GLAUCO AKELINGHTON FREIRE VITIELLO

MARCADORES MOLECULARES RELACIONADOS AO SISTEMA IMUNOLÓGICO NO CÂNCER DE MAMA FEMININO:

PAPEL DE POLIMORFISMOS NOS GENES *TGFB1*, *TGFBR2*, *IL7RA* E *APOBEC3B* NA SUSCEPTIBILIDADE E APRESENTAÇÃO CLÍNICA DA DOENÇA

Londrina
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Tese apresentada ao Programa de Pós-graduação em Patologia Experimental da Universidade Estadual de Londrina - UEL, como requisito para a obtenção do título de Doutor.

Orientadora: Profa. Dra. Maria Angelica Ehara Watanabe
Co-orientadora: Profa. Dra. Roberta Losi Guembarovski

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VITIELLO, Glauco Akelinghton Freire. **Marcadores moleculares relacionados ao sistema imunológico no câncer de mama feminino**: papel de polimorfismos nos genes *TGFB1*, *TGFBR2*, *IL7RA* e *APOBEC3B* na susceptibilidade e apresentação clínica da doença. 2020. 140 f. Tese (Doutorado em Patologia Experimental) – Universidade Estadual de Londrina, Londrina, 2020.

RESUMO

O câncer de mama (CM) é a neoplasia mais frequente e maior causa de mortalidade por câncer em mulheres no mundo, e apresenta-se como uma doença complexa e heterogênea, composta por diversos subtipos moleculares de relevância clínica. Sua evolução depende de interações com o estroma, incluindo células imunológicas, e polimorfismos genéticos modulando o estroma do câncer de mama já foram associados à doença. O presente trabalho contou com um total de 388 pacientes com CM e 405 mulheres livres de neoplasia que foram avaliadas quanto a polimorfismos funcionais nos genes *IL7RA*, *TGFB1*, *TGFBR2* e *APOBEC3B*. Além disso, os níveis plasmáticos da citocina TGFβ1 foram quantificados em 113 pacientes com CM livres de tratamento, 44 pacientes com CM tratadas e em 184 pacientes livres de neoplasia e a expressão de TGFβ1, TGFβRII e pSMAD2/3 no tecido tumoral foi avaliada por imunohistoquímica em 34 pacientes, todas genotipadas para os polimorfismos nos genes *TGFB1* e *TGFBR2*. Os resultados demonstraram que o polimorfismo rs6897932 (Thr244Ile) de *IL7RA* foi associado à susceptibilidade a cânceres do subtipo triplo negativos, enquanto indicou menor estadiamento da doença nesse mesmo subgrupo; esse polimorfismo também foi associado a parâmetros de pior prognóstico em tumores $HER2^+$ (maior proliferação celular, grau histopatológico e metástase em linfonodos). Com relação ao *TGFB1*, quatro variantes funcionais afetando a produção da citocina TGFβ1 foram avaliadas (rs1800468, rs1800469, rs1800470 e rs1800471) e suas estruturas haplotípicas inferidas; haplótipos associados a maior produção de TGFβ1 foram associadas a maior susceptibilidade a tumores do subtipo $HER2^+$ e correlacionaram-se com parâmetros de pior prognóstico nesse mesmo subgrupo e em tumores triplo-negativos, mas indicaram menor proliferação celular em tumores luminal-A, enquanto variantes de baixa produção demonstraram o padrão oposto. Com relação ao polimorfismo rs3087465 (G-875A) do *TGFBR2* os resultados demonstraram uma forte associação protetora contra o CM, especialmente do subtipo luminal-A, e uma correlação positiva com o grau histopatológico. O haplótipo ACTG de *TGFB1* indicou menores níveis de *TGFB1* quando comparado ao haplótipo de referência (GCTG) em controles saudáveis, mas não em pacientes com CM. Já a expressão de componentes da via de sinalização de TGFβ no tecido tumoral não foi associada aos polimorfismos analisados, mas se associou a parâmetros de melhor prognóstico em tumores Luminal-B e sem mutação em p53. Tais resultados são consistentes com os efeitos paradoxais descritos para a sinalização pelo TGFβ em CM atuando como supressor tumoral em tumores iniciais e pouco agressivos (como os luminais), mas induzindo a progressão de tumores mais agressivos (como os $HER2^+$ e os triplo-negativos). Com relação à deleção de *APOBEC3B* não foi observada associação com a susceptibilidade a nenhum subtipo da doença, mas a deleção desse gene foi um fator protetor independente contra metástases em linfonodo no subtipo luminal-A. Assim, os resultados descritos indicam associações subtipo-específicas para diversos genes relacionados ao sistema imune com a susceptibilidade e apresentação clínica do CM, e revelam marcadores promissores nessa doença.

Palavras-chave: Neoplasia mamária. Prognóstico. Polimorfismos genéticos. Susceptibilidade. Citocinas.

VITIELLO, Glauco Akelinghton Freire. **Molecular markers related to the immune system in female breast cancer:** roles for genetic polymorphisms in *TGFB1*, *TGFBR2*, *IL7RA* and *APOBEC3B* genes in disease susceptibility and clinical presentation. 2020. 140 p. Thesis (Doctorate degree in Experimental Pathology) – Universidade Estadual de Londrina, Londrina, 2020.

ABSTRACT

Breast cancer (BC) is the most common cancer and the leading cause of cancer mortality in women worldwide, and shows up as a complex and heterogeneous disease, composed of several molecular subtypes of clinical relevance. Its evolution depends on interactions with tumor stroma, which includes immune cells, and genetic polymorphisms modulating breast cancer microenvironment were associated with this disease. The present thesis evaluated a total of 388 breast cancer patients and 405 cancer-free women for functional polymorphisms in the *IL7RA*, *TGFB1*, *TGFBR2* and *APOBEC3B* genes, selected based on their relevance to breast cancer pathogenesis and on their potential to act both on neoplastic cells and modulate local immune microenvironment. In addition, TGFβ1 plasma levels were quantified in 113 treatment-free breast cancer patients, 44 patients with treated breast cancer, and 184 cancer-free patients, and the protein expression of TGFβ1, TGFβRII and pSMAD2/3 in tumor tissue was evaluated by immunohistochemistry in 34 patients, all genotyped for *TGFB1* and *TGFBR2* polymorphisms. Results have shown that *IL7RA* rs6897932 (Thr244Ile) polymorphism was associated with susceptibility to triple-negative BCs, while indicating lower disease staging in this same subgroup; this polymorphism was also associated with worse prognosis parameters in $HER2^+$ tumors (higher cell proliferation, histopathological grade and presence of lymph node metastasis). Regarding *TGFB1*, four functional variants affecting TGFβ1 production were evaluated (rs1800468, rs1800469, rs1800470 and rs1800471) and their haplotypic structures were inferred; haplotypes associated with higher TGFβ1 production were associated with increased susceptibility to $HER2^+$ cancers and correlated with worse prognosis parameters in this same subgroup and in triple-negative tumors, but indicated lower cell proliferation in luminal-A tumors; otherwise, low-production variants showed the opposite pattern. Regarding the *TGFBR2* rs3087465 (G-875A) polymorphism, a strong protective association against breast cancer was found, especially for luminal-A subtype, and a positive correlation with histopathological grade was noted. The *TGFB1* ACTG haplotype was associated with lower TGFβ1 levels when compared to the reference haplotype (GCTG) in healthy individuals, but not in BC patients. The expression of TGFβ1-signaling components in BC tissue otherwise was not associated with any polymorphism analyzed, but was associated with better prognostic parameters in Luminal-B and tumors without p53 mutations. These results are largely consistent with the paradoxical effects of TGFβ signaling in breast cancer, acting as a tumor suppressor in initial and mildly aggressive tumors (such as luminal BCs), but inducing progression of more aggressive cancers (such as HER2 + and triple-negative). Regarding *APOBEC3B* deletion, it was not associated with susceptibility to any breast cancer subtype. However, it was an independent protective factor against lymph node metastases in luminal-A subtype. Therefore, the results shown here point to subtype-specific associations between polymorphisms in immune-related genes and BC susceptibility and clinical presentation, and reveal promising markers for this disease.

Key words: Breast neoplasm. Prognosis. Genetic polymorphisms. Susceptibility. Cytokines.

LISTA DE ABREVIATURAS E SIGLAS

%	Por cento
A	Adenina
AID	(Activation-induced deaminase): Deaminase induzida por ativação
AJCC	(American Joint Committee on Cancer): Comitê Conjunto Americano para o Câncer
ASCO	(American Society of Clinical Oncology): Sociedade Americana de Oncologia Clínica
APOBEC	(apolipoprotein B mRNA-editing enzyme catalytic polypeptide like): enzima de edição do mRNA da apolipoproteína B
C	Citosina
CDI	Carcinoma ductal invasivo
CDIS	Carcinoma ductal in situ
CLI	Carcinoma lobular invasivo
CK	(cytokeratin): citoqueratina
DNA	Ácido desoxirribonucleico
EGFR	(epidermal growth factor receptor): receptor do fator de crescimento epidermal
G	Guanina
GH	Grau histopatológico
HER2	(Human Epidermal Growth Factor Receptor 2): Receptor do fator de crescimento epidermal humano
IFN	intérferon
IHQ	Imunohistoquímica
IL	Interleucina
IL-7Rα	Receptor alfa de interleucina 7
INCA	Instituto Nacional do Câncer
JAK	(Janus kinase): Janus quinase
MAPK	(Mitogen activated protein kinase): proteína quinase ativada por mitógeno
OMS	Organização Mundial da Saúde
PI3K	(phosphatidylinositol 3-kinase): fosfatidil-inositol 3-quinase
RE	Receptor de estrógeno
RNA	Ácido ribonucleico

RP	Receptor de progesterona
SNP	(Single nucleotide polymorphism): Polimorfismo de nucleotídeo único
STAT	(Signal transducer and activator of transcription): transdutor de sinal e ativador da transcrição
T	Timina
TCGA	(The Cancer Genome Atlas): O Atlas do Genoma do Câncer
TGFβ	(Transforming growth factor beta): Fator de crescimento e transformação beta
TGFβRII	(Transforming growth factor beta receptor 2): receptor 2 do fator de crescimento e transfoamação beta
UICC	(Union for International Cancer Control): União para o controle internacional do câncer

SUMÁRIO

1 INTRODUÇÃO

O câncer de mama, neoplasia mais frequente entre as mulheres no Brasil e no mundo e maior causa de morte por câncer entre as mulheres (BRAY et al., 2018; INCA, 2019), apresenta-se como uma doença extremamente complexa e heterogênea, apresentando múltiplos subtipos que variam na etiologia, apresentação clínica e prognóstico (EROLES et al., 2012).

Diversos fatores, tanto genéticos quanto ambientais, contribuem para a susceptibilidade ao câncer de mama e podem influenciar a progressão da doença: a arquitetura genética da susceptibilidade ao câncer é amplamente dependente de múltiplas variantes de baixa penetrância (FLETCHER; HOULSTON, 2010), e evidências demonstram que no câncer de mama essas variantes têm efeitos subtipo-específicos (BROEKS et al., 2011) e que não são restritos a célula tumoral diretamente, mas podem atuar na modulação do estroma tumoral (FLISTER; BERGOM, 2018).

De fato, a evolução do câncer de mama é amplamente dependente de interações do tumor com o estroma circundante (TATA; AL-ZUBEIDY; KULKARNI, 2019). Nesse contexto, o sistema imunológico desecandeia funções essenciais controlando o crescimento e a progressão tumoral por meio do reconhecimento e da eliminação de células neoplásicas, ou promovendo a evolução tumoral, por meio da secreção de fatores de crescimento que estimulam a metastização desses tumores (YANG; PANG; MOSES, 2010; CHEN; MELLMAN, 2013; BINNEWIES et al., 2018). Além disso, a composição do microambiente tumoral imunológico parece variar e ter relevância prognóstica diferenciada entre os subtipos de câncer de mama. Ainda, diversos fatores parecem modular o microambiente imunológico desses tumores, e o reconhecimento desses fatores pode viabilizar o emprego de imunoterapias promissoras, já em uso em outros cânceres (HAMMERL et al., 2018).

Assim, tendo em vista a importância do sistema imunológico na patogênese do câncer de mama e o papel de variantes genéticas na susceptibilidade à doença, o presente estudo objetivou avaliar a influência de variantes genéticas em moléculas que atuam tanto em células neoplásicas quanto na modulação do microambiente imunológico na susceptibilidade e apresentação clínica dos diferentes subtipos moleculares de câncer de mama. Foram selecionados os genes *IL7RA, TGFB1, TGFBR2* e *APOBEC3B* com base na relevância das moléculas codificadas por eles na patogênese do câncer de mama e no ineditismo de estudos de suas variantes genéticas em subtipos de câncer de mama ou na população brasileira.

