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Interleukin-1 gene locus polymorphisms are associated with risk to breast cancer in Croatian population

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Abstract

Breast cancer has a complex genetic susceptibility. Innate and adaptive immunity can additionally increase the genetic risk to breast cancer development. We typed polymorphisms in the genes of the interleukin(IL)-1 and IL-17 proinflammatory cytokines in a case-control study in Caucasian population from Croatia. We compared the allelic and genotypic frequencies between patients (n=194), healthy women (n=188) and general population (n=531). The risk for breast cancer has been significantly different at both allelic and genotypic levels for two polymorphisms: the IL1B gene single nucleotide polymorphism (SNP) at -511 (G>A; rs16944) and the IL1 Receptor antagonist gene (IL1RN) variable number of tandem repeats (VNTR). The major allele (G) of the IL1B rs16944 SNP was associated with susceptibility to breast cancer (P<0.01) in general population with odds ratio (OR) of 1.42 and 95 % confidence interval (CI) at 1.09–1.85. The IL1RN VNTR allele 3 (5 repeats) was correlated with predisposition to disease (P<0.01, OR: 9.71, 95 % CI: 1.34–198.51) in women. At the genotype level, G/G homozygosity at IL1B rs16944 was significantly associated with predisposition to disease (P<0.02, OR: 1.68, 95 % CI: 1.10–2.57), whereas the heterozygosity (G/A) was correlated with protection to disease (P<0.01, OR: 0.57, 95 % CI: 0.37–0.89) in women. The IL1RN VNTR genotype 1/3 was significantly associated with susceptibility to breast cancer (P<0.01, OR: 10.01, 95 % CI: 1.37–207.55). Genotypic differences were also significantly different in comparison with general population for IL1B SNP (P<0.001) and IL1RN VNTR (P<0.01). These results corroborate a premise that inflammatory factors play a role in pathogenesis to breast cancer.

INTRODUCTION

As the most common malignancy in women worldwide, breast cancer has an increasingly better prognosis in recent decades. Notwithstanding improved standard therapeutic procedures after surgery and chemotherapy, novel therapies based on immune modulation constitute a new hope for more successful treatment. These include monoclonal antibodies directed against tumor-promoting growth factors, their receptors and, recently, (anti-)cytokine treatments *(1)*.

The clinical research underlying such therapies is based on known facts, but many genetic risk factors still remain unknown. According to the immunosurveillance hypothesis *(2)* the immune system senses pre-cancerous, transformed and malignant cells [reviewed in *(3)*]. Humans with immunodeficiencies or after receiving post-transplantation immunosuppression therapy have increased incidence of cancers *(4–6)*. These observations were corroborated in experiments in various

