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Single Nucleotide Polymorphism in the Interleukin 12B Gene is Associated with Risk for Breast Cancer Development

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Abstract

We analysed the association of a single nucleotide polymorphism (SNP) in the gene encoding the IL-12 subunit p40 (IL12B, rs3212227, A>C) with breast cancer. The SNPs allelic and genotypic frequencies were compared between patients (n = 191) and healthy (n = 194) women in a case–control study from Croatia. The major allele (A) was associated with susceptibility to breast cancer (P = 0.003; OR = 1.67; 95% CI: 1.17–2.38). Likewise, the minor allele (C) was significantly correlated with protection (P = 0.003; OR = 0.60; 95% CI: 0.42–0.86). At the genotype level, AA homozygosity was significantly associated with predisposition to disease (P = 0.013; OR = 1.68, 95% CI: 1.09–2.59), whereas the minor allele homozygosity (CC) was correlated with protection to disease (P = 0.020, OR = 0.28, 95% CI: 0.09–0.91). The heterozygous genotype showed no significant correlation with disease. The product of the IL12B gene (IL-12 p40) can either form a homodimeric cytokine or be part of two pro-inflammatory (IL-12 and IL-23) cytokines. It is presently unclear whether the major allele is associated with higher or lower protein levels of IL-12 p40 and IL-12 p70, which are critical in inflammation and adaptive immune responses. However, as the A allele is high producer of IL12B (p40) mRNA, these results might imply that higher levels of IL-12 p40 (either as homodimers or joined with one or both of the other two subunits) predispose to breast cancer.

Introduction

Breast cancer is the most common malignancy in women, who have over 100-fold higher risk of developing disease than men. Despite the progress in treatment over the past years and relatively good overall survival at present (about 80% have an estimated 5-year survival rate), the disease has still caused over 450,000 deaths worldwide per year in the last decade. Recent novel therapies involve various cytokine treatments that promote anti-cancer-specific adaptive immunity. However, success of therapies might be greatly improved provided all risk factors were known.

Cancer has a multi-factorial genetic susceptibility. The immune system can contribute to genetic risk to cancer; first, innate immunity can provide stimulation of cell growth by inflammation, a well-known tumour promotion factor, and second, adaptive immunity can detect and eliminate cancer cells according to the immunosurveillance hypothesis [1]. An increased former capacity or a weakness in the latter ability would increase susceptibility to cancer. Evidence that adaptive immunity has a role in cancer development comes from experiments in various animal models [2–9] and from data showing an increased incidence of cancers in humans with immunodeficiencies [10] or after receiving post-transplantation immunosuppression therapy [11, 12]. In line with this, CD4 T cell and CD1a dendritic cell populations in axillary lymph nodes were highly correlated predictors of disease-free survival in patients with breast cancer [13].

The IL-12 cytokine is important for adaptive immunity with a principal role in the defence against intracellular micro-organisms [14, 15]. It is a 70-kDa heterodimeric protein (IL-12 p70) composed of p35

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(IL-12A) and p40 (IL-12B) subunits [16]. IL-12 was also shown to be important in host resistance to tumours [17–20]. The IL-12B subunit can form homodimers [21] and IL-23 cytokine by joining with another unrelated subunit, p19 (IL-23A). The IL-23 too plays an important role in the antimicrobial defence (for a review see [22, 23]).

Several molecular and genetic epidemiologic studies investigated the association between the IL12A and IL12B gene polymorphisms and risk for cancers. The significant associations were found in those that studied polymorphisms of the IL12A and IL12B genes in cervical cancers [24, 25], gliomas [25], non-Hodgkin lymphomas [26] and in lung cancer [27]. Furthermore, some studies showed non-significant correlation between various cancer types and IL12 gene polymorphisms like hepatocellular carcinoma [28], gastric cancer [29, 30], Kaposi sarcoma [31], colon carcinoma [32] and cutaneous malignant melanoma [33]. Despite the lack of association in the latter studies, interestingly, many found different pro-inflammatory, anti-inflammatory or immune-modulator cytokine gene polymorphisms to be associated instead, however, in combination with other cytokine genes' polymorphisms.

