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Interleukin 7 receptor alpha Thr244Ile genetic polymorphism is associated with susceptibility and prognostic markers in breast cancer subgroups

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ABSTRACT

Interleukin-7 (IL-7) exerts crucial functions on lymphoid cells' development and maintenance. In breast cancer (BC), IL-7 promotes growth of tumor cells in culture through the activation of JAK1/3-STAT5 and PI3K/AKT pathways, and expression of IL-7 signaling components was associated with worst prognosis. A C > T polymorphism (rs6897932; Thr244Ile) at exon 6 of IL-7 receptor alpha (IL-7Rα) gene (*IL7RA*) shifts the balance between the membrane-bound and soluble IL-7Rα splicing variants and was previously associated with autoimmune diseases, but has not been studied in cancer, including BC, so far. Therefore, the present study aimed to investigate the possible association of this polymorphism with the susceptibility and clinicopathological parameters of BC subgroups. *IL7RA* Thr244Ile was genotyped through PCR-RFLP in 403 women without neoplasia, no personal history of malignancy or family history of BC and in 338 BC patients with clinicopathological data available. BC patients were stratified according to their positivity for estrogen (ER) and/or progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Age-adjusted logistic regression was performed for case-control analyses, and correlations with clinicopathological parameters were assessed through Kendall's Tau-b coefficient. All analyses were two-tailed and had 95% confidence interval. In $ER^-PR^-HER2^-$ BCs, TT genotype was associated with increased susceptibility both in genotypic (TT vs. CC: OR = 3.07; CI = 1.01–9.38; $p = 0.05$) and recessive (TT vs. CC + CT: OR = 3.59; CI = 1.19–10.85; $p = 0.02$) models and negatively correlated with disease stage (Tau-b = −0.27; $p = 0.05$). Whereas T allele was positively correlated with histopathological grade (Tau-b = 0.29; $p = 0.03$) and lymph node metastasis (Tau-b = 0.35; $p = 0.02$) in $ER^-PR^+HER2^+$BCs and with Ki67 (Tau-b = 0.51; $p = 0.008$) in $ER^-PR^-HER2^+$ subgroup. These data indicate that IL-7Rα is involved in BC, and that *IL7RA* polymorphism may play distinct roles in breast carcinogenesis according to BC subtype, pointing this genetic variant as an interesting marker for breast carcinogenesis to be validated by further mechanistic and prospective studies with larger samples.

1. Introduction

Breast cancer (BC) is the most common malignant disease among women and the second more frequent neoplasia among all cancers worldwide. This neoplasia is the fifth in cause of death among all cancers and the leading cause of cancer death among women [1]. BC represents an extremely complex and heterogenous disease, presenting different classification schemes, molecular subtypes differing on etiology and clinical management and high degree of interindividual variance in clinical outcome and therapeutic response [2].

Gene expression profiling revealed at least 4 clinically relevant BC subtypes [3]. In clinical routine, BC subgroups are defined according to their positivity for hormonal receptor, namely estrogen (ER) and progesterone receptors (PR), to their cellular proliferation index (Ki67 staining) and to overexpression of human epidermal growth factor receptor 2 (HER2) into at least 4 major subgroups: Luminal-A ($ER/PR^+HER2^-Ki67^{low}$); Luminal-B (ER/PR^+HER2^+ or $ER/PR^+HER2^-Ki67^{high}$); HER2-enriched ($ER^-PR^-HER2^+$) and triple negative ($ER^-PR^-HER2^-$). Of these, Luminal A has the most favorable prognosis, Luminal B and HER2-enriched have an intermediate prognosis and triple negative has the worst prognosis [2]. Studies revealed that low penetrance *loci* have subtype-specific actions in BC,

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highlighting the different etiology of these tumors and the importance to consider these subtypes in association studies [4].

The interplay among cells and factors in tumor milieu, such as growth factors, cytokines, extracellular matrix, immune and tumor cells and fibroblasts, is crucial to BC development. Among cytokines, interleukin-7 (IL-7) has been recently suggested to play a role in BC pathogenesis promoting growth and survival of tumor cells in culture [5] and with the expression of its' signaling pathway molecules being also associated with worst prognosis in human BC samples [6]. Furthermore, peripheral blood mononuclear cells from BC cancer patients have lower expression of IL-7 receptor alpha chain (IL-7Rα; CD127) and show impaired IL-7 response and cytokine production when compared to those from healthy controls [7], suggesting that IL-7 defects is associated with BC or that cancer may modulate immune response through IL-7 pathway.

IL-7 is type 1 cytokine from hematopoietin family produced mainly by non-immune stromal cells which play important roles on immune system development and homeostasis by promoting lymphoid cell growth and survival. IL-7 functions are mediated through IL-7 receptor (IL-7R), which is composed by the common cytokine-receptor gamma-chain (γ_c; CD132) and the IL-7Rα, that leads to the activation of Janus Kinases (JAK) 1 and 3, promoting signal transducer and activator of transcription 5 (STAT5) function and modulation of gene expression, and also of phosphatidylinositol 3-Kinase (PI3K), activating anti-apoptotic and pro-survival signaling pathways [8].

IL-7Rα gene (*IL7RA*) is located on 5p13.2 chromosomic locus, and several single nucleotide polymorphisms (SNPs) have been described on this gene [9]. One of these SNPs (rs6897932, T244I) occurs at exon 6, changing a cytosine (C) to a thymine (T) in codon 244 (ACC > ATC) leading to a threonine to isoleucine change (Thr > Ile) on the border between extracellular and transmembrane regions.