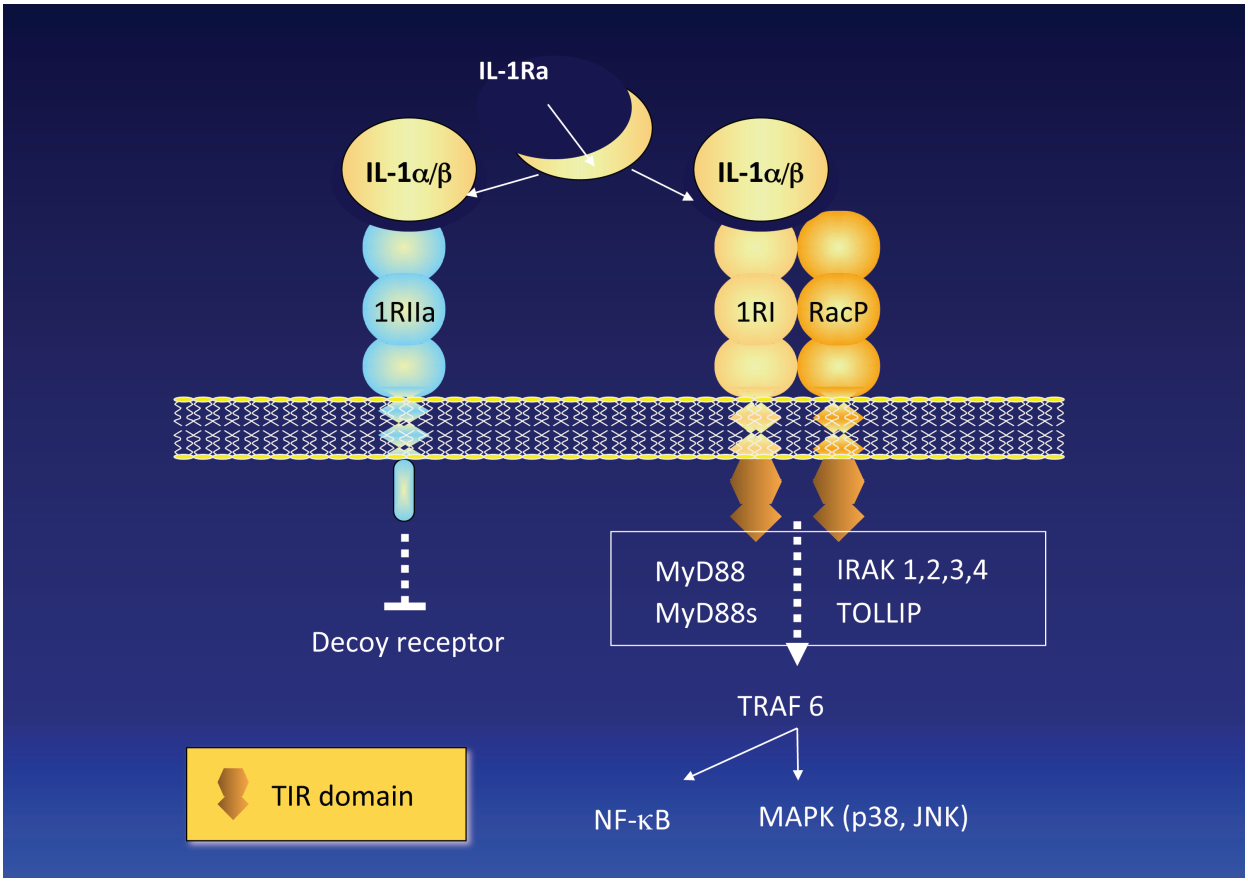

Figure 1. *The IL-1 cytokine action. The artwork is a schematic representation of proteins involved in the action and signal transduction of IL-1a and IL-1b cytokines. The IL-1 antagonist IL-1Ra (IL-1 receptor antagonist) binds IL-1 receptor (IL-1R, type-1; two chains IL-1RI and IL-1RacP) and the decoy IL-1 receptor (type-2; IL-1RIIa). A soluble form of the IL-1RacP chain can bind both IL-1 cytokines and additionally inhibit their actions. MyD88, IRAK, TOLLIP, and TRAF: factors and adaptors common to Toll-like receptor and IL-1 signalling pathway. TIR:* Toll *and IL-1 region. Signalling cascade ends with the nuclear factor -kappa B (NF-kB) and mitogen activated protein kinases (MAPKs) such as p38 and* jun*-activated kinase (JNK) that can promote expression of various genes.*

animal models, showing that immunity plays an important role in cancer development *(1, 7–15)*. Genes involved in the regulation of the anti-cancer immune attack could play an important role in tumor development *(1)*. Therefore, it is reasonable to assume that class-regulating aspects of the immune system largely characterized by cytokine actions (including inflammation) might constitute additional susceptibility to cancer. Genetic variation in these genes may play a role in determining predisposition not only to cancer, but to a range of common, chronic human diseases which have an inflammatory component *(16, 17)*.