No data have been reported for the IL12 genes' polymorphisms in analyzing the risk for breast cancer. We hypothesized that they might be correlated, and to analyse this, we have selected the IL12B gene SNP (A>C +1188, rs3212227), as it was recently found associated with autoimmune diseases like ankylosing spondylitis [34], insulin-dependent diabetes mellitus type I [35] and multiple sclerosis [36], but interestingly also with occurrence of cervical cancers, nasopharyngeal cancers, gliomas and non-Hodgkin lymphomas [24–26, 37].

Methods

Participants. Blood samples from patients with breast cancer at the Department for Medical Oncology, Division of Oncology, the Clinical Hospital Center 'Zagreb' (Croatia), were collected in EDTA containing tubes and kept frozen until DNA isolation. The control group included 194 healthy women from Rijeka and Zagreb (Croatia), and their blood samples were similarly preserved for DNA isolation.

Patients were diagnosed by medical, radiological and biochemical assessment. Most presented at early stages of breast cancer (*carcinoma ductale invasivum* and *carcinoma lobulare invasivum* at approximately 5:1 ratio) and the initial treatments were started at the Department. All patients (n = 191) provided oral and written informed consent. Present study involved additional 70 patients to the previous case–control report by our group [38]. With these additions, the mean age for patients at the time of cancer diagnosis was 56.4 ± 11.4 (±SD). There was an average of 2.9 ± 4.3 years time span between diagnosis and sample collection (approximately 40% of patients had disease diagnosed 5–20 years earlier). Control individuals were healthy women (n = 194) from Rijeka and Zagreb, Croatia: 102 came from the same hospital as patients, and the rest (92) were reported previously and included healthy blood donors [39, 40]. The control population had a mean age of 44.9 ± 9.2 (±SD) years at the time of sample collection. The study was approved by the ethics committees of the Medical Research Council at the Medical faculty at the Universities of Zagreb and Rijeka.

DNA isolation and SNP analysis. The blood was taken from patients and genomic DNA extracted as described previously [38–41]. Genotyping of the IL12B gene SNP (A>C) rs3212227 (exon 8, position 1188 of the transcript) was performed by real-time PCR using the Taqman method for allele-specific detection (Applied Biosystems, San Diego, CA, USA) according to the instructions of the manufacturer in the MX3005 PCR machine with its own analysis software (Stratagene, San Diego, USA). The rs3212227-specific probe and primer mixture was ordered from Applied Biosystems using assays-on-demand service, which included the synthesis of the following four oligonucleotides: The genomic DNA was amplified using the forward primer 5′-AGGATCAC AATGATATCTTTGCTGTATTTGT and the reverse primer 5′-GCAACTTGAGAGCTGGAAAATCTATACA TAAA. SNP was detected using allele-specific probes that could detect complementary nucleotides at the SNP site and were modified with a minor-groove-binder to increase the specificity of allele discrimination. The probes were 5′ labelled with VIC or FAM dyes and had the following sequences 5′-AGCATTTAGCATCT AACTAT or 5′-CATTTAGCATCGAACTAT, respectively (with alternative bases in bold representing the polymorphism).

Statistical analyses. We compared the allelic and genotypic frequencies of the polymorphic locus between cases and controls by using chi-squared-method (STATCALC program, ACASTAT software; and internet-based application: http://statpages.org/ctab 2x2.html). The genotype frequencies were in Hardy–Weinberg equilibrium.

A minimum sample size of 366 total subjects (183 cases and 183 controls) was calculated as needed to be analysed to detect a relevant difference in allelic frequency (i.e. 9% difference between the groups ranging from 73% to 82%) according to the power analysis based on following parameters: type II error rate (β = 0.2), type I error rate (α = 0.05). We used odds ratio (OR) as an indication for the relative risk, and 95% confidence interval (CI) to describe the limitations within which the estimated calculations should be valid.