2 REVISÃO DE LITERATURA

2.1 CÂNCER: EPIDEMIOLOGIA E ETIOLOGIA GERAL

O câncer tem se consolidado cada vez mais como um grave problema de saúde pública mundial: apresentando rápido crescimento na incidência e mortalidade no mundo todo – devido, principalmente ao aumento da população mundial e da expectativa de vida populacional - e representando a principal ou segunda principal causa de morte antes dos 70 anos na maioria dos países, o câncer representa a maior barreira para o aumento na expectativa de vidal global atualmente (BRAY et al., 2018).

No mundo todo, excluindo-se os casos de câncer de pele não melanoma, foram estimados 17 milhões de novos casos de câncer e 9,5 milhões de mortes por câncer para o ano de 2018 segundo os dados da Organização Mundial da Saúde (OMS) (BRAY et al., 2018). Para efeito de comparação, a estimativa anterior da OMS previa 14,1 milhões de novos casos e 8,2 milhões de mortes relacionadas ao câncer para o ano de 2012. No Brasil, dados do Instituto Nacional do Câncer José Alencar Gomes da Silva (INCA) estimam cerca de 450 mil novos casos de câncer, excetuando-se câncer de pele não melanoma, para cada ano do triênio 2020-2022 (INCA, 2019).

O câncer pode ser caracterizado como um conjunto de doenças heterogêneas e complexas de etiologia genética, mas não necessariamente hereditárias, capazes de acometer praticamente todos os tecidos do organismo. O desenvolvimento de um câncer passa, necessariamente, pela transformação genética de uma célula, que então prolifera-se indefinidamente (VOGELSTEIN; KINZLER, 1993). Durante esses ciclos proliferativos, as células geradas acumulam novas mutações, originando tumores com milhões de células, que, embora derivadas de uma célula comum, têm sua própria gama de mutações. Isso permite que subpopulações celulares sejam selecionadas com base em suas alterações e evoluam, eventualmente ganhando a capacidade de migrar e invadir tecidos adjacentes (MCGRANAHAN; SWANTON, 2017).

Assim, o desenvolvimento de um câncer depende que células adquiram, por meio de mutações, características que permitam sua evolução. Essas características são conhecidas como as marcas distintivas do câncer, e incluem: instabilidade genômica, suficiência em relação a fatores de crescimento, capacidade replicativa ilimitada, insensibilidade a fatores de inibição de crescimento, evasão à morte celular programada,

alteração do metabolismo energético, angiogênese aumentada, indução de processos inflamatórios, evasão a resposta imunológica, invasão tecidual e disseminação à distância (HANAHAN; WEINBERG, 2011).

Diversas dessas características dependem da interação de células neoplásicas com o estroma circundante. Assim, embora células transformadas constituam a principal população celular em um câncer, é preciso pensar o câncer como um tecido vivo, no qual células neoplásicas e estromais colaboram para a progressão da doença. Dentre as pricipais células do estroma tumoral podem-se citar: fibroblastos, células endoteliais e células do sistema imunonológico, que variam em proporção e função em diferentes cânceres (BALKWILL; CAPASSO; HAGEMANN, 2012).

Virtualmente todos os tecidos do organismo podem desenvolver câncer; entretanto, observa-se uma grande variação na incidência dessa doença entre diferentes órgãos, que pode ser explicada pela exposição desses a fatores de risco conhecidos (carcinógenos), como fuligem, tabaco, álcool, vírus, radiação ultravioleta, radiação ionizante e contaminantes alimentares (DANAEI et al., 2005), e pela taxa de replicação celular nesses tecidos, que favorece o aparecimento de mutações aleatórias que podem ocorrer naturalmente a cada ciclo de divisão celular (TOMASETTI; VOGELSTEIN, 2015).

2.2 CÂNCER DE MAMA FEMININO:EPIDEMIOLOGIA

Dentre todas as neoplasias, exceto tumores de pele não melanoma, o câncer de mama feminino é a neoplasia mais frequente em mulheres no mundo todo: dados da OMS estimaram 2,1 milhões de novos casos desse câncer no mundo para o ano de 2018 com uma incidência de 46,3 novos casos a cada cem mil mulheres. Dessa forma, o câncer de mama é responsável por um a cada quatro casos de câncer em mulheres e é a neoplasia mais frequentemente diagnosticada em 154 dos 185 países analisados (BRAY et al., 2018), incluindo o Brasil, onde a estimativa é de aproximadamente 66 mil novos casos para cada ano do triênio 2020-2022 (INCA, 2019).

Em relação à mortalidade, essa também é a neoplasia que mais mata mulheres no mundo, respondendo por cerca de 627 mil mortes para o ano de 2018 e apresentando uma mortalidade de 13 a cada 100 mil mulheres. Embora a incidência de câncer de mama seja maior em países desenvolvidos (54,4/100.000 mulheres, em média) que em países subdesenvolvidos ou em desenvolvimento (31,3/100.000 mulheres), a taxa de mortalidade demonstra um padrão oposto sendo de 14,9/100.000 em países subdesenvolvidos e em desenvolvimento e de

11,6/100.000 em países desenvolvidos (BRAY et al., 2018).

Assim como outros cânceres, o câncer de mama é uma neoplasia de etiologia complexa e diversos fatores de risco, tanto genéticos quanto ambientais, contribuem para o desenvolvimento da doença. A participação de fatores de risco genéticos é evidenciada pela maior incidência da doença em indivíduos com histórico familiar de câncer de mama ou ovário; entretanto, apenas de 5% a 10% dos casos de câncer de mama podem ser explicados pela presença de mutações em genes fortemente associados ao câncer, como *BRCA1*, *BRCA2*, *CHEK2* e outros (ELLISEN; HABER, 1998).

Estudos demonstram que em imigrantes vindos de países de baixa incidência para países de alta incidência de câncer de mama, a incidência da doença cresce a cada geração, evidenciando o maior peso de fatores ambientais na etiologia da doença (ZIEGLER et al., 1993). Dentre esses fatores destacam-se aqueles associados à sinalização hormonal reprodutiva feminina, que influenciam diretamente na fisiologia mamária, como: uso de terapia de reposição hormonal (NELSON et al., 2002), menarca precoce, menopausa tardia (BRINTON et al., 1988; MSOLLY; GHARBI; BEN AHMED, 2013), idade avançada na primeira gravidez ou nuliparidade (WHITE, 1987) e maior densidade (BOYD et al., 2011) e anormalidades histológicas do tecido mamário (DUPONT et al., 1993). Outros fatores de risco associados fortemente ao câncer de mama incluem exposição a radiações (BOICE et al., 1991), idade avançada (MCPHERSON; STEEL; DIXON, 2000), dieta rica em gordura, sobrepeso e obesidade (TRETLI, 1989). Ainda, alguns estudos sugerem que o consumo de álcool (ELLISON et al., 2001) e cigarros (CATSBURG; MILLER; ROHAN, 2015) e infecções virais (LAWSON; SALMONS; GLENN, 2018; AMARANTE et al., 2019) também podem estar associadas ao desenvolvimento da doença.

A maior incidência de câncer de mama em países desenvolvidos parece estar associada primariamente ao contato com fatores de risco associados ao estilo de vida, como dieta, sobrepeso, uso exógeno de hormônios reprodutivos, e nuliparidade ou retardamento da gravidez (ZIEGLER et al., 1993). Enquanto a menor mortalidade ocasionada por esse câncer nesses mesmos países parece decorrer da maior conscientização, rastreamento populacional mais eficiente empregando técnicas como mamografia e ultrasonografia, que auxiliam o diagnóstico precoce, e uso de terapias mais efetivas contra a doença (SHARMA, 2019).

2.3 HISTOPATOLOGIA E PARÂMETROS CLINICOPATOLÓGICOS DE CARCINOMAS MAMÁRIOS

Os tumores de mama acometem a unidade terminal ductolobular da mama feminina, que constitui a porção secretora da glândula (WELLINGS, 1980). O desenvolvimento da doença segue estágios bem definidos, iniciando-se pela hiperplasia de células mamárias, dando origem a atipias celulares que eventualmente originarão carcinomas *in situ*, quando células atípicas, proliferativas e potencialmente invasivas estão confinadas ao lúmem da glândula, sem romper a membrana basal. Essas lesões pré-malignas podem então evoluir a carcinomas invasivos que irrompem o estroma glandular e, finalmente, a carcinomas metastáticos, caracterizados pela migração, invasão e colonização de tecidos distantes (POLYAK, 2007).

A apresentação do câncer de mama é bastante heterogênea, variando entre subtipos histológicos, moleculares, estágios clínicos e de paciente para paciente, já que cada tumor possui um conjunto de mutações específicas e que fatores genéticos e ambientais inerentes ao portador da doença podem influenciar o comportamento tumoral. Assim, é necessária a classificação desses tumores de modo a auxiliar a compreensão da doença e o delineamento de estratégias terapêuticas adequadas a cada paciente (GRUVER; PORTIER; TUBBS, 2011; MALHOTRA et al., 2014).

Histologicamente, os tumores de mama podem ser categorizados em diferentes tipos, dentre os quais os carcinomas ductais e os carcinomas lobulares constituem os principais, e outros tipos (como carcinomas tubulares, medulares, mucinosos, etc) são menos frequentes. É importante lembrar que tanto os tumores chamados de ductais quanto aqueles chamados de lobulares ocorrem na unidade terminal ductolobular mamária, constituindo subtipos diferenciados pela morfologia ao microscópio (histologicamente), mas não ao exame macroscópico e anatômico, como o nome poderia sugerir (WELLINGS, 1980).

Embora as etapas de progressão do câncer de mama acima citadas sejam bem estabelecidas (POLYAK, 2007), a ausência de marcadores que diferenciem hiperplasia (típica e atípica), carcinomas *in situ* e carcinomas invasivos, dificulta a caracterização clínica dessas etapas. Amplamente, tumores podem ser divididos entre carcinomas *in situ* (restritos a membrana basal) e invasivos, sendo esses classificados, principalmente, entre lobulares e ductais (MALHOTRA et al., 2014).

Carcinomas lobulares *in situ* são, quase sempre, achados acidentais, já que não provocam alterações teciduais palpáveis ou detectáveis em exames de imagem, e apresentam baixa variação histológica (MALHOTRA et al., 2014). Já os carcinomas lobulares

invasivos (CLI), apresentam-se como massas palpáveis ou regiões densas de formato irregular à mamografia e caracterizam-se por crescimento não coesivo, em fileiras, apresentando núcleos arredondados pela deficiência da molécula de adesão intercelular E-caderina. Esses, correspondem a cerca de 10% dos casos de carcinomas mamários invasivos (SCHIMITT; GOBBI, 2011).

Já os carcinomas ductais *in situ* (CDIS) apresentam grande variabilidade histológica, podendo ser classificados de acordo com a arquitetura tecidual em: comedocarcinoma; carcinoma cribiforme; micropapilar; papilar e sólido (MALHOTRA et al., 2014). Carcinomas ductais invasivos sem outra especificação (CDI) são os carcinomas mamários mais frequentes, correspondendo a até 80% de todos os tumores mamários invasivos (LI; URIBE; DALING, 2005). Tais tumores constituem um grupo heterogêneo a nível molecular e não apresentam achados morfológicos que permitam sua classificação em subtipos histológicos especiais. São detectados como massas palpáveis e densas em exames de imagens, frequentemente apresentando microcalcificações (SCHIMITT; GOBBI, 2011). Outros subtipos histológicos raros de carcinomas invasivos incluem carcinomas medulares, mucinosos e tubulares (MALHOTRA et al., 2014).

Embora a classificação histológica dos tumores seja de grande valia na compreensão da biologia tumoral mamária, ela possui pouco impacto no manejo clínico da doença. Assim, outras formas de classificar os tumores de mama com base em seu comportamento e apresentação clínica são necessárias e rotineiramente utilizadas juntamente com sua classificação histológica e molecular, que será discutida posteriormente.

A classificação histopatológica indica o quão diferente do tecido original (não neoplásico), um tumor é. Essa classificação leva em conta o índice de formação de túbulos e glândulas, o grau de pleomorfismo nuclear e o número de células em mitose por campo analisado para categorizar tumores em três graus: grau I, apresentando tumores mais semelhantes a glândula mamária; grau II, com um fenótipo intermediário; e grau III, no qual as células tumorais sofreram anaplasia, perdendo suas as características epiteliais e adquirindo aspectos mesenquimatosos. Esse processo está intimamente ligado a transição-epitélio-mesenquimal, que também ocorre em outros processos patológicos, como em cicatrizações, e permite que células epiteliais adquiram capacidade migratória, favorecendo o desenvolvimento de metástases (RAKHA et al., 2010).