On the other hand, sites of chronic inflammation are often associated with the establishment and growth of various malignancies including breast cancer. A quintessential proinflammatory cytokine is Interleukin(IL)-1. IL-1 has a key role in the activation of macrophages at the receiving end of the cell-mediated immunity with pro--inflammatory character exemplified with CD4+ Th1 cells and NK cells that have a principal role in the defense against intracellular microorganisms (18). IL-17 is another pro-inflammatory cytokine secreted by Th17 cell subtype also implicated in the activation of tissue macrophages as well as recruitment of neutrophils [for a review see *(19)*].

Proinflammatory cytokines such as IL-1 and IL-6 were reported to be associated with disease free survival or the risk to breast cancer, respectively, in Tunisian women *(20)*. However, IL6 gene (but not the IL1 gene locus) polymorphisms were found associated with higher risk for the disease development in German and Austrian female populations *(21)*. Interestingly, in Japanese women, a single nucleotide polymorphism (SNP) in the IL1B gene (-31 C>T) was associated with a decreased risk for breast cancer *(22)*. Variability in genetic susceptibility to a disease, in general, is known to vary across ethnic boundaries, and perhaps might explain these findings. There were no studies about IL-17 cytokine in assessing the risk for breast cancer development. However, in animal model with metastatic breast cancer, treatment with anti-IL17 and with celecoxib, an anti-inflammatory drug, completely abrogated the development of metastases *(23)*. In humans, a recent genome-wide study found SNPs associated with musculoskeletal adverse

events in women treated with aromatase inhibitors for early breast cancer close to a gene (T-cell leukemia 1A) which, in turn, was related to the IL-17 cytokine *(24)*.

We thus hypothesized that polymorphisms in pro-inflammatory cytokine genes were associated with breast cancer development in Caucasian subpopulation from Croatia. We analyzed frequencies of three IL1 gene locus SNPs (IL1A -889 C>T, IL1B -511 G>A, IL1B +3954 C>T), the IL-1 receptor antagonist gene (IL1RN) variable number of tandem repeats (VNTR), and the IL17 gene SNP (-197 G>A) in the breast cancer case-control study.

METHODS

Participants

The study included 191 breast cancer patients initially treated at the Department for Medical Oncology, Division of Oncology, the Clinical Hospital Center "Zagreb", Faculty of Medicine, University of Zagreb (Croatia). They were diagnosed by medical, radiological, and biochemical assessment and mostly comprised early stages of breast-cancer (*carcinoma ductale invasivum* and *carcinoma lobulare invasivum*, 5:1 ratio). All patients provided oral and written informed consent. The mean age for patients at the time of cancer diagnosis was 56,4 ± 11,4 (±SD). The control population was divided in two groups: (1) healthy women (n=194) from Rijeka and Zagreb, Croatia, as reported previously *(25)* that had a mean age of 44,9 ± 9,2 years (at the time of sample collection), and a (2) mixed gender healthy population (n=531) that included healthy blood donors (mean age: 42.3 ± 11.9). The study was approved by the ethics committees of the Medical Research Council at the Medical faculties at the Universities of Zagreb and Rijeka.

DNA isolation

The blood was taken from patients and genomic DNA extracted as described previously (25). Genomic DNA from healthy blood donors was isolated by the salting out procedure using commercially available kit (Qiagen, Germany) as reported earlier *(26)*.

SNP analyses

The gene cluster of inerleukin-1 on chromosome 2q13–14 consists of 9 members, encoding IL-1α, IL-1β (proinflammatory) and IL-1 receptor antagonist (IL-1Ra, anti-inflammatory) cytokines among other genes. Genotyping of the IL1 gene locus polymorphisms was performed by PCR as described previously *(26)*. In short, for IL1A (-889 C>T, rs1800587) and IL1B (-511 G>A, rs16944; and +3954 C>T, rs1143634) SNPs we used the TaqMan method for allele-specific detection in the Mx3005P Real-Time PCR System (Stratagene, Agilent Technologies Inc., Santa Clara, CA, USA) according to manufacturer's instructions (Applied Biosystems Inc., Foster City, CA, USA). The IL-1 receptor antagonist (IL1RN) gene VNTR was typed by PCR with following conditions: 40 cycles of denaturation (95 °C, 10 s), annealing (59 °C, 20 s) and elongation (72 °C, 90 s) after initial preheating at 95 °C for 1 minute. We analyzed PCR products by 2 %-agarose-gel electrophoresis (stained with ethidium bromide). As markers for fragment length, we loaded a 100bp DNA ladder (GeneRuler™, Fermentas, Fisher Scientific, Pittsburg, USA) in adjacent lanes on the gel. Genotyping of the promoter specific SNP of the IL17A gene at -197 G>A (rs2275913) was performed according to the TaqMan protocol using the Assays-on-Demand kit (the primers and probes detecting the rs2275913 SNP were selected and synthesized by the Applied Biosystems Inc).

