)	0.105
	Allelic	A allele carriers	157	1		1		1	
		A allele non carriers	45	1.568 (0.727-3.383)	0.251	1.585 (0.697-3.604)	0.272	2.322 (0.973-5.543)	0.058
		C allele carriers	146	1		1		1	
		C allele non carriers	56	0.613 (0.266-1.415)	0.252	0.791 (0.336-1.864)	0.592	0.651 (0.240-1.766)	0.399
VEGFA rs833061	Genotype	CC	56	1	0.295	1	0.430	1	0.115
		TC	103	1.445 (0.600-3.499)	0.410	1.081 (0.429-2.703)	0.875	1.124 (0.377-3.344)	0.842
		TT	43	2.189 (0.813-5.892)	0.121	1.801 (0.652-4.974)	0.257	2.694 (0.880-8.250)	0.083
	Allelic	C allele carriers	159	1		1		1	
		C allele non carriers	43	1.709 (0.792-3.687)	0.172	1.718 (0.755-3.905)	0.197	2.511 (0.952-5.994)	0.058
		T allele carriers	146	1		1		1	
		T allele non carriers	56	0.609 (0.264-1.405)	0.245	0.786 (0.334-1.853)	0.583	0.649 (0.239-1.760)	0.395
VEGFA rs1005230	Genotype	TT	57	1	0.438	1	0.657	1	0.268
		CT	99	1.553 (0.646-3.755)	0.327	1.153 (0.465-2.891)	0.763	1.204 (0.402-3.593)	0.740
		CC	46	1.894 (0.702-5.083)	0.207	1.584 (0.571-4.355)	0.381	2.281 (0.753-6.974)	0.149
	Allelic	C allele carriers	57	1		1		1	
		C allele non carriers	145	0.604 (0.262-1.393)	0.237	0.779 (0.331-1.835)	0.568	0.647 (0.238-1.753)	0.392
		T allele carriers	156	1		1		1	
		T allele non carriers	46	2.034 (0.857-4.844)	0.110	1.440 (0.634-3.269)	0.384	2.031 (0.851-4.844)	0.110

Table V. Continued.

Reference SNP ID	Model	Genotype/allele/haplotype	Patients, n	PFS		MFS		OS	
				Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value	Univariate hazard ratio (95% CI)	P-value
VEGFA rs25648	Genotype	CC	131	1	0.425	1	0.727	1	0.602
		CT	65	0.604 (0.271-1.345)	0.217	0.796 (0.348-1.820)	0.589	0.706 (0.274-1.819)	0.471
		TT	6	1.373 (0.185-10.181)	0.756	1.706 (0.228-12.794)	0.603	1.861 (0.246-14.087)	0.548
	Allelic	T allele carriers	71	1		1		1	
		T allele non carriers	131	1.553 (0.721-3.343)	0.261	1.181 (0.534-2.612)	0.681	1.291 (0.526-3.168)	0.577
		C allele carriers	196	1		1		1	
		C allele non carriers	6	1.593 (0.217-11.703)	0.647	1.835 (0.248-13.556)	0.552	2.083 (0.280-15.494)	0.474

Unadjusted hazard ratios for PFS, MFS and OS with each of the SNPs in genotype and allelic model. *VEGFA*, vascular endothelial growth factor A; SNP, single nucleotide polymorphism; PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival.

Table VI. Cox's multivariate model.

		PFS		MFS		OS	
Variable	Patients, n	Multivariate hazard ratio (95% CI)	P-value	Multivariate hazard ratio (95% CI)	P-value	Multivariate hazard ratio (95% CI)	P-value
Age at diagnosis, years							
≥50	71	1		1		1	
<50	131	7.523 (0.990-56.690)	0.049[a]	6.081 (0.786-46.619)	0.082	9.237 (0.983-57.232)	0.091
Tumor size, cm							
<2	129	1		1		1	
2-5	73	1.102 (0.583-2.399)	0.795	1.018 (0.522-1.824)	0.631	1.021 (0.529-1.919)	0.669
Lymph node status							
Negative	111	1		1		1	
Positive	91	2.331 (1.086-4.989)	0.030[a]	2.413 (1.051-5.587)	0.039[a]	2.151 (0.832-5.560)	0.115
Grade							
G1 and G2	158	1		1		1	
G3	44	2.281 (0.916-5.719)	0.079	2.145 (0.815-5.628)	0.123	1.904 (0.636-5.645)	0.247
Estrogen receptor status							
Positive	137	1		1		1	
Negative	65	1.182 (0.482-2.939)	0.721	1.304 (0.496-3.455)	0.594	1.580 (0.545-4.555)	0.395
Progesterone receptor status							
Positive	121	1		1		1	
Negative	82	3.088 (1.193-7.993)	0.020[a]	3.284 (1.164-9.228)	0.025[a]	4.670 (1.315-16.435)	0.018[a]
Human epidermal growth factor receptor 2 status							
Negative	164	1		1		1	
Positive	38	2.393 (0.841-6.817)	0.104	1.904 (0.641-5.621)	0.246	1.718 (0.518-5.735)	0.376
IL-6 haplotype							
AC haplotype carriers (GG/AC+ AC/AC)	128	-		1		1	