Já o estadiamento tumoral estabelece graus que indicam o quão avançada a doença se encontra. O sistema preconizado pela União Internacional de Controle do Câncer (UICC) e adotado globalmente para o estadiamento de tumores malignos é o sistema Tumor-

Nódulo-Metástase (TNM), que se baseia em três parâmetros que afetam o prognóstico e sobrevida dos portadores desses tumores: o tamanho tumoral (T); a presença de metástases em linfonodos (N); e a presença de metástases à distância (M) para classificar a doença em cinco estágios que vão de 0, para carcinomas *in situ*, a IV para carcinomas metastáticos (AJCC, 2010; GANNON; COTTER; QUINN, 2014).

Assim, por meio da avaliação desses parâmetros clinicopatológicos (grau histopatológico, tamanho tumoral, presença de metástases em linfonodo e em órgãos distantes e estadiamento tumoral), é possível inferir a severidade da doença de cada paciente e delinear uma conduta terapêutica mais adequada a cada caso.

2.4 Classificação Molecular de Carcinomas Mamários

Diferentemente da classificação histológica, histopatológica e clínica dos tumores de mama apresentada na seção anterior, a classificação molecular do câncer de mama, como o nome sugere, baseia-se em móleculas presentes no tecido tumoral para classificar essas neoplasias. Dentre as grandes vantagens dessa classificação pode-se citar que a identificação de moléculas associadas a tumores específicos auxilia na compreensão da biologia tumoral identificando os fatores mais profundos que governam sua patogênese, e permite o desenvolvimento de terapias direcionadas contra tais fatores, ou moléculas, chamadas de terapias alvo-direcionadas.

A primeira molécula identificada como chave no desenvolvimento de alguns tumores de mama foi o receptor de estrógeno (RE), na década de 70 (LEMON, 1970). Esse é um receptor envolvido na sinalização hormonal reprodutiva feminina, e sua presença no tecido tumoral mamário foi associada a menor proliferação celular (MEYER et al., 1977) e melhor sobrevida livre de recidiva (KNIGHT et al., 1977), além de conferir sensibilidade à terapia com tamoxifeno (KIANG et al., 1978; BYAR; SEARS; MCGUIRE, 1979).

A seguir relatou-se a presença de receptor de progesterona (RP) no tecido tumoral (HORWITZ; MCGUIRE, 1975) e identificou-se que a expressão dessa proteína também seria um importante preditor na resposta à terapia hormonal no câncer de mama (OSBORNE et al., 1980). A expressão de RP é regulada por RE, e, portanto, a expressão do primeiro raramente ocorre sem a expressão do segundo (HORWITZ; KOSEKI; MCGUIRE, 1978). Aproximademente 80% dos cânceres de mama são positivos para RE, e cerca de 70% dos tumores mamários são positivos para RE e RP simultaneamente. A presença de receptores hormonais está associada à expressão de genes característicos de células do lúmem mamário e

os tumores que expressam ao menos um desses receptores são, assim, chamados de luminais (GANNON; COTTER; QUINN, 2014).

Já na década de 1980, foi demonstrado que a amplificação do gene do receptor de fator de crescimento epidermal humano 2 (HER2/ERBB2) em cânceres de mama, estaria associada a pior sobrevida global e livre de doença e apresentava relevância prognóstica mais forte que os outros parâmetros clinicopatológicos ou a expressão de RE ou RP (SLAMON et al., 1987). Esses achados abriram caminho para o desenvolvimento, já na década de 1990, de um anticorpo monoclonal que tem como alvo essa proteína, o que aumentou consideravelmente a sobrevida de mulheres portadoras de câncer de mama com essa alteração (SLAMON et al., 2001). A amplificação desse oncogene é observada em aproximadamente 15% dos carcinomas mamários (GANNON; COTTER; QUINN, 2014).

Também na década de 80, a proteína Ki67 foi descrita como um indicador da atividade replicativa celular, sendo expressa durante todas as fases da mitose, mas não na intérfase, e seu uso foi sugerido como um indicador da taxa proliferativa de diversos tumores, incluindo tumores de mama, com potencial prognóstico (BARNARD et al., 1987; BOUZUBAR et al., 1989).

Na mesma época, notou-se que o acúmulo de p53 em células tumorais estava correlacionado a negatividade para receptores hormonais e maior grau histopatológico (CATTORETTI et al., 1988). A p53 está envolvida na promoção de morte celular induzida por danos no DNA atuando como um supressor tumoral, e mutações nessa proteína promovem sua ligação a chaperonas e aumentam sua meia vida, fazendo que a proteína seja detectada pelos métodos de imunohistoquímica; assim a detecção por imunohistoquímica é uma medida indireta de mutações em p53 e indicam maior agressividade tumoral (ELLEDGE et al., 1994).

A partir do desenvolvimento de técnicas de alto rendimento para a análise da expressão gênica global no fim da década de 1990, como os microarranjos de cDNA, foi possível caracterizar os tumores mamários a partir da expressão de todos os seus genes e identificar assinaturas de expressão gênica nesses cânceres, gerando um retrato mais fiel da biologia desses tumores. Em um importante trabalho, PEROU et al. (2000) utilizaram essa técnica para caracterizar 38 amostras de câncer de mama. A partir desses dados, foi possível realizar agrupamento hierárquico das amostras, uma técnica na qual as amostras são agrupadas com base em seu perfil global de expressão gênica, que pode ser considerado o perfil molecular desses tumores e é intimamente relacionado a célula de origem tumoral.

Assim, esses autores identificaram quatro subgrupos de câncer de mama principais que diferiam significativamente em seu comportamento clínico e prognóstico: grupo

luminal (expressando genes característicos de células luminais, governados por RE), grupo HER2-superexpresso (expressando genes relacionados a ativação da via das MAPKs), grupo basaloide (expressando genes característicos de células basais da glândula mamária) e grupo normal (com perfil semelhante ao da mama normal) (PEROU et al., 2000).

Um trabalho subsequente pelo mesmo grupo utilizando a mesma abordagem mas incluindo um número maior de amostras identificou que os tumores luminais poderiam ser divididos em dois grupos: luminal A e luminal B, sendo que tumores do grupo luminal A seriam aqueles de melhor prognóstico dentre todos os subtipos, enquanto tumores luminais B, apesar de conservarem as características luminais, apresentavam alta expressão de genes relacionados à proliferação celular, predizendo pior prognóstico em comparação aos tumores luminais A (SORLIE et al., 2001).

Tais subgrupos apresentam boa correlação com os marcadores acima citados: a presença de receptores hormonais caracteriza subtipos luminais, sendo que tumores do subtipo luminal B apresentam alto índice de proliferação celular (Ki67) e podem ou não apresentar amplificação de HER2; subtipos HER2-superexpresso apresentam amplificação de HER2 na ausência de receptores hormonais; e o subtipo basaloide também é conhecido como triplo negativo, por não apresentar expressão de receptores hormonais ou amplificação de HER2 (GANNON; COTTER; QUINN, 2014). Juntos, esses quatro marcadores constituem o painel IHC4, que tem validade semelhante a uma assinatura de expressão de 21 genes para a estimativa de risco de recorrência da doença (CUZICK et al., 2011). O consenso de St. Gallen de 2011 validou o uso desses marcadores por imunohistoquímica para a definição de subgrupos moleculares, já que a análise de expressão gênica raramente é possível na rotina clínica e patológica (GOLDHIRSCH et al., 2011).

Tumores triplo-negativos constituem um grupo heterogêneo: O subtipo basaloide, inicialmente descrito por PEROU et al. (2000), além de não apresentar expressão de receptores hormonais e amplificação de HER2, apresenta expressão de marcadores de células basais da mama como citoqueratinas (CKs) 5, 6, 14 e 16 e receptor do fator de crescimento epidermal (EGFR); no entanto, esses marcadores podem estar ausentes em tumores classificados como triplo negativos. Um subgrupo desses tumores foi caracterizado pela baixa expressão de proteínas de junção celular, como claudinas, ocludinas e E-caderinas, constituindo um novo subtipo denominado claudina baixa (HERSCHKOWITZ et al., 2007).

O quadro 1 resume as características principais de cada um desses subtipos moleculares de câncer de mama.

Quadro 1. Classificação molecular dos carcinomas mamários.

Subtipo	Freq. (%)	Perfil IHQ	Ki67	Mutações em p53	GH	Prognóstico
Luminal A	50-60	RE/RP^{+} $HER2^{-}$	Baixo	Baixo	Baixo	Bom
Luminal B	10-20	$RE/RP^{+}HER2^{+/-}$	Alto	Mod.	Mod.	Intermediário
HER2	10-15	$RE^{-}RP^{-}HER2^{+}$	Alto	Alto	Alto	Ruim
Basaloide	10-20	$RE^{-}RP^{-}HER2^{-}$ $CK5/6^{+}$	Alto	Alto	Alto	Ruim
Claudina baixa	12-14	$RE^{-}RP^{-}HER2^{-}$ $Claudina^{-}$	Alto	Alto	Alto	Ruim

Freq: Frequência; **IHQ:** Imunohistoquímico; **RE:** Receptor de estrógeno; **RP:** Receptor de progesterona; **HER2:** Receptor 2 do fator de crescimento epidermal humano; **CK:** citoqueratina; **Mod.:** Moderado; **GH:** Grau histopatológico.
Fonte: Adaptado de EROLES et al. (2012).

A classificação em subtipos moleculares permite prever a evolução clínica da doença e delinear o tratamento mais adequado para cada subtipo: tumores que expressam receptores hormonais são sensíveis à terapia hormonal com antiestrógenos como o tamoxifeno (KIANG et al., 1978), inibidores de aromatase (FABIAN, 2007) e fulvestranto (WAKELING, 2000), enquanto tumores que superexpressam HER2 são sensíveis à terapia com o anticorpo monoclonal trastuzumab (SLAMON et al., 2001; DAHABREH et al., 2008). Já para os tumores triplo-negativos não há terapias alvo-direcionadas disponíveis; entretanto, esses tumores frequentemente apresentam mutações em *BRCA1*, o que os torna sensíveis a terapias com inibidores de poli-ADP ribose polimerase (PARP) (PEROU, 2010).

Além da classificação molecular, diversos testes comerciais baseados na expressão de múltiplos genes foram desenvolvidos e validados para auxiliar na conduta terapêutica de tumores, auxiliando em decisões clínicas sobre a necessidade e extensão de quimioterapia adjuvante a ser aplicada em tumores iniciais (REIS-FILHO; PUSZTAI, 2011). Alguns desses testes já são validados em um amplo número de pacientes e receberam recomendação de uso da ASCO, AJCC e aprovação do FDA (VIEIRA; SCHMITT, 2018).

Recentemente, o projeto integrativo global "*The Cancer Genome Atlas*" (TCGA) traçou o perfil molecular de diversos cânceres, incluindo os de mama, através de múltiplas tecnologias de alto rendimento para avaliar mutações somáticas, expressão de mRNA, expressão de micro-RNAs, análise de metilação de DNA, alteração de número de cópias, e expressão proteica de milhares de pacientes com informações clínicopatológicas e de sobrevida disponíveis (CHIN; ANDERSEN; FUTREAL, 2011; TOMCZAK; CZERWINSKA; WIZNEROWICZ, 2015).

No câncer de mama os resultados desse projeto demonstraram uma boa correlação entre os subtipos baseados no perfil de expressão gênica e aqueles baseados nas

múltiplas tecnologias simultaneamente. Além disso foram identificadas que algumas mutações acontecem de maneira subtipo específica, como observado pela alta prevalência de mutações em *PIK3CA* e no fator de transcrição *GATA3* em tumores luminais A e de p53 em tumores triplo-negativos (CANCER GENOME ATLAS, 2012).

Os dados do TCGA demonstraram também que embora carcinomas lobulares tenham perfil de expressão gênica semelhante a tumores ductais luminal-A apresentando expressão de receptor de estrógeno, esses possuem mutações, específicas desse subtipo histológico além daquelas em E-caderina (*CDH1*), principalmente no fator de transcrição FOXA1 e no supressor tumoral PTEN, levando a hiperativação da via pró-sobrevivência AKT. Três subtipos de relevância prognóstica puderam ser definidos dentro dos CLIs com base em seu perfil de expressão gênica (CIRIELLO et al., 2015). No entanto, tais achados ainda não foram incorporados à rotina clínica.

Além das assinaturas de expressão gênica, a análise de assinaturas mutacionais constitui outra informação de grande valia na compreensão da etiologia de tumores diversos: cada processo mutacional envolvido na carcinogênese, gera uma "assinatura mutacional" diferente, caracterizada por trocas de nucleotídeos específicas, às vezes em sequências de DNA específicas. Assim, ao identificar essas assinaturas, é possível inferir o mecanismo carcinogênico envolvido em um dado tumor (como tabaco, radiação UV, falha em mecanismos de reparo, etc) (HOLLSTEIN et al., 2017).