Statistical analyses

We compared the allelic and genotypic frequencies of the polymorphic locus between cases and controls by using contingency table analysis and Chi-square method (Statcalc program, Acastat software; and web-based java program at statpages.org/ctab2x2.html). All genotype frequencies were in Hardy-Weinberg equilibrium, as analyzed by the Arlequin software v3.5 (Genetics and Biometry Laboratory, University of Geneva, Switzerland). A statistically significant difference was pronounced when p was < 0.05, if 95 % confidence interval (CI) for odds ratio (OR) did not cross the value of 1.

RESULTS

We have genotyped four SNPs: IL1A (rs1800587), IL1B (rs16944), IL1B (rs1143634) and IL17 (rs2275913), and the IL1RN gene VNTR in female breast cancer patients. Comparison of allelic frequencies showed that only two polymorphisms had significant differences (Table 1). The allelic frequency of a major allele (G) of the IL1B SNP (rs16944) in women with breast cancer was 71.76 % (n=277), which was remarkably higher when compared to healthy women (65,43 %, n=246) or mixed-gender healthy population (64,20 %, n=651). However, only the latter comparison resulted with a statistically significant difference (P=0.007, OR: 1.42, 95 % CI: 1.09–1.85). The former difference had a trend for statistical significance with p=0.06, OR: 1.34 (95 % CI 0.98–1.85). The minor allele (A) was found in 28.24 % (n=109) of breast cancer cases (Table 1), and comparison with mixed-gender control population (35.80 %; n=363) revealed a similarly high significance (p=0.007, OR: 0.71, 95 % CI: 0.54–0.92).

The IL1RN VNTR-polymorphism frequencies were also different at allelic level. A minor allele 3 (that has 5 tandem repeats) was significantly increased in women with breast cancer (p=0.007, OR: 9,71, 95 % CI: 1.34–198.51; Table 1). The comparison with general population however revealed only a trend towards significant difference (p=0.07).

The allelic frequencies of other tested SNPs in the IL1B, IL1A and IL17 genes were not significantly different between cases and controls (Table 1).

TABLE 1

Allelic association analysis of IL1 gene locus and IL17 gene polymorphisms in patients with breast cancer in the Croatian population.

Allelic frequencies and number										
Protein	**Gene polymorphism**	**Alleles**	**Controls (all, FM)**		**Controls (women; F)**		**Patients (BC)**		**p**	**p**
			Frequency	N	Frequency	N	Frequency	N	(F vs BC)	(FM vs BC)
IL-1α	IL1A (-889) SNP	C	70.52 %	739	76.50 %	179	72.94 %	283	0.32	0.37
	rs 1800587	T	29.48 %	309	23.50 %	55	27.06 %	105		
IL-1β	IL1B (-511) SNP	G	64.20 %	651	65.43 %	246	71.76 %	277	0.06[1]	*0.007*[2]
	rs 16944	A	35.80 %	363	34.57 %	130	28.24 %	109		
	IL1B (+3954) SNP	C	74.11 %	787	76.92 %	180	76.96 %	294	0.99	0.27
	rs 1143634	T	25.89 %	275	23.08 %	54	23.04 %	88		
IL-1Ra (IL-1 Receptor antagonist)	IL1RN VNTR	1 (4x)	70.93 %	732	76.69 %	181	71.69 %	271	0.17	0.78
		2 (2x)	26.74 %	276	22.88 %	54	24.34 %	92	0.68	0.36
		3 (5x)	2.23 %	23	0.42 %	1	3.97 %	15	*0.0073*	0.07
		4 (3x)	0.10 %	1	0.00 %	0	0.00 %	0	–	1
IL-17	IL17 (+192) SNP	G	62.80 %	260	60.99 %	111	67.10 %	259	0.15	0.20
	rs 2275913	A	37.20 %	154	39.01 %	71	32.90 %	127		