Results

As shown in Table 1, the frequency of major IL12B SNP allele (A; rs3212227) was 81.4% in patients with breast cancer compared with 72.4% in healthy women (P = 0.003; OR = 1.67, 95% CI: 1.17–2.38; χ^2: 8.75). This difference implies an association with 67% higher predisposition to the disease. On the other hand, carriers of the minor allele (C) had an association with 40% reduced disease risk, as it had a frequency of 18.6% in patients, compared with 27.6% in controls (P = 0.003; OR = 0.60; 95% CI: 0.42–0.86).

The genotypic analysis of IL12B SNP frequencies showed also significant differences between cases and controls. As shown in Table 2, individuals homozygous for the major allele (AA) had an association with higher risk for breast cancer (P = 0.013; OR = 1.68, 95% CI: 1.09–2.59). High risk was also found for all carriers of the major allele (AA and AC), as 96.9% women with breast cancer (185/191) had the major allele compared with 91.2% women in healthy population (177/194), which was a statistically significant difference (P = 0.020; OR = 2.96, 95% CI: 1.07–8.62). In contrast, those that were homozygous for the minor allele (CC) had an association with protection from disease (P = 0.02; OR = 0.36, 95% CI: 0.13–0.94). However, heterozygous individuals had no significant association with either of the risks (P < 0.16), perhaps because both alleles neutralized each other's association.

Discussion

Patients with breast cancer had significantly higher allelic frequencies of the major (A) allele of IL12B SNP (A>C, rs3212227) suggesting that this allele conferred increased risk for disease development in Croatian Caucasian population. Having only the major allele (homozygous AA carriers) implicated a 1.7-fold increased risk, whereas having only the minor allele (CC) reduced the risk by 64%, suggesting a co-dominant effect.

It is likely that this association indicates the involvement of the IL12B gene in the risk for breast cancer development. However, we cannot exclude a possibility that this SNP is only a marker for other kind of genomic influence like micro-RNA (miR), CpG islands or adjacent genes. We have, therefore, screened the micro-RNA database at the Next Bio internet site (https://www.nextbio.com/) and found 31 hsa-miR within the IL12B gene (ranging from hsa-miR-129-1 to hsa-miR-653). Among hundreds of target genes in the genome that these micro-RNAs might regulate (by searching GeneCopoeia™, http://www.genecopoeia.com/product/mirna/targets.php), the one of interest might be hsa-miR-653 that targets the BRCA2 gene, which is implicated in the early breast cancer onset. Two CpG islands border with the IL12B gene, and within genomic area of 100 kb on chromosome 5, two genes flank the IL12B gene. One is 25 kb downstream of the SNP (the nuclear proteasome phosphatase gene UBLCP1) and the other one 2 kb upstream of its promoter (an unknown gene). To clarify the relationship between hsa-miR-653 with IL12B SNP, further research is required.

The frequencies of IL12B in our subpopulation are different from frequencies listed for Asian, African and some other Caucasian subpopulations, according to various databases. In the HapMap database, they vary from 42% in Japanese (n = 100), 57% in Han Chinese (n = 86), 67% in Nigerian (n = 111), 70% in Kenyan (n = 143) population, to 81% in population from Utah, USA (n = 113) that has descended from a mix of Northern-and-Western-European ancestors with unknown population in the USA. In the SNP database, we found another Caucasian population also from Utah (USA) of Western European descent (n = 23) who had comparable frequency of major allele (76.4%) to our control population (n = 194; 72.4%). At a subpopulation level, our study has the superior number of typed chromosomes (n = 388) in healthy controls for this SNP compared with all listed studies. Many if not all SNPs in the genome show similar variability among human populations. Thus, it is possible that breast cancer as a complex disease might have various associated markers in different populations.