Investigando o genoma de um grupo de 21 cânceres de mama, uma nova assinatura mutacional foi descoberta: caracterizada pela troca de citosinas (C) por timinas (T) em nucleotídeos antecedidos por T, e ocorrendo em muitos nucleotídeos próximos em regiões do genoma, essa assinatura mutacional foi atribuída à atividade de citidina deaminases da família das AID/APOBECs, enzimas envolvidas principalmente na imunidade inata antiviral (NIK-ZAINAL et al., 2012). A alta prevalência dessa assinatura em cânceres de mama e outros cânceres, e a participação das enzimas APOBEC3A e APOBEC3B nesse processo foi confirmada utilizando dados do TCGA (BURNS et al., 2013; BURNS; TEMIZ; HARRIS, 2013; ROBERTS et al., 2013; CHAN et al., 2015) .

Outras assinaturas mutacionais encontradas frequentemente em cânceres de mama incluem, principalmente, aquelas atribuídas à deficiência nos mecanismos de reparo por recombinação homóloga, ocasionada por mutações em *BRCA1* e *BRCA2*, à idade e à deaminação de metil-citosinas em ilhas CpG (NIK-ZAINAL; MORGANELLA, 2017).

2.5 Resposta Imunológica no Câncer de Mama

A progressão do câncer depende da interação das células neoplásicas com o estroma tumoral, incluindo fibroblastos, células endoteliais e células do sistema imunológico (HANAHAN; WEINBERG, 2011). O microambiente tumoral imunológico apresenta componentes da imunidade adaptativa (linfócitos T e B) e inata (macrófagos, células dendríticas, células *natural killer* e neutrófilos, principalmente) (BINNEWIES et al., 2018).

Respostas imunes adaptativas são desencadeadas a partir do reconhecimento de antígenos associados a tumores (aqueles que são altamente expressos em tumores mas não em outros tecidos) e antígenos específicos de tumores (aqueles com expressão específica em células tumorais, como proteínas mutadas e proteínas de vírus oncogênicos ou retrovírus endógenos reativados especificamente em tumores)(GILBOA, 1999; SMITH et al., 2019).

A resposta imunológica efetora antitumoral se dá principalmente por células T citotóxicas ($CD8^+$), que reconhecem antígenos via MHC de classe I diretamente em células tumorais, promovendo sua morte, e por células T auxiliares ($CD4^+$) do perfil Th1, que reconhecem antígenos via MHC de classe II em células apresentadoras de antígenos, como macrófagos e células dendríticas, nos linfonodos e secretam intérferon gama (IFNγ), ativando essas mesmas células apresentadoras de antígenos, células *natural killer* e as células citotóxicas, que migram para o tumor como células efetoras para desempenhar sua função e eliminar células tumorais carregando os antígenos por elas reconhecidos (CHEN; MELLMAN, 2013).

Por outro lado, como uma doença crônica, mecanismos de tolerância e exaustão imunológica são induzidos. Esses processos são mediados por citocinas secretadas pelo próprio tumor e células do estroma, como o fator de crescimento e transformação beta 1 (TGFβ1), que induzem diferenciação de macrófagos no subtipo M2, inibem a maturação de células dendríticas, induzem expressão de moléculas de exaustão celular em células T (como CTLA4 e PD1) e ativam, em conjunto com os antígenos próprios (não mutados), células T regulatórias (Tregs), que são células chave na manutenção da tolerância imunológica (YANG; PANG; MOSES, 2010; CHEN; MELLMAN, 2013; LIU; WORKMAN; VIGNALI, 2016)

A compreensão do microambiente imunológico tumoral torna-se especialmente relevante em face da recente implementação das imunoterapias na rotina clínica, alternativas terapêuticas promissoras que se baseiam na ativação de respostas imunológicas anti-tumorais, principalmente através da inibição de moléculas envolvidas na tolerância e exaustão imunológica por anticorpos monoclonais para promover a eliminação de tumores (BINNEWIES et al., 2018). Tais terapias hoje são aplicáveis a tumores ditos imunogênicos,

que possuem alta carga mutacional e apresentam alta densidade de neoepítopos, sendo associados a maior ativação imunológica (KHALIL et al., 2016).

O câncer de mama não é considerado um tumor imunogênico em geral. Assim, a taxa de respostas a imunoterapia nesses tumores não é tão alta quanto a observada em outros tumores. Entretanto, o sistema imunológico participa ativamente na patogênese de cânceres de mama, estando presente, inclusive, na glândula mamária normal, onde desempenha a função de imunovigilância contra agentes infecciosos (DEGNIM et al., 2014) e participa da organogênese normal da mama (ATABAI; SHEPPARD; WERB, 2007) sofrendo alterações com o avanço da doença (HUSSEIN, 2006).

Estabelecer a relação entre o sistema imunológico e o câncer de mama é um problema complexo, já que, tanto a doença quanto a composição do infiltrado, são extremamente heterogêneas e a composição desse infiltrado varia entre os subtipos moleculares de câncer de mama, de acordo com o estágio da doença, podendo ter relevância prognóstica diferenciada em cada uma dessas situações.

Em uma recente revisão de literatura sobre o tema, HAMMERL et al. (2018) observaram que, em geral, tumores RE^- apresentam maior infiltração por células T que tumores RE^+, e que células T citotóxicas, células dendríticas e macrófagos inflamatórios (M1) predizem bom prognóstico tanto em tumores ER^+ quanto em ER^-; já macrófagos M2, neutrófilos e células supressoras derivadas da linhagem mieloide (MDSC, do inglês *myeloid-derived suppressor cells*) estão associadas a pior prognóstico no câncer de mama, enquanto células T regultórias parecem ter um efeito ambíguo, sendo associadas a pior prognóstico em tumores RE^+ mas a bom prognóstico em tumores RE^-. Esse efeito ambíguo foi discutido com base na presença de células T indicando um mecanismo compensatório pós-ativação de respostas efetoras em tumores ER^-, que são mais imunogênicos.

Os autores observaram ainda que a presença de neoantígenos e antígenos associados a tumores entre os subtipos tumorais cresce na seguinte ordem: Luminal-A, Luminal-B, HER2-superexpresso e tumores basaloides; e discutem que quimioterápicos convencionais podem agir como sensibilizadores desses tumores à imunoterapia, já que terapias citotóxicas têm o potencial de expor antígenos tumorais aumentando a imunogenicidade desses tumores (HAMMERL et al., 2018). Assim, as evidências recentemente revistas destacam o papel do sistema imunológico no câncer de mama e demonstram que embora, em geral, tumores de mama não apresentem alta imunogenicidade, alguns tumores podem, sim, ser imunogênicos. A identificação desses tumores por meio de marcadores, bem como estratégias terapêuticas que visem ao aumento da imunogenicidade dos tumores, podem permitir o uso de imunoterapias

também no câncer de mama.

Dentre os fatores que influenciam a resposta imunológica tumoral, demonstrou-se que a expressão de elementos retrovirais endógenos está associada a maior potencial citotóxico em tumores de mama (ROONEY et al., 2015). Outro estudo recente demonstrou que a maioria dos antígenos reconhecidos pelo sistema imunológico em tumores, provém de regiões tidas como não codificantes, e mapeia em retroelementos no genoma humano (LAUMONT et al., 2018). A expressão desses elementos retrovirais ativam vias de intérferon tipo I, que também estão associadas a aumento de imunogenicidade em tumores (CHIAPPINELLI et al., 2017).

Ainda, a ativação dessas vias induz a expressão da família das enzimas da APOBEC3 como um mecanismo antiviral (KOITO; IKEDA, 2013), e a expressão dessas enzimas está associada a uma assinatura mutacional também associada a um maior infiltrado imunológico e a um maior potencial citolítico; mecanisticamente, as mutações geradas por essa assinatura tendem a afetar aminoácidos neutros (glutamato e aspartato) e aumentar a carga elétrica dos peptídeos mutados, o que está correlacionado com maior ativação de linfócitos T no câncer de mama (SMID et al., 2016).

Assim, o sistema imunológico está intimamente relacionado à glândula mamária feminina modulando desde sua organogênese a processos patológicos que ocorrem na mama. Durante a carcinogênese mamária, a composição do infiltrado imunológico é alterada e atua de forma diferente em cada subtipo molecular e estágio da doença, e diversos fatores parecem afetar a dinâmica das respostas imunológicas na mama. A compreensão desses fatores auxilia no entendimento da patogênese da doença e pode permitir a otimização de protocolos terapêuticos baseados em imunoterapias para o câncer de mama.

2.6 Variações Genéticas em Moléculas Imunológicas e a Patogênese do Câncer de Mama

Como previamente discutido, o câncer de mama é uma doença de etiologia complexa, na qual participam fatores de risco ambientais e genéticos. Embora componentes genéticos e hereditários correspondam a aproximadamente 30% da susceptibilidade à doença (MOLLER et al., 2016), apenas de 5 a 10% dos casos de câncer de mama são associados a um forte componente hereditário, e apenas de 4 a 5% dos casos são explicados por mutações em genes de alta penetrância na doença (APOSTOLOU; FOSTIRA, 2013), como os genes *BRCA1*, *BRCA2* e *CHEK2* (HALL et al., 1990; MIKI et al., 1994; MEIJERS-HEIJBOER et al., 2003).

Esses dados sugerem que a maior parte da susceptibilidade genética ao câncer de mama seja explicada por variantes de baixa penetrância. De fato, esse modelo é observado na maioria dos cânceres, nos quais múltiplas dessas variantes, com baixo efeito individual, se somam para explicar a susceptibilidade à doença (FLETCHER; HOULSTON, 2010).

A identificação dessas variantes e a elucidação de seus papéis na carcinogênese mamária é alvo de intensas pesquisas e diversas variantes associadas ao câncer de mama foram reveladas. Observou-se que essas variantes de baixa penetrância se associam ao câncer de mama de maneira subtipo-específica, confirmando a etiologia diferencial dos diversos subtipos de câncer de mama observados na clínica (BROEKS et al., 2011). Além disso, os *loci* de susceptibilidade podem variar de população para população e apresentar importância prognóstica na doença (LILYQUIST et al., 2018).

A maioria dos *loci* investigados e identificados como associados ao câncer de mama desempenham papel nas células neoplásicas, aumentando sua resposta a fatores de crescimento, promovendo instabilidade genômica, aumentando sua resistência à apoptose ou ativando vias de proliferação celular (FLETCHER; HOULSTON, 2010). Entretanto, evidências recentes revelam que algumas variantes têm efeito na modificação do estroma tumoral, e não na biologia das células neoplásicas diretamente (FLISTER; BERGOM, 2018).

Assim, o estudo de variantes genéticas com potencial de modulação tanto na célula tumoral diretamente quanto no estroma tumoral, em especial em seu componente imunológico, podem auxiliar na compreensão da doença e revelar novos marcadores de susceptibilidade e prognóstico do câncer de mama, e a elucidação de seus papéis na carcinogênese mamária pode revelar alvos terapêuticos diretos ou marcadores para delineamento terapêutico na doença.

2.6.1 Sinalização por IL-7 e Gene IL7RA

A interleucina 7 (IL-7) é uma citocina do tipo 1, da família das hematopoietinas que desempenha funções essenciais no desenvolvimento e homeostase do sistema imunológico, participando em todas as etapas de desenvolvimento de células T: no timo essa citocina é necessária na maturação de timócitos e na periferia é necessária para a sobrevivência de linfócitos T virgens e de memória. Por seu papel na manutenção da sobrevivência de células T, seu uso tem sido sugerido na imunoterapia de cânceres, principalmente aqueles que apresentam deleção de linfócitos pela terapia citotóxica (ELKASSAR; GRESS, 2010; LIN et al., 2017).

Essa citocina atua por meio da ligação ao receptor de IL-7 (IL-7R) na membrana celular. Esse receptor é composto por duas cadeias alfa (IL-7Rα, ou CD127) e uma cadeia gama-comum (γc) que é compartilhada por outras citocinas da mesma família da IL-7 (IL-2, IL-4, IL-9, IL-14, IL-15 e IL-21), e ativam proteínas janus quinase 1 e 3 (JAK1 e JAK3), que ficam associadas às suas caudas citoplasmáticas. Uma vez ativadas, as proteínas JAK1 e JAK3 fosforilam essas caudas citoplasmáticas e os sítios fosforilados servem de ancoragem para o fator de transcrição "transdutor de sinal e ativador da transcrição" 5 (STAT5, do inglês *signal transducer and activator of transcription 5*), que também são fosforilados pelas proteínas JAK e, então, translocam-se para o núcleo, onde governam a expressão de seus genes alvo. A sinalização da IL-7 por meio de seus receptores também ativam a via fosfatidil-inositol 3-quinase (PI3K)/Akt, que está associada a sinais pró-sobrevivência e antiapoptóticos (JIANG et al., 2005).