[1] OR: 1.34 (95 % Confidence interval 0.98–1.85)
[2] OR: 1.42 (95 % Confidence interval 1.09–1.85) – associated with predisposition
[3] OR: 9.71 (95 % Confidence interval 1.34–198.51) – associated with predisposition

The genotypic analysis showed statistically significant differences of IL1B rs16944- and IL1RN VNTR-polymorphism frequencies between cases and both control populations (Table 2). Women bearing homozygous (G/G) genotypes of rs16944 had frequencies that were associated with predisposition to breast cancer (p=0.012, OR: 1.68, 95 % CI: 1.10–2.57). Interestingly, the minor allele homozygosity (A/A) had no association with the disease. However, heterozygous women (G/A) had a significant association with protection to development of breast cancer (p=0.009, OR: 0.57, 95 % CI: 0.37–0.89). These differences were more pronounced when mixed-gender controls were compared with women with breast cancer (Table 2); for major allele p=0.0003 (OR: 1.85, 95 % CI: 1.31–2.63) and for minor allele p=0.0002 (OR: 0.53, 95 % CI: 0.37–0.75). The interpretation for these findings might involve a genetic factor that is not modified by a sex difference in the predisposition to breast cancer.

As shown in Table 2, another genotype was found associated with breast cancer: the IL1RN VNTR genotype 1/3 was significantly correlated with susceptibility to disease (p=0.007, OR:10.01, 95 % CI: 1.37–207.55). This difference was also significant in comparison with general population (p=0.004, OR: 2.84, 95 % CI: 1.28–6.28; Table 2).

The genotypic frequencies for the IL1A (-889, C>T; rs1800587), IL1B (+3954, C>T; rs1143634) and the IL17 (-197, G>A; rs2275913) SNPs were not significantly different between cases and controls (Table 2).

DISCUSSION

We have demonstrated associations of two polymorphisms within the IL-1 gene locus with breast cancer in a case-control study in Croatian women: the IL1B (-511 G>A) SNP rs16944 and IL1RN VNTR. Therefore, IL1 gene locus alleles marked by these SNP and VNTR could be associated with the increased risk for developing breast cancer in this subpopulation of Caucasians.

The major allele (G) of the rs16944 SNP could increase genetic risk for acquiring the disease by approx. 42 %, whereas the minor allele (A) might decrease the risk by 29 %. The allelic frequency of the major allele (G) of the IL1B SNP (rs16944) in women with breast cancer was significantly higher when compared to general mixed-gender healthy population. However, a similar comparison with healthy women revealed a difference that had only a trend for statistical significance. The reason for it may lay in the small number of samples tested, as the general population had 507 individuals, whereas the women-only study had 193 patients and 188 control persons. Interestingly, the HapMap-CEU population frequencies of the IL1B rs16944 alleles for Caucasians of the Western and Northern European origin (G= 64.2 %, A=35.8 %; n=226 chromosomes) were identical to a two-decimal point with our results for general healthy controls. The healthy control women in our study had slightly higher frequency (65,43 %), which could perhaps explain this discrepancy. Thus, it is likely that with more samples of healthy women we would also see a significant difference at this locus as we observed using

TABLE 2

Genotype association analysis of IL1 gene locus and IL17 gene polymorphisms in patients with breast cancer in the Croatian population.