The IL12B gene encodes p40, which partakes in the generation of at least two important heterodimeric cytokines regulating T-helper cell development: IL-12 and

Table 1 Allelic frequency analysis of IL12B SNP (rs3212227) in patients with breast cancer in the Croatian population.

	rs3212227	Patients[a]	Controls[a]			
IL12B[b]	SNP (A>C)	n = 382	n = 388	P	OR	Association
Allelic frequencies and number						
1	A	0.814 (311)	0.724 (281)	0.003	1.67 (1.17–2.38)	Predisposition
2	C	0.186 (71)	0.276 (107)	0.003	0.60 (0.42–0.86)	Protection

SNP, single nucleotide polymorphism.
[a]Frequency (number).
[b]Allele designation (1 – major, 2 – minor allele).

Table 2 Genotype analysis of IL12B SNP (rs3212227) in patients with breast cancer in the Croatian population.

	rs3212227	Patients[a]	Controls[a]			
IL12B[b,c]	SNP (A>C)	n = 191	n = 194	P	OR	Association
Genotypic frequencies and number						
1/1	A/A	0.660 (126)	0.536 (104)	0.013	1.68 (1.09–2.59)	Predisposition
1/2	A/C	0.309 (59)	0.376 (73)	0.16		
2/2	C/C	0.031 (6)	0.088 (17)	0.02	0.36 (0.13–0.94)	Protection

SNP, single nucleotide polymorphism.
[a]Frequency (number).
[b]Allele designation (1 – major; 2 – minor allele).
[c]Genotype designation.

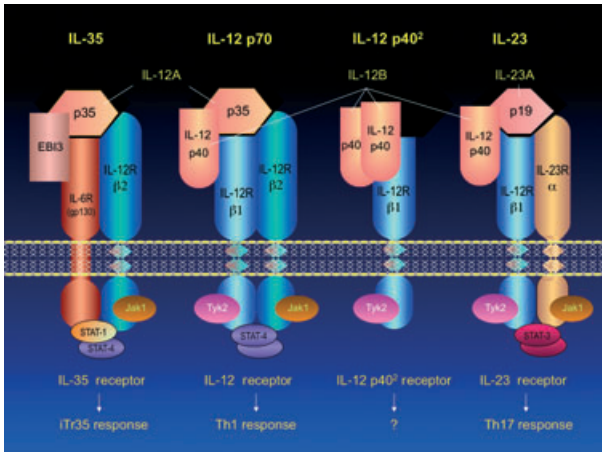

Figure 1 The structure and function of cytokines formed by IL-12B (p40) and the relationship to IL-35. The p40 forms 3 cytokines: IL-12, IL-23 and homodimers ($p40^2$), and by interaction with receptors, it can generate various T cell responses. In humans (and in mice), one of its counterparts – p35 can form with EBI3 subunit IL-35 cytokine. The artwork is a schematic representation of proteins involved in the action and signal transduction of these cytokines. Th1 = CD4 T-helper cell-1 population (secreting IFN-γ); Th17 = T-helper cell-17 population (secreting IL-17); iTr35 = induced T regulatory cell ($CD4^+$ $FoxP3^-$) population (secreting IL-35, but neither IL-10 nor TGFβ).

IL-23 (Fig. 1). IL-12 initiates differentiation of naïve CD4 T-helper (Th) population into pro-inflammatory Th1 type cells, which together with B cell response including antibody production helps in fight against cancer (as proposed in immunosurveillance hypothesis, reviewed in [42]). On the other hand, IL-23 stimulation (in concert with IL-6 and TGFβ1) of resting memory T cells leads to formation of Th17 subset defined by the production of IL-17, another pro-inflammatory cytokine. Human Th17 cells can develop *ex vivo* either into effector population of T cells that can secrete IL-1 and IFN-γ upon stimulation with a specific tumour antigen [43] or, if Th17 were cultured by the TGFβ, into a regulatory Th17 type (reviewed in [23]). The IL-23/IL-17 axis seems to be an important pro-inflammatory pathway in predisposition to rheumatoid arthritis [44]. It is assumed that Th17 too might help in fight against cancer. Recently, a study [21] revealed that IL-12 p40 homodimers (Fig. 1.) might not be biologically inactive as previously thought, however, with so far unknown relationship to cancer.