Além do seu efeito em células T, alguns estudos demonstram que a IL-7 pode atuar em células neoplásicas, provocando inibição de apoptose e induzindo sobrevivência dessas células, e estimulando a progressão da doença (AL-RAWI; MANSEL; JIANG, 2003; UJIIE et al., 2015; QU et al., 2016; LIN et al., 2017). No câncer de mama, a alta expressão de componentes da via da IL-7 foi correlacionada com pior prognóstico (AL-RAWI et al., 2004b) e ensaios *in vitro* demonstraram que a IL-7 promove crescimento e sobrevivência de células de câncer de mama pela ativação de STAT5 e PI3K (AL-RAWI et al., 2004a); além disso, deficiência na expressão de IL-7Rα foi demonstrada em células imunológicas circulantes de pacientes com câncer de mama (VUDATTU et al., 2007).

O gene do IL-7Rα (*IL7RA*) localiza-se no braço curto cromossomo 5 (*locus* 5p13.2), e apresenta diversos polimorfismos de base única (SNP, do inglês *single nucleotide polymorphism*). Entre esses, uma transição de C para T no éxon 6 (rs6897932), promovendo a alteração de uma treonina por uma isoleucina na porção extracelular da proteína, próximo a membrana celular. Esse polimorfismo é um *tag* SNP para outros polimorfismos no gene, e foi associado à proteção contra diversas doenças autoimunes (MAZZUCCHELLI; RIVA; DURUM, 2012). Essa associação ocorre porque esse polimorfismo diminui a expressão da variante de *splicing* solúvel do RNA de IL-7Rα (sIL-7Rα) (GREGORY et al., 2007; HOE et al., 2010), que aumenta a biodisponibilidade de IL-7 circulante e promove maior ativação imunológica crônica (LUNDSTROM et al., 2013).

Assim, tendo em vista os efeitos da IL-7 no sistema imunológico e no câncer de mama, e os efeitos desse polimorfismo na biologia da IL-7, no presente trabalho foi investigada, pela primeira vez na literatura, a possível associação desse polimorfismo com a

susceptibilidade e apresentação clínica dos subtipos moleculares de câncer de mama (Seção 3.1 Artigo 1: “Interleukin 7 receptor alpha Thr244Ile genetic polymorphism is associated with susceptibility and prognostic markers in breast cancer subgroups”).

2.6.2 Sinalização Por TGF-Beta: Genes TGFB1 e TGFBR2

O fator de crescimento e transformação beta 1 (TGFβ1) é o membro mais abundante e ubíquo da subfamília TGFβ, que inclui também o TGFβ2 e TGFβ3, expressos em contextos mais restritos e específicos durante a embriogênese (CHANG; BROWN; MATZUK, 2002). Todas as três isoformas atuam pelo mesmo conjunto de receptores transmembrana e ativam as mesmas vias de sinalização celular, desencadeando efeitos contexto-específicos (CHEIFETZ et al., 1987).

A sinalização do TGFβ se inicia pela ligação a receptores serina/treonina quinase transmembrana chamados de receptor de TGFβ do tipo II (TGFβRII), que possuem sítios de reconhecimento à citocina no seu domínio extracelular. Esses receptores ligados à citocina então se ligam a um segundo receptor com atividade serina-treonina quinase, o receptor de TGFβ do tipo I (TGFβRI); uma vez oligomerizados, o TGFβRII promove fosforilação em resíduos de serina na cauda citoplasmática de TGFβRI, que agora é ativado e promove fosforilação em proteínas sinalizadoras citoplasmáticas, as SMADs associadas a receptores (R-SMADs) SMAD2 e SMAD3, que estão associadas ao receptor por meio de proteínas de ancoragem (KUBICZKOVA et al., 2012).

As proteínas SMAD2 e 3 fosforiladas então se associam a outra proteína de sinalização citoplasmática, a SMAD4, e o complexo SMAD2/3-4 é translocado para o núcleo celular, onde se ligam a sequências conservadas no DNA, chamadas de elementos de resposta a SMADs por meio de seu domínio de homologia a *mad* 1 (MH1, do inglês *mad homology domain*) e a outros fatores de transcrição por meio de seu domínio MH2, atuando como co-ativador ou co-repressor desses fatores e desempenhando, dessa forma, efeitos dependentes dos fatores de transcrição que estão ativos na célula alvo no momento da sinalização (MASSAGUE, 2000). Além dessa via de sinalização, chamada de via clássica, diversas outras vias são ativadas por TGFβ, incluindo a via das proteínas quinases ativadas por mitógenos (MAPK), que são ativadas pela proteína Ras, da PI3K/Akt e das GTPases pequenas da família Rho (Rho GTPases) (MU; GUDEY; LANDSTROM, 2012).

A ativação da via clássica do TGFβ está associada principalmente a efeitos antiproliferativos e pró-apoptóticos em células epiteliais e hematopoiéticas; já as vias

alternativas do TGFβ induzem sobrevivência, proliferação e motilidade celular, e estão associadas à transição epitélio mesenquimal (EMT), um processo pelo qual células epiteliais adquirem um fenótipo mesenquimal e podem migrar no tecido (SIEGEL; MASSAGUE, 2003; PARVANI; TAYLOR; SCHIEMANN, 2011). Esses efeitos têm profundas implicações na patogênese do câncer, onde o TGFβ atua como supressor tumoral por meio da via clássica em tumores iniciais, mas induz progressão em metástase em tumores avançados e agressivos que adquiriram resistência aos efeitos apoptóticos do TGFβ e que apresentam hiperativação das vias das MAPK, PI3K e das Rho GTPases (BIERIE; MOSES, 2006). Além dos efeitos diretos nas células neoplásicas, a indução de tolerância imunológica induzida por TGFβ1 também promove a progressão tumoral (YANG; PANG; MOSES, 2010; JIANG; LI; ZHU, 2015).

No câncer de mama esses efeitos se refletem entre os diferentes subtipos da doença, com tumores de perfil menos agressivo, como os do subtipo luminal-A, apresentando principalmente respostas citostáticas a essa citocina, enquanto tumores mais agressivos como os que hiperexpressam HER2 e os triplo-negativos apresentando maior potencial metastático (WILSON et al., 2005; PARVANI; TAYLOR; SCHIEMANN, 2011). No caso de tumores $HER2^{+}$ a sinalização cruzada de TGFβ leva a hiperativação da via das MAPK e a hiperativação do programa de EMT (WANG, 2011), enquanto em tumores triplo-negativos a sinalização por TGFβ está relacionada com a geração e manutenção de células tronco tumorais, que são associadas a resistência ao tratamento e recidiva (ASIEDU et al., 2011; BHOLA et al., 2013).

Além disso, a sinalização de TGFβ induz a expressão de diversos receptores de quimiocinas, levando ao desenvolvimento de metástases órgão-direcionadas: a expressão de TGFβ1 correlaciona-se positivamente com a expressão de CXCR4 tumoral, que direciona metástases para o fígado e medula óssea (BIERIE et al., 2009; ODA et al., 2012), e a ativação de TGFβ e MAPK simultaneamente é necessária para a indução do receptor CCR7, que direciona metástases ao linfonodo (PANG et al., 2015). A ativação de TGFβ1 também favorece metástases mamárias para o pulmão (PADUA et al., 2008).

O gene do TGFβ1 (*TGFB1*) localiza-se no braço longo do cromossomo 19 (*locus* 19q13.1) e diversos polimorfismos funcionais com potencial de alterar qualitativamente ou quantitativamente o produto desse gene são descritos (MARTELOSSI CEBINELLI et al., 2016). Dentre esses, dois polimorfismos na região promotora (rs1800468, G-800A e rs1800469, C-509T) e dois no peptídeo sinal desse gene (rs1800470, T29C e rs1800471, G74C) têm sido amplamente estudados na patogênese dessa doença.

O polimorfismo G-800A encontra-se situado em uma região de ligação a fatores de transcrição da família dos elementos de resposta ao AMP-cíclico (CREB, do inglês

cyclic AMP response element binding protein) (GRAINGER et al., 1999) e seu alelo A foi associado a menor secreção de TGFβ1 por leucócitos de sangue periféricos estimulados *in vitro* (COTTON et al., 2002). Estudos de associação na população europeia (DUNNING et al., 2003) e indiana (POOJA et al., 2013) não demonstraram associação desse polimorfismo com o câncer de mama.

O polimorfismo C-509T foi identificado como um fator genético afetando os níveis plasmáticos de TGFβ1 em um estudo com gêmeas (GRAINGER et al., 1999), e diversos ensaios *in vitro* confirmaram maior produção de TGFβ1 com ligação diferencial de fatores de transcrição ao alelo T desse polimorfismo (LUEDECKING et al., 2000; SILVERMAN et al., 2004; SHAH; HURLEY; POSCH, 2006; CAO et al., 2014). Estudos associando esse polimorfismo ao risco de câncer de mama são controversos, com resultados demonstrando associação à proteção (NIU et al., 2010) ou ao risco dessa doença (DUNNING et al., 2003).

Já para o polimorfismo T29C, o alelo C foi associado a uma maior produção de TGFβ1 em ensaios com células transfectadas com vetores para cada um dos alelos (DUNNING et al., 2003). Uma meta-análise relatou aumento do risco de câncer de mama para portadores do alelo C desse polimorfismo, mas resultados de estudos individuais mostram-se controversos, e seu efeito pode ser diferente em diferentes populações (QIU et al., 2010).

Em relação ao polimorfismo G74C, o alelo C foi associado a menor produção de TGFβ1 por leucócitos periféricos estimulados *in vitro* (AWAD et al., 1998). Um estudo de associação na população europeia não encontrou qualquer associação entre esse polimorfismo e o câncer de mama (JIN et al., 2004), enquanto um estudo na população indiana demonstrou uma associação protetora para o alelo C (POOJA et al., 2013).

Assim, os resultados de associação entre esses polimorfismos e o câncer de mama são controversos na literatura. Tais contradições podem decorrer do fato de esses polimorfismos terem sido analisados em amostras de câncer de mama geral, não estratificadas por subtipos moleculares, nos estudos citados. Dada a variação de efeitos do TGFβ1 entre diferentes subtipos, é razoável supor que suas variantes genéticas também apresentem associações subtipo-específicas. Além disso esses polimorfismos são herdados em conjuntos, em estruturas haplotípicas, e o efeito de um polimorfismo pode mascarar o efeito de outros; apesar disso, apenas um estudo avaliou as estruturas haplotípicas compostas por esses quatro polimorfismos no câncer de mama (JIN et al., 2004). Dessa forma, o presente trabalho avaliou, pela primeira vez na literatura, a associação desses polimorfismos e suas estruturas haplotítpicas com a susceptibilidade e apresentação clínica de tumores mamários em uma amostra brasileira (seção 3.2 Artigo 2: “Transforming growth factor beta 1 (TGFβ1) polymorphisms and

haplotype structures have dual roles in breast cancer pathogenesis").

O gene do TGFβRII (*TGFBR2*), encontra-se no braço curto do cromossomo 3 (*locus* 3p22) e apresenta um polimorfismo em sua região promotora que leva ao aumento de sua expressão, como comprovado em um ensaio reporter em células HeLa transfectadas com vetores para ambos alelos (SEIJO et al., 2001). Pesquisas subsequentes sugeriram a ligação diferencial de fatores de transcrição entre os dois alelos (CHOE et al., 2012).

Esse polimorfismo foi estudado em diversos cânceres, e uma meta-análise desses estudos sugeriu que o alelo A estaria associado à proteção contra o câncer de forma geral, consistente com os efeitos do TGFβ como um supressor tumoral em fases de iniciação (HUANG et al., 2014). No câncer de mama esse polimorfismo foi associado a proteção contra tumores positivos para receptores hormonais na população chinesa (ZHANG et al., 2011), mas esse estudo possuia baixo poder estatístico para detectar efeitos em outros subtipos.

Assim, no presente trabalho foi avaliada a associação desse polimorfismo com a susceptibilidade e apresentação clínica de diferentes subgrupos de câncer de mama na população brasileira (seção 3.3. Artigo 3: "Transforming growth factor beta receptor II (TGFBR2) promoter region polymorphism in Brazilian breast cancer patients: association with susceptibility, clinicopathological features, and interaction with TGFB1 haplotypes").