Genotypic frequencies and number													
Gene			Controls (all) (men & women; FM)		Controls (women; F)		Patients Patients (BC)		Statistical analysis				
Protein	Polymorphism	Genotypes	Freq.	*N*	Freq.	*N*	Freq.	*N*	p (F vs BC)	OR (95 % CI) (F vs BC)	p (FM vs BC)	OR (95% CI) (FM vs BC)	Association
IL-1α	IL1A (-889)	C/C	51.34 %	269	58.97 %	69	52.58 %	102	0.27		0.77		
	SNP	C/T	38.36 %	201	35.04 %	41	40.72 %	79	0.32		0.56		
	rs1800587	T/T	10.31 %	54	5.98 %	7	6.70 %	13	0.81		0.14		
IL-1β	IL1B (-511)	G/G	39.64 %	201	42.02 %	79	54.92 %	106	*0.012*	1.68 (1.10–2.57)	*0.0003*	1.85 (1.31–2.63)	predisposition
	SNP	G/A	49.11 %	249	46.81 %	88	33.68 %	65	*0.009*	0.57 (0.37–0.89)	*0.0002*	0.53 (0.37–0.75)	protection
	rs16944	A/A	11.24 %	57	11.17 %	21	11.40 %	22	0.95		0.95		
IL-1β	IL1B (+3954)	C/C	54.99 %	292	58.12 %	68	57.59 %	110	0.93		0.53		
	SNP	C/T	38.23 %	203	37.61 %	44	38.74 %	74	0.84		0.90		
	rs1143634	T/T	6.78 %	36	4.27 %	5	3.66 %	7	0.79		0.12		
IL-1Ra		1(4x)/1(4x)	51.72 %	267	58.47 %	69	48.68 %	92	0.09		0.37		
	IL1RN	1(4x)/2(2x)	35.27 %	182	35.59 %	42	38.10 %	72	0.66		0.57		
	VNTR	2(2x)/2(2x)	8.72 %	45	5.08 %	6	5.29 %	10	0.94		0.13		
		1(4x)/3(5x)	2.91 %	15	0.85 %	1	7.94 %	15	*0.007*	10.01 (1.37–207.55)	*0.004*	2.84 (1.28–6.28)	predisposition
IL-17	IL17 (+192)	G/G	39.61 %	82	37.36 %	34	46.11 %	89	0.16		0.19		
	SNP	G/A	46.38 %	96	47.25 %	43	41.97 %	81	0.40		0.38		
	rs2275913	A/A	14.01 %	29	15.38 %	14	11.92 %	23	0.42		0.53		

the mixed gender controls. The other human subpopulations (Asians, Africans) have all even lower frequencies of the major allele (dbSNP at http://www. ncbi.nlm.nih.gov). In addition, as the breast cancer is not gender specific (albeit rare in men) it seems reasonable to suggest that the observed association of the IL1B rs16944 SNP with breast cancer might be valid for both sexes.

Regarding the genotype of the IL1B SNP (rs16944) the risk might be 68–85 % higher for the homozygous carriers of major (G/G) alleles, whereas the heterozygous G/A carriers could have a 43–47 % reduced risk in developing breast cancer. However, the homozygous individuals with only the minor allele have not been significantly associated with either of the outcomes. This seems paradoxical, as the minor allele was associated with protection. Perhaps additional factors within the IL1 gene locus might play a role in the risk for breast cancer development that might counteract its protective role. Because, additionally, these associations were more pronounced when mixed-gender controls were compared with breast cancer patients (Table 2), we suggest that this is an influence of a gender-unrelated factor (such as inflammation) that is implicated in the susceptibility to breast cancer.

There is a controversy regarding the expression of IL-1β cytokine and the IL1B (-511 G>A; rs16944) SNP. Major allele G/G homozygote carriers had a higher IL-1β production in Caucasian population (*27*). Furthermore, another study showed that mononuclear blood cells with G-allele produced increased levels of IL-1β upon stimulation *in vitro* compared to those with a minor allele *(28)*. In contrast, an earlier study showed that a haplotype containing the minor A-allele at IL1B -511 has been associated with increased IL-1β production *in vitro (29)*.