The anti-tumour role of IL-12 in mammary carcinomas was shown in mice. Mouse transgenic for the rat HER2/neu oncogene spontaneously develops multifocal mammary carcinomas. Experimental immunotherapy of such mice with allogeneic mammary carcinoma cells (expressing HER2/neu) and systemic administration of IL-12 greatly prevented occurrence of such tumours [19]. According to this result, administration of IL-12 cytokine might potentially be beneficial in treatment of breast cancer in humans. In addition, IL-12 is also anti-angiogenic, in murine breast cancer model [45]. Because of its anti-tumour and anti-angiogenic activities in animal model [46] and promotion of cellular immunity of the Th1 type, there is a recent interest in testing IL-12 as a possible anti-cancer drug.

Unfortunately, administering IL-12 might also increase the chance of developing autoimmunity. In fact, excessive IL-12 production was found in several organ-specific autoimmune diseases, including rheumatoid arthritis [47], type I diabetes mellitus [35], multiple sclerosis [36] and Crohn's disease [48].

In addition, the IL12B rs3212227 SNP has recently been found associated with autoimmune diseases like ankylosing spondylitis [34], and previously with insulin-dependent diabetes mellitus type I [35] and multiple sclerosis [36]. Interestingly, the allele (A) that was associated with increased risk in all of these studies was the same as the one found in this study. This is counterintuitive, as it was believed that a risk for autoimmunity might be a beneficent factor in cancer, and *vice versa*.

On the other hand, the minor allele-containing two genotypes (CC/AC) of the IL12B rs3212227 SNP (in combination with IL12A SNP) have been found to pose a higher risk for occurrence of cervical cancer and gliomas in Chinese women [25]. Similarly, a Korean study identified minor (C) allele of this SNP within a particular IL12B haplotype in patients with cervical cancer that conferred susceptibility to cervical cancer [24]. A recent

Tunisian study also identified minor (C) allele with nasopharyngeal cancer risk [37]. By contrast, in this study, the minor allele homozygous genotype (CC) is associated with a lower risk (protection) to breast cancer. The dissimilarities between our present study and the published ones [24, 25, 37] possibly reflect differences in disease aetiology including complex inheritance variability, the basic molecular mechanisms and possibly environmental exposures in diverse populations. Lastly, the results that would seem to corroborate the protective role of the C allele seen in our present study appeared from a case–control study with non-Hodgkin lymphoma, where the CC/AC heterozygous genotype of IL12B SNP was associated with the longer survival [26].

Thus, it seems that major allele (A) of the IL12B rs3212227 poses increased risk for autoimmune diseases in reported studies [34–36] as well as for breast cancer in this study. What is the mechanism by which the SNP could affect these risks? This SNP lies in exon 8 in the 3′ untranslated region of the transcript, possibly affects exon silencers and thus could regulate the level of IL-12B mRNA expression. It was found that major (A) allele correlates with 50% increased transcript levels of p40 in unstimulated purified peripheral lymphocytes isolated from persons with three different genotypes, estimated by quantitative PCR method [49]. This polymorphism affected different levels of expression in EBV-transformed cell lines with major allele having up to five-fold increased p40 transcript expression, measured by densitometry of Northern blot hybridized with labelled cDNA probe [35]. By contrast, a study at the protein level in monocytes and macrophages homozygous for minor allele showed higher IL-12 p70 secretion upon stimulation with LPS and IFN-γ, but, with no effect on p40 secretion [50].