Table VI. Continued.

Variable	Patients, n	PFS		MFS		OS	
		Multivariate hazard ratio (95% CI)	P-value	Multivariate hazard ratio (95% CI)	P-value	Multivariate hazard ratio (95% CI)	P-value
AC haplotype non carriers (GG/GG)	63	-	-	2.750 (1.184-7.690)	0.048	2.631 (1.042-7.670)	0.049
IL-1α rs1800587		1		1		1	
T allele carriers (CT+TT genotypes)	105	2.704 (1.280-5.683)	0.009[a]	3.442 (1.484-7.994)	0.004[a]	3.181 (1.252-8.134)	0.016[a]
T allele non carriers (CC genotype)	97	-	-	2.750 (1.184-7.690)	0.048[a]	2.631 (1.042-7.670)	0.049[a]

[a]Significant. -, not significant in univariate analysis. Adjusted hazard ratios for PFS, MFS and OS. PFS, progression-free survival; MFS, metastasis-free survival; OS, overall survival.

Figure 3. Kaplan-Meier survival curves for the *IL-6* haplotype (rs1800797, rs1800795). (A) MFS and (B) OS. IL-6, interleukin-6; MFS, metastasis-free survival; OS, overall survival.

of BC metastasis and our study results, we propose the hypothesis that IL-6 polymorphisms may predict the development of metastasis rather than loco-regional relapse.

It is important to mention that, in contrast with the findings of the above-mentioned studies, Markkula *et al* (29), who analysed a Swedish cohort of patients with any primary stage non-metastatic BC, demonstrated that the carriers of the C allele in rs1800795 SNP had a higher risk of early BC events. The authors, however, conducted no DFS, MFS or OS analyses. Furthermore, none of the GWAS studies showed a statistically significant association between *IL-6* polymorphisms and BC survival (23,24). The contrasting findings in *IL-6* polymorphism studies may be due to sampling errors or differences in patient ethnicity. Nevertheless, most of the experimental data and the possible biological pathway support our findings.

IL-6 also acts as a regulator of oestrogen synthesis and may modulate the tumour cell growth related to the hormone receptor status (40). Hormone-sensitive cells exhibit a higher response to IL-6, while ER-negative cells are suppressed by IL-6 (41). However, there is no clear mechanism of association between IL-6 SNPs and the oestrogen receptor positive, progesterone receptor positive and HER2 negative status. Therefore, further studies are needed.

Table VII. Studies evaluating the assocaitions of IL-6 rs1800795, IL-6 rs1800797 and IL-1α rs1800587 single nucleotide polymorphisms with breast cancer survival.

Author, year	Polymorphism	Study design	Number (inclusion criteria)	Ethnicity	Finding	(Refs.)
Abana *et al*, 2017	*IL-6* rs1800795	Case/control	157/158 (stage IV vs. stage I-III)	Non-Hispanic white	GG genotype with distant metastasis	(26)
DeMichele *et al*, 2003	*IL-6* rs1800795	Cohort	80 (stage I-III)	Various	GG genotype with worse DFS and OS	(27)
DeMichele *et al*, 2009	*IL-6* rs1800795, rs1800797	Cohort	346 (non-metastatic, ≥10 positive nodes)	Various	GG genotypes of both SNPs with worse DFS in estrogen receptor positive subgroup	(28)
Markkula *et al*, 2014	*IL-6* rs1800795	Cohort	634 (stage I-III)	No data	C allele in estrogen receptor negative cases with early events	(29)
Snoussi *et al*, 2005	*IL-6* rs1800795, rs1800797 *IL-1α* rs1800587	Cohort	305 (stage I-III)	North African	GG genotypes in both SNPs with worse DFS, but not OS TT genotype with worse DFS and OS	(32)
Grimm *et al*, 2009	*IL-1α* rs1800587	Cohort	262 (stage I-III)	Caucasian	C allele with OS in the univariate analysis only	(31)
Escala-Garcia *et al*, 2019	GWAS	Cohort	96.661 (stage I-IV)	Various	No association of IL-1α and IL-6 SNPs with breast cancer-specific mortality	(23)
Khan *et al*, 2017	GWAS	Meta-analysis	3.136 (stage I-IV estrogen receptor positive)	Various	No association of IL-1α and IL-6 SNPs with breast cancer-specific mortality	(24)
Present study	*IL-6* rs1800795, rs1800797 *IL-1α* rs1800587	Cohort	202 (early stage I-II)	Eastern-European (Lithuanian)	GG/GG diplotype with worse MFS and OS CC genotype with worse DFS, MFS and OS	-