Because of this controversy, we have two possible interpretations for our finding that G/G is associated with predisposition to breast cancer. Higher propensity of mammary tissue for inflammatory response (if G allele correlates with higher expression of the IL-1β) could be directly related to cancer-cell transformation perhaps as a consequence of tissue stress and damage during physiologic (i.e. breastfeeding) or pathophysiologic (i.e. infections) processes. Alternatively, a higher IL-1 production might be instrumental for immune surveillance and anti--cancer immune attack. In such scenario, tumor-associated macrophages would be stimulated to secrete IL-1 (among other cytokines), and with its action kill adjacent cancer cells. Such tumor-associated macrophages could be activated via CD4 T cells specific for tumor antigens on MHC-class-II negative cancer cells, as suggested and shown in the mouse myeloma model *(14, 15)*. Perhaps, in other cancers, CD4 Th1 tumor-specific cells might hypothetically enhance licensing of tumor-specific MHC--class-I-restricted CD8 T cells that would in turn be able to eradicate their target (cancer) cells. Therefore, if the G allele of the rs16944 SNP represents the allele with a low IL-1β production, it could diminish the anti-cancer immunosurveilance, and hence lead to increased risk for developing tumors.

We found that individuals harboring the IL1RN VNTR allele 3 (5 copies) had 2.8- to 10-fold increased risk for breast cancer. The IL-1 Receptor Antagonist (IL-1Ra) is the modulator of the IL-1 action (Figure 1) as it inhibits binding of both IL-1a and IL-1b to the receptor of the IL-1. IL-1Ra also inhibits interaction of both IL-1 cytokines with the decoy receptor (Figure 1), which in turn, can additionally modulate IL-1 actions by sequestering local concentration of IL-1 cytokines. It is unclear which of the IL-1Ra actions might diminish or increase the risk for breast cancer. Nevertheless, it is possible that the allele 3 of the IL1RN VNTR polymorphism is associated with a weaker inhibition of the IL-1 actions resulting with higher proinflammatory response (and increased risk). Alternatively, a stronger inhibition of the IL-1 action might reduce tumor immunosurveillance. Notwithstanding these uncertainties, our data collectively suggest that IL-1 cytokine actions modulate the risk for breast cancer, despite a distant possibility that observed associations might involve other genomic or epigenetic influences (i.e. methylation of CpG islands and microRNA).

In other populations, studies from two case-control studies in women of Tunisian and German/Austrian origin have shown no association with the IL1 gene locus *(20, 21)*. Both studies found association with another pro-inflammatory-cytokine gene polymorphism, IL-6. On the other hand, in Japanese population a case-control study reported a reduction of the risk for breast cancer for carriers of a SNP in the IL1B gene (at -31) *(22)*. We believe that the differences between our study and published ones possibly reflect variations in disease etiology, population history that can produce characteristic patterns of SNP allele frequencies, linkage disequilibrium and haplotypes when ethnic groups are compared, and perhaps environmental exposures in diverse populations.

Our IL-17 data revealed no associations with the risk for breast cancer development in Croatian population. Interestingly, recent genome-wide study found a correlation with the SNP near the T-cell leukemia 1A gene (that was related to the IL-17 receptor A gene expression, and hence IL-17) with musculoskeletal adverse events in women treated with aromatase inhibitors for early breast cancer *(24)*. Perhaps, some characteristics other than the risk of developing disease might be correlated with polymorphisms in the IL-17 cytokine gene. Indeed, it was recently reported that a metastatic potential of breast cancer is influenced by the IL-17 in an animal model *(23)*.

In conclusion, we showed a correlation of polymorphic markers within the proinflammatory-cytokine IL-1 gene locus with the risk in developing breast cancer. Taken together with our recent finding that a IL12B gene SNP is also associated with the risk for the disease *(25)*, we suggest that inflammation via innate and adaptive immunity contributes to multifactorial hereditary predisposition to pathogenesis of the breast cancer.

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