Além disso, estudos avaliando quantitativamente moléculas relacionadas às vias do TGFβ sistemicamente ou no microambiente tumoral em relação ao seu valor prognóstico produziram resultados conflitantes na literatura, provavelmente resultando dos efeitos subtipo-específicos dessas moléculas no câncer de mama, uma vez que subtipos moleculares raramente foram considerados nesses estudos. Além disso, apesar de ter sido demonstrado que os polimorfismos nos genes *TGFB1* e *TGFBR2* alteram suas expressões em contextos específicos, estudos avaliando os níveis TGFβ1 em indivíduos genotipados para alguns desses polimorfismos também produziram resultados controversos. Assim, objetivou-se também avaliar os níveis plasmáticos de TGFβ1 em pacientes com câncer de mama e controles livres de neoplasia com haplótipos determinados para esses quatro polimorfismos (seção 3.4 Artigo 4: "Transforming growth factor beta 1 (TGFβ1) plasmatic levels in breast cancer patients and healthy women: association with patients' characteristic and *TGFB1* haplotypes") bem como a expressão proteíca intratumoral de TGFβ1, TGFβRII e a ativação de SMADs em tumores de pacientes genotipadas para esses polimorfismos (seção 3.5 Artigo 5: "Transforming growth factor beta 1 (TGFβ1) pathway components in breast cancer tissue from aggressive subtypes: correlation with single nucleotide polymorphisms, plasmatic TGFβ1 and clinicopathological features").

2.6.3 APOBECs e Deleção APOBEC3A/B

As APOBECs (do inglês: *Apolipoprotein B mRNA-editing enzyme catalytic polypeptide like*) da subfamília 3 são um grupos de sete enzimas citidina deaminases (APOBEC3A, APOBEC3B, APOBEC3C, APOBEC3D, APOBEC3F, APOBEC3G e APOBEC3H) que participam da imunidade antiviral e são codificadas por um agrupamento genômico no braço longo do cromossomo 22. Essa subfamília é parte da superfamília AID/APOBEC que também inclui a deaminase induzida por ativação (AID), responsável pelo fenômeno de hipermutação na geração de anticorpos em células B; a APOBEC1, responsável pela edição do mRNA da Apoliproteína-B, gerando a isoforma curta da proteína que atua no transporte de lipídeos; e as APOBECs 2 e 4, que não demonstram atividade catalítica e são, provavelmente, pseudogenes (SMITH et al., 2012).

As enzimas da família APOBEC3 são induzidas por respostas imunológicas antivirais a partir de IFNs do tipo I, principalmente. Essas respostas se iniciam pelo reconhecimento de produtos da replicação viral, como RNAs de fita dupla e RNAs virais citosólicos pela proteína RIG-I (do inglês: *Retinoic acid inducible gene I*) ou DNA e híbridos de RNA-cDNA citosólicos pela proteína cGAS (do inglês: *cyclic GMP-AMP synthase*), provenientes de vírus exógenos e retroelementos endógenos, levando à ativação de fatores de transcrição como o IRF3 (do inglês: *interferon response factor 3*) que promovem a expressão de intérferons (HURST; MAGIORKINIS, 2015; ZEVINI; OLAGNIER; HISCOTT, 2017). As enzimas APOBEC3 reconhecem DNA de fita simples e promovem mutações nesses ácidos nucleidos, promovendo, dessa forma, inativação viral (CHIU; GREENE, 2008).

Por seu potencial mutagênico as APOBECs foram implicadas no desenvolvimento de cânceres em diversos relatos iniciais (HENDERSON; FENTON, 2015). Mais recentemente, foi identificado um padrão mutacional consistente com a atividade de APOBEC3 em amostras de câncer de mama (STEPHENS et al., 2005; NIK-ZAINAL et al., 2012), e relatos posteriores identificaram as enzimas APOBEC3A e APOBEC3B como as enzimas responsáveis pela geração desse padrão (BURNS et al., 2013; BURNS; TEMIZ; HARRIS, 2013; ROBERTS et al., 2013; CHAN et al., 2015). Esse padrão mutacional foi associado a maior infiltração de células T e maior potencial citolítico no câncer de mama (SMID et al., 2016), indicando que esse pode ser um modulador da resposta imune no microambiente tumoral.

Uma deleção de linhagem germinativa de aproximadamante 29,5 kilobases unindo a porção codificante da APOBEC3A com a região 3'-UTR (do inglês: 3'-*untranslated*

region) da APOBEC3B foi associada a maior carga mutacional mediada por APOBEC3 e a maior susceptibilidade para o câncer de mama (NIK-ZAINAL et al., 2014). Foi sugerido que essa deleção aumenta a estabilidade do transcrito por ligação diferencial de micro-RNAs, aumentando a expressão proteica e a carga mutacional (REVATHIDEVI et al., 2016). Esses dados são consistentes com o maior potencial mutagênico da APOBEC3A e, consequentemente, do alelo híbrido, em relação a APOBEC3B, demonstrado em ensaios *in vitro* (BURNS et al., 2013; SOMMER et al., 2013; CAVAL et al., 2014) e em genomas de pacientes (CHAN et al., 2015; CHEN et al., 2019).

Entretanto, estudos de associação relacionando essa deleção com o risco de câncer de mama são controversos: enquanto estudos em populações orientais, onde essa deleção é frequente, demonstraram associação com risco aumentado de câncer de mama para essa deleção, estudos em populações caucasoides produziram resultados controversos, com uma meta-análise demonstrando ausência de associação nessa população (KLONOWSKA et al., 2017; HASHEMI; MOAZENI-ROODI; TAHERI, 2019). Os efeitos da expressão de APOBEC3A, APOBEC3B e da deleção envolvendo esses dois *loci* no prognóstico da doença também são controversos, e podem variar de população para população (CESCON; HAIBE-KAINS; MAK, 2015; TOKUNAGA et al., 2016; CHEN et al., 2019).

Assim, no presente trabalho foi investigada a frequência dessa deleção em pacientes com câncer de mama e controles livres de neoplasia na população brasileira, e a associação dessa deleção com a susceptibilidade a doença e com parâmetros clinicopatológicos foi testada (seção 3.5 Artigo 5: “Germline APOBEC3B deletion influences clinicopathological parameters in luminal-A breast cancer: evidences from a southern Brazilian cohort”).

3 PRODUÇÃO BIBLIOGRÁFICA

Esta seção contém os seis artigos científicos produzidos no desenvolvimento desta tese. São eles:

1) "*Interleukin 7 receptor alpha Thr244Ile genetic polymorphism is associated with susceptibility and prognostic markers in breast cancer subgroups*". Publicado no periódico ***Cytokine***.

2) "*Transforming growth factor beta 1 (TGFβ1) polymorphisms and haplotype structures have dual roles in breast cancer pathogenesis*". Publicado no periódico ***Journal of Cancer Research and Clinical Oncology***.

3) "*Transforming growth factor beta receptor II (TGFBR2) promoter region polymorphism in Brazilian breast cancer patients: association with susceptibility, clinicopathological features, and interaction with TGFB1 haplotypes*". Publicado no periódico ***Breast Cancer Research and Treatment***.

4) "*Transforming growth factor beta 1 (TGFβ1) plasmatic levels in breast cancer patients and healthy women: association with patients' characteristic and TGFB1 haplotypes*". Publicado no periódico ***Cytokine***.

5) "*Transforming growth factor beta 1 (TGFβ1) pathway components in breast cancer tissue from aggressive subtypes: correlation with single nucleotide polymorphisms, plasmatic TGFβ1 and clinicopathological features*". A ser submetido ao periódico ***Pathology & Oncology Research.***

6) "*Germline APOBEC3B deletion influences clinicopathological parameters in luminal-A breast cancer: evidences from a southern Brazilian cohort*". Publicado no periódico ***Journal of Cancer Research and Clinical Oncology***.

3.1 ARTIGO 1: "INTERLEUKIN 7 RECEPTOR ALPHA THR244ILE GENETIC POLYMORPHISM IS ASSOCIATED WITH SUSCEPTIBILITY AND PROGNOSTIC MARKERS IN BREAST CANCER SUBGROUPS"

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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

samples were sequenced. First, PCR products were purified using the PureLink™ PCR Purification Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. Sequencing reaction was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA). Amplicons were analyzed in a 24-capillary 3500xl Genetic Analyzer (Applied Biosystems®) and sequence was assembled using clustalW multiple alignment algorithm, confirming the amplification of desired *IL7RA* fragment. PCR-RFLP was also repeated for at least 5% of total sample obtaining 100% concordance between results.

2.4. Statistical analyses

Statistical analyses were performed in IBM® SPSS® Statistics software version 22 (IBM®, Armonk, New York City, NY, USA). All tests had significance level of 5% and were two-tailed.

For case-control analyses, odds ratio (OR) with its' 95% of confidence interval (CI) was calculated through binary logistic regression setting case-control status as dependent variable and age and models of association as predictor variables in forced entry method. Models of association tested were: genotypic (CT or TT versus CC), dominant (CT and TT versus CC) and recessive (TT versus CT and CC).

For correlation analyses between *IL7RA* polymorphism and clinicopathological parameters, the Kendall's tau-b rank correlation coefficient was calculated considering additive (genotypic) model (CC = 0; CT = 1; TT = 2), dominant model (CC = 0; CT or TT = 1) and recessive model (CC or CT = 0; TT = 1).

3. Results

3.1. Sample characterization

Total BC sample (n = 338) was grouped based on tumor positivity for hormonal receptors (ER/PR) and HER2 overexpression into 4 subgroups: Luminal-HER2$^-$ (L-HER2$^-$; ER/PR$^+$HER2$^-$; n = 212), Luminal B-HER2$^+$ (L-HER2$^+$; ER/PR$^+$HER2$^+$; n = 42), HER2-enriched (HER2; ER$^-$PR$^-$HER2$^+$; n = 24) and triple negative (TN; ER$^-$PR$^-$HER2$^-$; n = 60). Clinicopathological parameters for patients within each subgroup are shown in Table 1. For control group mean age was 54 years (S.D. = 13.97) while median was 55 (I.Q. range = 15.25).

As expected tumors from L-HER2$^+$, HER2 and TN subgroups tended to have worst prognosis parameters when compared to L-HER2$^-$ tumors, as evidenced by higher proportion of tumors occurring at earlier ages (< 50), with higher tumor size, histopathological grade, Ki67, p53 mutation, LN metastasis and with higher disease stages (III or IV) at diagnosis (Table 1).

Table 2 shows genotypes distribution for BC subgroups and controls. Both BC patients and controls were tested for Hardy-Weinberg equilibrium and no deviation from expected genotype distribution was found for any group ($p > 0.05$). Genotype frequencies were significantly different between controls and TN subgroup, although this difference was marginal ($\chi^2 = 6.01$; $p = 0.049$).

Both BC patients and controls were tested for Hardy-Weinberg equilibrium and no deviation from expected genotype distribution was found for any group ($p > 0.05$). Genotype distribution from our control population was compared to that deposited on the recently-published ABraOM databank (http://www.abraom.ib.usp.br/) of genetic variations from 609 elderly health Brazilian individuals from São Paulo [21], and genotype frequencies were found to be similar ($\chi^2 = 1.07$; $p = 0.58$).

Allele frequency was compared through Fisher's exact test between control group and diverse populations worldwide using data from 1000 genomes project, freely made available through the web-based application LDlink (https://analysistools.nci.nih.gov/LDlink/) [22]. T allele was significantly more frequent in our control group than in all African subpopulations ($p < 0.05$) except for Americans with African Ancestry in USA (ASW, $p = 0.25$). Among Admixed American subpopulations, T allele frequency was shown to be slightly higher in control group than in Puerto Rico population ($p = 0.047$), but was similar to other subpopulations (Mexicans, Peruvians and Colombians). Our allele frequency was also similar to all East Asia subpopulations ($p > 0.05$). Among European populations, T allele frequency from our control group was significantly smaller than all but Italian population from Toscana. Finally, when comparing to South Asian populations, only Indian population from UK showed an increased T allele frequency when compared to our control group.

3.2. IL7RA Thr244Ile and breast cancer susceptibility

Table 3 summarizes the results for association studies between *IL7RA* and BC subgroups. As shown, no association was found for general BC, neither for L-HER2$^-$, L-HER2$^+$ or HER2 subgroups. However, TT genotype was associated with increased susceptibility only for TN BCs both in genotypic (TT vs CC: OR = 3.07; CI = 1.01–9.38) and recessive (TT vs CC and CT: OR = 3.59; CI = 1.19–10.85) models. ER/PR$^+$HER2$^{+/-}$ and ER$^{+/-}$PR$^{+/-}$HER2$^+$ tumors were also tested as subgroups for association with *IL7RA* polymorphism, but no significant association was found (data not shown).