GWAS, genome-wide association studies. IL, interleukin-1.

As far as *IL-1α* SNP is concerned, Um *et al* (18) demonstrated that the *IL-1α* rs1800587 CC genotype is associated with higher transcriptional activity of the IL-*1α* gene. Overexpression of the *IL-1α* promotes tumour invasiveness and metastasis by inducing the expression of angiogenic genes and growth factors (12). A large meta-analysis performed by Xia *et al* (30) showed that the *IL-1α* rs1800587 C allele and CC genotype are associated with increased cancer risk in general. The studies which investigated the associations between *IL-1α* rs1800587 polymorphism and BC survival are presented in Table VII. Grimm *et al* (31) analysed 262 Caucasian BC patients and found that the *IL-1α* rs1800758 C allele in the univariate model is associated with OS; however, the multivariate model failed to repeat the association. In our study, the multivariate survival analysis confirmed the statistically significant impact of the CC genotype on DFS, MFS and OS. Contrasting data is provided by Snoussi *et al* (32), who analysed North African BC patients and found that the rs1800587 homozygous TT genotype showed a significant association with reduced DFS and OS rate. However, the allele and genotype frequency of rs1800587 SNP in African population differs substantially from that in the population with European ancestry. This difference may be the cause of the observed inconsistency between these studies. We demonstrated that in an Eastern-European population the *IL-1α* rs1800758 C allele is associated with more aggressive local disease (i.e., larger tumour size) and for the first time we proposed that the *IL-1α* rs1800758 CC genotype is an independent negative prognostic biomarker for early-stage BC. As the allele frequencies of the *IL-1α* rs1800758 SNP in our study correspond to those of the 1000 Genomes project phase 3 database for European population, these findings could potentially be replicated for European population, but larger confirmatory studies are warranted.

Although experimental data suggests that *VEGFA* and *IL-1β* play a role in BC, our findings, along with the results from several other studies (22-24), do not suggest that several common polymorphisms in these genes are associated with BC clinical and morphological variables and BC survival rates.

Potential limitations of our study include a limited sample size, lack of access to tumour and/or tumour stromal tissue (which would have allowed for the assessment of the presence of SNPs in those tissues), the risk of other confounders, possible gene-gene and gene-environment interactions, and non-random sampling. We also acknowledge that *VEGFA*, *IL-1α*, *IL-1β and IL-6* measurements were not available in the current study. However, our study supports the relevance of functional *IL-6* and *IL-1α* germline polymorphisms to BC prognosis. Further investigations, preferable on larger cohorts with different ethnic origins, are needed to confirm the results of the current study.

In conclusion, we found an association between the IL-*1α* rs1800587 C allele and larger primary tumour size. The *IL-6* rs1800797 A allele, GA genotype, *IL-6* rs1800795 C allele and *IL-6* (rs1800797-re1800795) AC diplotype were linked to hormonal receptor positive BC. Additionally, *IL-6* rs1800797 A was associated with HER2 negative status. The multivariate *IL-1α* rs1800587 CC genotype was confirmed as an independent negative prognostic factor for DFS, MFS and OS, and the IL6 GG/GG diplotype for MFS and OS in early-stage BC patients. Our findings confirm the hypothesis that functional SNPs in angiogenesis- and inflammation-associated genes are associated with early-stage BC prognosis in Lithuanian population.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

EK, RU and EJu designed the research study. EK, RU, RI, LP, VR, EJa and EJu performed the research. EK and RU analyzed the data. RU and RI contributed essential reagents or tools. EK wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by Kaunas Regional Ethics Committee for Biomedical Research (protocol no. BE-2-10). Informed consent for participation in the study and use of tissue was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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