T (Ile) allele for Thr244Ile polymorphism has been shown to a be a Tag allele for a protective haplotype in multiple sclerosis [10] and has been also associated with several other immune-related disorders [11]; mechanistically, this allele was shown to be associated with decreased expression of soluble IL-7Rα (sIL-7Rα) isoform, which lacks exon 6, in detriment of membrane-bound IL-7Rα [10,12], and sIL-7Rα was shown to increase IL-7 bioavailability and activity [13], probably enhancing immune function cronically during lifetime and promoting autoimmunity in susceptible individuals.

Despite evidences indicating the influence of IL-7 in several cancers, including BC [14], and the documented functional implications of *IL7RA* Thr244Ile polymorphism in immune function and autoimmune diseases [11], there is no study on the literature investigating this polymorphism on BC. Therefore, the aim of the present study was to investigate the possible association of this polymorphism with susceptibility and clinicopathological features in BC subgroups.

2. Material and methods

2.1. Sample characterization

All procedures in the present study were approved by Londrina State University Ethics Committee for Research Involving Human Subjects (CEP/UEL 189/2013 – CAAE 17123113400005231) and all individuals were informed about the research and freely signed a consent term prior to biological material collection.

Peripheral blood, surgically excised breast tissue or paraffin-embedded tissue was obtained from 338 women diagnosed for BC with clinicopathological data available from Londrina Cancer Hospital, Londrina, Parana, Brazil. For control group, peripheral blood was collected from 403 women from the same geographic region with no evidence of mammary tumors proved by recent physical and mammograph examination (within past two years from sample collection), no self-reported personal history of any malignancy nor family history for BC. Invitation, questionnaire and sample collection were carried out during routine medical examination at primary health care unities and at Londrina State University Clinical Hospital. Both BC and control groups were attended by the Brazilian public health system (SUS).

Due to high miscegenation rates observed in Brazilian populations, it is not reliable to classify individuals into ethnic groups by physical examination [15,16]. However, both BC and control individuals were from the geographical region of Londrina, located in southern Brazil, which displays high degree of European inheritance (Caucasoid ethnicity) [15,17,18].

For subgroup analyses, BC patients were grouped according to their immunohistochemistry (IHC) profile for estrogen and/or progesterone receptor (ER and PR, respectively) and human epidermal growth factor receptor 2 (HER2). IHC scores were assessed according to the American Society of Clinical Oncology (ASCO) recommendations. Samples with inconclusive HER2 staining (2, in a scale ranging from 0 to 3) were submitted to fluorescence *in situ* hybridization (FISH) analysis to check for *HER2* genetic amplification.

BC clinicopathological staging was determined following Union for International Cancer Control (UICC) criteria. Other clinicopathological features included age at diagnosis, tumor size on its' larger extension, histopathological grade, lymph node (LN) metastasis, cellular proliferation index (assessed through Ki67 IHC staining), p53 mutation (assessed through p53 IHC staining).

2.2. DNA extraction

For blood samples, DNA was obtained using Biopur Mini Spin kit (Biometrix Diagnostica®, Curitiba, PR, Brazil). Surgical excision tissues were macerated mechanically and DNA was extracted through a salting-out method using Proteinase-K. For paraffin embedded tissues, DNA was extracted using the protocol proposed by Isola et al. [19].

DNA samples were quantified using a NanoDrop2000c Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, EUA) at 260 nm. The 260/280 nm and 260/230 nm absorbance ratios were measured to assess protein and organic compound contamination, respectively.

2.3. IL7RA rs6897932 (Thr244Ile) genotyping

IL7RA rs6897932 polymorphism was genotyped through polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis, using primers described by Čierny et al. [20].

Briefly, approximately 3 ng/μL of DNA template was amplified through PCR for a 500 base-pair (bp) fragment sequence from *IL7RA* comprising the polymorphic *locus* performed on a final volume of 15 μL per reaction. All PCR reagents were purchased from Invitrogen™ (Carlsbad, CA, USA) and their concentration for each PCR reaction was as follows: 1× PCR Buffer (20 mM of Tris-HCl pH 8.4; 50 mM of KCl), 1.5 mM of $MgCl_2$, 0.1 mM of dNTP, 0.2 μM of each primer and 0.05 U/μL of Taq DNA polymerase diluted in ultra-pure water. PCR conditions were as follows: 5 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 55 °C and 45 s at 72 °C, and a final extension phase of 10 min at 72 °C.

PCR product (500 bp) was then subjected to enzymatic restriction with *Bcc*I enzyme (New England Biolabs®, Ipswich, MA, USA). Amplified fragment encompassed a nonpolymorphic cleavage site, serving as an internal cleavage control, and a cleavage site that is eliminated by the C to T transition, thus generating 314 bp and 186 bp fragment for T allele and 280 bp, 186 bp and 34 bp fragments for C allele, permitting the correct identification of individuals as prevalent homozygotes (CC), heterozygotes (CT) and variant homozygotes (TT).

Both PCR-amplified and enzyme cleaved fragments were visualized confirmed through electrophoresis on 10% polyacrylamide gel stained with silver nitrate ($AgNO_3$).

To validate specificity of primers and accuracy of the method, some