3.3. IL7RA Thr244Ile correlation with breast cancer clinicopathological parameters

Results for correlation analyses are shown in Table 4. Although there was no significant correlation between clinicopathological parameters and *IL7RA* polymorphism in total BC sample, in subgroup-stratified analyses TT genotype indicated later age at diagnosis for L-HER2$^-$ tumors; in L-HER2$^+$, *IL7RA* polymorphism was positively correlated with histopathological grade and lymph node metastasis, both in the additive and dominant models; in HER2-enriched tumors, allele T carriers presented significant positive correlations with cellular proliferation index (Ki67) and in TN cancers, TT genotype negatively correlated with disease stage.

4. Discussion

In the present study, it was found that *IL7RA* Thr244Ile polymorphism is associated with TN BC susceptibility and related to prognosis parameters in different BC subgroups. The association between this *IL7RA* Thr244Ile and autoimmune disorders has been established by previous literature [11]. However, the present study is the first to investigate this polymorphism in general BC or its' clinical subgroups, to our knowledge.

The rs6897932 is described as a C to T transition on codon 244 (exon 6), leading to a threonine to isoleucine on the amino acid chain. This alteration shifts the balance between membrane-bound (IL-7Rα) and soluble (sIL-7Rα) isoform of this receptor towards the first, probably by altering an exonic splicing silencer [12], making T allele carriers produce more membrane-bound IL-7Rα compared. By competing for IL-7 binding, sIL-7Rα diminishes early IL-7 signaling intensity but increases IL-7 half-life, leading to prolonged exposure to IL-7 [13].

We found our control group genotype frequency to be similar to those from Brazilian population from São Paulo [21]. When comparing to other populations around the world [22], we found that T allele frequency from control group (17.87%) was similar to the majority of Asian and Admixed American populations, but was significantly higher than African populations (pooled frequency = 6.58%; $p < 0.0001$) and smaller than European populations (pooled frequency = 27.14%; $p < 0.0001$). This data indicate that T allele has an European Ancestry, and differences between Admixed American populations (including Brazilian population) and European and African may be explained by the high miscegenation hates observed in those populations.

Table 1
Clinicopathological features distribution among BC subtypes.

Parameter	General BC	L-HER2$^-$	L-HER2$^+$	HER2	TN
Age (years)					
Median (IQ range)	53 (19)	54 (18.5)	47.5 (11.5)	51 (10.5)	53.5 (19)
Mean (SD)	54.5 (12.9)	55.7 (12.7)	49.2 (10.8)	53 (14.03)	54.6 (13.4)
< 40 [n (%)]	38 (11.28)	20 (9.48)	7 (16.67)	3 (12.5)	8 (13.33)
40–49 [n (%)]	98 (29.08)	59 (27.96)	17 (40.48)	7 (29.17)	15 (25.0)
50–59 [n (%)]	85 (25.22)	52 (24.64)	10 (23.81)	8 (33.33)	15 (25.0)
60–69 [n (%)]	65 (19.29)	44 (20.85)	7 (16.67)	4 (16.67)	10 (16.67)
70–79 [n (%)]	41 (12.17)	29 (13.74)	1 (2.38)	1 (4.17)	10 (16.67)
> 80 [n (%)]	10 (2.97)	7 (3.32)	0 (0.0)	1 (4.17)	2 (3.33)
Unknown [n (%)]	1	1	0	0	0
Tumor size					
Median (IQ range)	2.45 (1.8)	2 (1.5)	2.5 (1.2)	2.2 (1.88)	3.2 (2.6)
Mean (SD)	2.85 (1.87)	2.6 (1.62)	3.01 (2.01)	2.84 (1.48)	3.64 (2.6)
0–1.5 cm [n (%)]	44 (13.21)	30 (14.29)	4 (9.76)	2 (9.09)	8 (13.33)
1.51–3.0 cm [n (%)]	159 (47.75)	112 (53.33)	19 (46.3)	12 (54.55)	16 (26.67)
> 3.0 cm [n (%)]	130 (39.04)	68 (32.38)	18 (43.9)	8 (36.36)	36 (60.0)
Undocumented	5	2	1	2	0
Histopathological grade [n (%)]					
I	36 (11.32)	35 (17.68)	1 (2.44)	0 (0.0)	0 (0.0)
II	135 (42.45)	103 (52.02)	14 (34.15)	7 (33.33)	11 (18.97)
III	147 (46.23)	60 (30.3)	26 (64.41)	14 (66.67)	47 (81.03)
Unknown	20	14	1	3	2
Ki67 [n (%)]					
Low	58 (24.79)	51 (34.93)	4 (15.38)	0 (0.0)	3 (6.12)
Intermediate	103 (44.02)	73 (50.0)	12 (46.15)	5 (38.46)	13 (26.53)
High	73 (31.2)	22 (15.07)	10 (38.46)	8 (61.54)	33 (67.35)
Unknown	104	66	16	11	11
p53 mutation [n (%)]					
Positive	94 (37.6)	35 (22.58)	15 (60.0)	11 (64.7)	33 (62.26)
Negative	156 (62.4)	120 (77.42)	10 (40.0)	6 (35.29)	20 (37.74)
Unknown	88	57	17	7	7
Lymph node metastasis [n (%)]					
Positive	158 (48.77)	98 (47.34)	19 (48.72)	11 (50.0)	30 (53.57)
Negative	166 (51.23)	109 (52.66)	20 (51.28)	11 (50.0)	26 (46.43)
Unknown	14	5	3	2	4
Tumor stage [n (%)]					
0	9 (3.32)	6 (3.41)	1 (3.03)	2 (10.0)	0 (0.0)
I	50 (18.45)	41 (23.3)	3 (9.09)	1 (5.0)	5 (11.9)
II	117 (43.17)	79 (44.89)	14 (42.42)	6 (30.0)	18 (42.86)
III	78 (28.78)	40 (27.73)	12 (36.36)	9 (45.0)	17 (40.48)
IV	17 (6.27)	10 (5.68)	3 (9.09)	2 (10.0)	2 (4.47)
Unknown	67	36	9	4	18

Table 2
Genotype frequencies among controls and BC subgroups.

IL7RA genotype	Control [n (%)]	BC subtypes [n (%)]				
		BC	L-HER2$^-$	L-HER2$^+$	HER2	TN
CC	272 (67.5)	212 (62.7)	129 (60.8)	22 (52.4)	17 (70.8)	44 (73.3)
CT	118 (29.3)	110 (32.5)	75 (35.4)	18 (42.9)	6 (25.0)	11 (18.3)
TT	13 (3.2)	16 (4.7)	8 (3.8)	2 (4.8)	1 (4.2)	5 (8.3)
p-value[#]	–	0.31	0.26	0.144	0.89	0.049[*]

[#] Obtained by Fisher's exact test compared to control group.
[*] Significant ($p < 0.05$) difference in genotype distribution.

IL-7 is a pleiotropic cytokine that exert main functions on immune system by promoting the development and homeostasis of lymphoid cells [23]. Upon ligation with its' receptor complex, composed by the common cytokine-receptor γ chain (CD132) and the IL-7Rα (CD127), IL-7 activates JAK1 and 3, which in turn phosphorylates the signal transducer STAT5 and PI3K pathway, activating pro-survival responses in lymphoid cells [8].

Beyond its' functions on immune system, IL-7 has been shown to act on tumor cells themselves [14]. In prostate cancer, Qu et al. [24] found that the expression of IL-7 and IL-7Rα were both upregulated and IL-7 dose-dependently promoted invasion and migration of cancer cells, whereas knockdown of IL-7Rα attenuated the effect of IL-7. Aiming to characterize the tumor immune microenvironment and identify immune-related prognostic markers, it was also verified by Ujiie et al. [25] that IL-7Rα expression by tumor cells predicted worse patient survival.

In BC, elevated expression of IL-7 signaling components in tumor milieu was associated with worst BC prognosis [6]. *In vitro* assays with BC cells have shown that IL-7 is capable to promote cell growth and survival, and that this effect was dependent on both STAT5 and PI3K activation, as shown by inhibition analyses of these molecules [5].

STAT5, represented by two homologue isoforms (STAT5A and STAT5B), is crucial to mammary gland and BC development. In the developing breast, STAT5 is activated by prolactin (mainly) and other signaling pathways, such as hormonal receptors and EGFR, and promotes its' terminal differentiation and lactation [26]. In BC STAT5 activation is also associated with well differentiated phenotypes [27], better prognosis [28] and better response to endocrine therapy [29].

In this study, TT genotype was significantly associated with increased susceptibility for TN tumors, both in genotypic (OR = 3.07; IC: 1.01–9.38) and recessive (OR = 3.59; IC: 1.19–10.85) models (Table 2). As this genotype is associated with increased proportion of

Table 3
Subgroup association analyses for *IL7RA* polymorphism.

BC subtype	Model	OR	95% CI	*p*-value
General BC	CT	1.09	0.79–1.51	0.6
	TT	1.65	0.76–3.55	0.2
	Dominat	1.15	0.84–1.57	0.39
	Recessive	1.6	0.75–3.44	0.26
L-HER2$^-$	CT	1.21	0.84–1.75	0.31
	TT	1.34	0.53–3.28	0.53
	Dominat	1.23	0.86–1.76	0.25
	Recessive	1.27	0.51–3.18	0.6
L-HER2$^+$	CT	1.71	0.86–3.39	0.12
	TT	2.34	0.47–11.6	0.3
	Dominat	1.76	0.91–3.42	0.09
	Recessive	1.91	0.4–9.25	0.95
HER2	CT	0.76	0.29–2.02	0.58
	TT	1.3	0.16–10.73	0.81
	Dominat	0.81	0.32–2.04	0.66
	Recessive	1.4	0.17–11.4	0.75
TN	CT	0.51	0.25–1.04	0.06
	TT	3.07	1.01–9.38	0.05*
	Dominat	0.71	0.38–1.32	0.8
	Recessive	3.59	1.19–10.85	0.02*

* Significant factor ($p < 0.05$) in logistic regression analysis adjusted by age.

Table 4
Correlation analyses for *IL7RA* polymorphism and BC clinicopathological parameters.

BC subtype	Parameter	Models for *IL7RA* Thr244Ile [Tau-b (*p*-value)]		
		Additive	Dominant	Recessive
General BC	Age	−0.02 (0.68)	−0.04 (0.48)	0.08 (0.08)
	Tumor size	0.03 (0.61)	0.02 (0.64)	0.02 (0.68)
	Hist. grade	−0.07 (0.18)	−0.08 (0.15)	0.02 (0.73)
	Ki67	0.05 (0.4)	0.06 (0.37)	0.08 (0.91)
	LN	0.04 (0.48)	0.04 (0.47)	0.06 (0.92)
	Stage	0.04 (0.5)	0.04 (0.42)	−0.03 (0.56)
L-HER2$^-$	Age	0.03 (0.65)	0.01 (0.84)	0.13 (0.05)*
	Tumor size	0.03 (0.68)	0.02 (0.78)	0.1 (0.21)
	Hist. grade	−0.1 (0.1)	−0.11 (0.09)	0.03 (0.54)
	Ki67	0.09 (0.28)	0.12 (0.14)	−0.08 (0.38)
	LN	0.002 (0.97)	−0.007 (0.92)	0.04 (0.6)
	Stage	0.02 (0.8)	0.02 (0.78)	−0.01 (0.92)
L-HER2$^+$	Age	0.003 (0.98)	0.01 (0.92)	−0.07 (0.34)
	Tumor size	−0.02 (0.91)	0.03 (0.87)	0.16 (0.3)
	Hist. grade	0.29 (0.03)*	0.28 (0.05)*	0.12 (0.31)
	Ki67	0.32 (0.07)	0.31 (0.09)	NA
	LN	0.32 (0.03)*	0.35 (0.02)*	0.17 (0.3)
	Stage	0.12 (0.46)	0.1 (0.52)	0.28 (0.3)
HER2	Age	−0.09 (0.63)	−0.08 (0.68)	−0.16 (0.31)
	Tumor size	0.24 (0.2)	0.28 (0.16)	−0.11 (0.34)
	Hist. grade	−0.04 (0.86)	0.00 (1.0)	−0.32 (0.29)
	Ki67	0.51 (0.008)*	0.51 (0.008)*	0.23 (0.29)
	LN	−0.12 (0.56)	−0.1 (0.65)	−0.22 (0.29)
	Stage	−0.05 (0.81)	−0.05 (0.81)	0.06 (0.79)
TN	Age	−0.14 (0.22)	−0.17 (0.16)	0.05 (0.62)
	Tumor size	0.06 (0.66)	0.09 (0.46)	−0.16 (0.29)
	Hist. grade	−0.11 (0.44)	−0.1 (0.5)	−0.16 (0.34)
	Ki67	−0.1 (0.46)	−0.11 (0.43)	0.01 (0.96)
	LN	0.07 (0.61)	0.08 (0.56)	−0.02 (0.89)
	Stage	0.13 (0.38)	0.21 (0.13)	−0.27 (0.05)*

* Significant ($p < 0.05$) tau-b coefficient for correlation. NA: not analyzed.

membrane-bound IL-7Rα, increasing IL-7 signaling intensity, and promoting tumor cell growth, it may be reasonable to suppose that it may favor tumor development. Since TN is considered the most aggressive BC subtype, this hypothesis is consistent with previous data indicating higher expression of IL-7Rα in aggressive tumors [6]. However, conclusions in this regard should be viewed with caution, due to the absence of data investigating IL-7Rα expression in specific BC subgroups.