How can we reconcile these seemingly contradictory data? We should perhaps start by asking which cell types can produce the highest level of the IL-12 p40 in humans. The striking result is that the highest level of IL12B transcript is found in the immature dendritic cell (DC) of the peripheral blood with relative gene expression above 1200. This is 12 times higher than the median expression, which is treated as the 'normal/control' expression level. For the IL12B, median is 101 (measured by Affymetrix Human U133 Plus2/U133A platforms for human studies and listed in Body Atlas at the Next Bio site). The Th1 cell (of the cord blood) follows with 700 relative expression units (REU). Then, next in line are freshly isolated cells like basophiles with 280, memory Th cells with 250 and myeloid cells with 250 REU. Interestingly, peripheral (not immature) DCs including plasmacytoid ones had about 100 REU. Of pathological cell lines, Hodgkins lymphoma cells (900 REU), and some other B-cell malignancies had the highest levels of IL-12B transcript. Some cancer cell lines from a number of lung, colon, pancreatic and brain cancers have doubled or moderately increased transcript levels. Breast tumour cell lines produce IL-12 p40 as a monomer or homodimers at median levels, but, interestingly, do not make complete IL-12 p70 molecule [51].

Could the influence of p40 alone on the immune system be similar to an action of immature DC yielding suppression rather than activation? Intriguingly, if it did, then it would counteract anti-tumour effects reported for IL-12 p70 [17–20]. Perhaps, $p40^2$ might antagonize p70 by acting via different receptor (Fig. 1.). Alternatively, it might prevent formation of both IL-12 and IL-23 cytokines within a cell during protein synthesis. For example, p40 homodimers might be favourably assembled if overexpressed, even though p35 or p19 would be present. This could explain the controversial finding of the minor allele having secreted higher p70 levels without changing the p40 protein levels [50] as follows: Let's assume that minor allele would yield 'normal' levels of p40 and that would suffice in formation of 'normal' protein levels of IL-12 and IL-23 cytokines. We propose that this process would be changed in cells homozygous for the major allele (A). Namely, as the higher transcript producer (A) allele, it should yield more p40 protein than the minor allele. This overexpression might preferentially promote self-joining rather than fusing with other subunits. Consequently, less p70 (or IL-23) would be secreted compared with minor allele. On the other hand, increased levels of p40 homodimers might hypothetically allow p35 to join more frequently with another unrelated subunit (EBI3) and make IL-35 cytokine (Fig. 1). The latter is produced by the regulatory T cell ($CD4^+FoxP3^-$) subset iTr35 that can suppress T cell proliferation and convert conventional naïve T cells ($CD4^+CD25^-CD45RB^{hi}$) into iTr35 cells [52]. Thus, IL-35 might potentially inhibit immune response against cancer cells. However, these suggestions would need to be experimentally verified. Furthermore, secreting a lot of $p40^2$ homodimers might also correlate with ability of B-cell malignomas and breast cancer cells to dodge T cell attack against them. It is also conceivable that T cells, dendritic cells and macrophages have different IL12B regulation and thus various levels of its transcript.

In conclusion, our data strongly suggest the involvement of the IL12B rs3212227 SNP major allele (A) in conferring the increased risk in developing human breast cancer, whereas the minor (C) allele might be implicated in a protective role in Croatians. It is currently uncertain if the major allele is correlated with higher or lower protein levels of extracellular IL-12 p40 and IL-12 p70, which are essential in inflammation and adaptive immune responses. Despite the multitude of reasons that might explain these associations, we favour the simplest. We suggest that pro-inflammatory activity of the IL-12 p40 might be the probable cause of higher risk for developing

breast cancer, although we cannot exclude involvement of p40 alone or in cytokines such as IL-12 and IL-23. Perhaps, in support to our suggestion, recent study of mortality in patients with breast cancer in UK showed an interesting correlation between aspirin intake and reduction in mortality [53]. This might be due to aspirin effect on inhibition of prostaglandins, which in turn would have a calming effect on inflammation.

As novel immune-based cancer therapies aim to deliver IL-12 cytokine into patients with breast cancer, it is possible that carriage of different alleles coding for the IL-12 p40 might affect such therapy perhaps leading to adverse or detrimental effects.

Nevertheless, the suggestion that the immune system senses precancerous, transformed and malignant cells should still generate excitement about novel cytokine therapies in cancer treatment. Learning more about regulation of the immune response should eventually give us the ability to avoid possible adverse effects of such treatments.

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