Despite association with increased TN BC susceptibility, TT individuals in this subgroup tended to have lower disease stages (tau-b = −0.27). TN tumors are associated to BC stem cells, showing the less differentiated and more aggressive phenotype among BC subtypes. Thus, the activation of STAT5 by enhanced IL-7Rα and IL-7 signaling caused by this genotype may promote phenotype changes associated with decrease of stem- and mesenchymal-cell markers in TN tumor cells and indicate a better prognosis. Indeed, lower expression of CD44, a major stem cell marker, in TN tumors is associated with better prognosis [30].

By the other side, *IL7RA* polymorphism was positively correlated with histopathological grade and lymph node metastasis in L-HER2$^+$ tumors and with KI67 in HER2-enriched BCs. Increasing in these prognosis parameters are often associated to epithelial-to-mesenchymal transition (EMT) in BC, a process by which epithelial cells lose their phenotypic differentiation and gain mesenchymal properties, becoming able to migrate and metasize [31]. Since both subgroups are characterized by HER2 overexpression, it could be of interest to study the possible cross-talking of IL-7Rα and HER2, searching for possible synergistic effect between them. PI3K activation is required for EMT [32], HER2$^+$ BCs have high PI3K activation mediated by HER2/HER3 heterodimer [33] and PI3K is associated with resistance to Trastuzumab treatment in HER2$^+$ BCs [34], making PI3K an attractive possible crossing-point between IL-7 and HER2 pathways.

5. Conclusions

In conclusion, the present results indicate, for the first time, that *IL7RA* Thr244Ile polymorphism is associated specifically with increased susceptibility for TN tumors, and may exert subgroup-specific roles in BC depending on the molecular pathways activated in each tumor microenvironment. These data shed lights on the comprehension of breast carcinogenesis and suggest this genetic variant and the IL-7Rα as interesting candidates for susceptibility and prognostic markers in BC to be further evaluated by prospective and retrospective studies with larger samples and different populations.

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The authors declare no conflicts of interest.

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3.2 Artigo 2: "Transforming growth factor beta 1 (TGFb1) polymorphisms and haplotype structures have dual roles in breast cancer pathogenesis"

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ORIGINAL ARTICLE – CANCER RESEARCH

Transforming growth factor beta 1 (TGFβ1) polymorphisms and haplotype structures have dual roles in breast cancer pathogenesis

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Abstract

Purpose Despite the documented dual role of TGFβ1 in breast cancer (BC) pathogenesis, the subtype-specific influences of its polymorphisms remain undocumented. The present study investigated the effects of the *TGFB1* promoter region (rs1800468 or G-800A and rs1800469 or C-509T) and signal peptide (rs1800470 or C29T and rs1800471 or G74C) single nucleotide polymorphisms (SNPs) and their haplotype structures on the susceptibility and clinicopathological presentation of BC subtypes.

Methods *TGFB1* genotypes were assessed by PCR-RFLP and haplotype structures were inferred for 323 BC patients and 405 neoplasia-free women, and case–control analyses were performed by logistic regression adjusted by age. Clinicopathological parameters (age at diagnosis, tumor size, histopathological grade, lymph node metastasis, proliferation index and disease stage) were tested for correlation with *TGFB1* variants. All statistical analyses were two-tailed with an alpha level of 0.05.

Results Variants related to increased TGFβ1 production (C-509T SNP and GTCG haplotype) were associated with increased susceptibility to $HER2^+$ tumors and correlated with worse prognostic parameters in $HER2^+$ and triple-negative (TN) BCs, but correlated negatively to Ki67 in ER/PR^+HER2^- tumors. Conversely, low TGFβ1 production variants (C29T SNP and GCTG haplotype) were protective against $HER2^+$ tumors and correlated negatively with prognostic parameters in $HER2^+$ and TN BCs, while indicating higher proliferation rates in ER/PR^+HER2^- tumors. Furthermore, the GCCG haplotype was associated with decreased susceptibility to ER/PR^+HER2^- tumors, but correlated positively with Ki67 in this subgroup.

Conclusion The present study indicates that *TGFB1* variants have subtype-specific roles in BC and may switch from tumor suppressor to promoter during tumor development, consistent with TGFβ1 dual role in BC pathogenesis.

Keywords Breast cancer · Subtypes · TGFβ1 · Polymorphisms · Haplotypes · Prognosis

Introduction

Transforming Growth Factor beta 1 (TGFβ1), the most abundant member of the TGFβ subfamily of growth factors (which also comprises TGFβ2 and TGFβ3), is a pleiotropic

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cytokine that exerts important functions during embryogenesis and in diverse physiological and disease contexts by coordinating cell cycle, apoptosis and differentiation (Blobe et al. 2000).

In cancer, the dual role of TGFβ1 has been extensively documented, and the term "TGFβ paradox" has been coined. It states that TGFβ is able to induce cell cycle arrest and apoptosis in initial or poorly aggressive neoplasia and in normal cells, but induces cell growth, motility and invasiveness by promoting epithelial-to-mesenchymal transition (EMT) and suppression of local immunosurveillance, favoring tumor progression, in advanced and highly invasive tumors (Bierie and Moses 2006; Yang et al. 2010).

In BC these paradoxical effects are evident among different subtypes. Good prognosis subtypes, such as Luminal A (LA) tumors, show cytostatic and pro-apoptotic TGFβ responses, while highly invasive subtypes, such as $HER2^{+}$ and triple-negative (TN) BCs, display enhanced aggressiveness upon TGFβ stimulation (Parvani et al. 2011; Wilson et al. 2005).

The *TGFB1* gene locates on 19q13.1 locus (*RefSeqGene* ID on NCBI Gene Bank: NG_013364.1). Several polymorphisms with functional implications have been described and studied for their contribution to cancer susceptibility in this gene (Martelossi Cebinelli et al. 2016). Among them, the single nucleotide polymorphisms (SNPs) rs1800468 (G-800A, g.4245G>A) and rs1800469 (C-509T, g.4536C>T), both located on the *TGFB1* promoter region, and rs1800470, on codon 10 (C29T, Pro10Leu, g.5911C>T), and rs1800471, on the codon 25 (G74C, Arg25Pro, g.5956G>C), both on the signal peptide sequence, have been widely studied in BC (Dunning et al. 2003; Jin et al. 2004; Niu et al. 2010; Pooja et al. 2013; Qiu et al. 2010).

Considering their location on essential regions for gene expression regulation and protein secretion, efforts have been made to identify possible influences of these polymorphisms on TGFβ1 production, and a role was identified for C-509T and C29T SNPs: Variant T from C-509T polymorphism might increase TGFβ1 levels (Grainger et al. 1999), while allele T from C29T downregulates TGFβ1 secretion (Dunning et al. 2003).

Regarding association studies, data remain controversial, and few studies have addressed association between *TGFB1* haplotype structures and BC and the effects of these variants on BC clinical outcome. Furthermore, despite previous research suggesting that low penetrance loci have subtype-specific associations with BC (Broeks et al. 2011), these polymorphisms were never investigated for susceptibility to different BC subtypes.

Therefore, the present study aimed to investigate possible effects of these four *TGFB1* variants and their haplotype structures on susceptibility and clinical presentation of BC subtypes.

Methods

Human subjects

Sample collection and study cohort were previously described in Vitiello et al. (2017). The research protocol was approved by the Ethics Committee for Research Involving Human Subjects of Londrina State University (CEP/UEL 189/2013—CAAE 17123113400005231) and all volunteer donors signed a free informed consent form prior to biological material collection.

Blood samples or surgical excision material tissues were obtained from 323 patients with breast cancer with clinical and pathological data available from the Londrina Cancer Hospital during the period of 2009–2012. Clinical staging was determined according to the Union for International Cancer Control (UICC) criteria. Pathological features analyzed included: tumor size, histopathological grade, proliferation index, p53 immunostaining and lymph node metastasis.

For subgroup analyses, samples were grouped according to their immunohistochemistry (IHC) profile for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) retrieved from patients' medical forms. IHC scores were assessed according to the American Society of Clinical Oncology (ASCO) recommendations. For HER2, samples with dubious IHC staining (grade II, in a scale ranging from 0 to III) were submitted to fluorescence in situ hybridization (FISH) analysis to check for *HER2* amplification.

For the control group, approximately 5 mL of peripheral blood was obtained from 405 women (collected from 2011 to 2015) with no signs of mammary alterations proved by recent (within the past 2 years) mammographic examination, no self-reported personal history of any cancer and no familial history of BC reported. Invitation, questionnaire and sample collection were carried out during routine medical exams at municipal primary health care unities and at Clinical Hospital from Londrina State University (HC-UEL).

Both BC and control populations were from the geographical region of Londrina, located in southern Brazil (Paraná State), and were attended by the Brazilian public health system (SUS). Due to high miscegenation observed in Brazilian population, it is not reliable to classify individuals into ethnic groups by physical examination or self-declaration of ethnicity for genetic studies (Pena et al. 2009; Pimenta et al. 2006). The use of ancestry-informative markers (AIMs) reveals that the majority of individuals of Brazilian population are admixed, showing varying proportions of Caucasoid, African and Amerindian ancestry (Naslavsky et al. 2017). Thus, for the purposes of the

present research, the study population was not stratified into ethnic groups. Londrina locates almost on the border between Southeastern and Southern regions of Brazil, which display the higher degree of European inheritance (Caucasoid ethnicity) among the Brazilian regions (Kehdy et al. 2015; Lins et al. 2009; Parra et al. 2003; Pena et al. 2009).

To validate our findings, haplotype distribution was compared between our control population and populations around the globe using data from 1000 Genomes project made freely available through LDlink application (https://analysistools.nci.nih.gov/LDlink/) (Machiela and Chanock 2015) and genotype frequencies from our control group were compared to those from a cohort of elderly healthy individuals from the city of São Paulo, located on the Southeastern region of Brazil, using data from the ABraOM database (http://abraom.ib.usp.br/) (Naslavsky et al. 2017).

DNA extraction

DNA was extracted from blood samples using Biopur Mini Spin kit (Biometrix Diagnóstica®, Curitiba, Paraná, Brazil). Tissues from surgical excision were mechanically macerated and DNA was obtained by salting-out method. DNA quantification was performed in a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific, Wilmington, DE, EUA) at 260 nm wavelength and the absorbance ratios at 260/280 and 260/230 nm were used to assess DNA purity.

Genetic polymorphisms genotyping

Genetic polymorphisms were analyzed by Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) analysis, according to that described by Jin et al. (2004) with modifications. All PCR reagents were purchased from Invitrogen™ (Carlsbad, CA, USA), and all restriction enzymes were from New England Biolabs® (Ipswich, USA).

Briefly, two primer pairs were designed based on *TGFB1* gene reference sequence (NCBI gene bank accession number NG_013364.1): one encompassing the two promoter region polymorphisms (G-800A and C-509T) and the other encompassing the signal peptide polymorphisms (C29T and G74C) (Jin et al. 2004). The PCR conditions for the two reactions were the same. Both reactions were performed in a final volume of 25 µL using 2.5 µL of 10× PCR buffer, dNTP (0.1 mM), 0.2 µM of primers, $MgCl_2$ (1.0 mM), Taq DNA polymerase (1U/reaction) and approximately 3 ng/µL of genomic DNA. PCR reactions were performed along with a negative control with no DNA addition to check for exogenous DNA contamination.

The sequences of the primers flanking the promoter region polymorphisms were: 5′-GCAGTTGGCGAGAACAGTTG-3′ and 5′-CCAGAACGGAAGGAGAGTCAG-3′, generating a 597 base-pairs (bp) amplicon (annealing temperature: 59 °C). The restriction enzyme *HpyCH4IV* was used for enzymatic restriction of G-800A polymorphisms generating 402 and 195 bp fragments for G allele and *Bsu36I* restriction enzyme was used for C-509T genotyping, generating 488 and 109 bp fragments for C allele. Restriction conditions followed the manufacturer's protocol.

Primer sequences for the signal peptide region were: 5′-TTCCCTCGAGGCCCTCCTA-3′ and 5′-GCCGCAGCTTGGACAGGATC-3′. Annealing temperature was set at 62 °C. *MspA1I* restriction enzyme was used to determine C29T genotypes, cleaving the 294 bp amplicon in 161, 67, 40 and 26 bp fragments for T allele, and in 149, 67, 40, 26 and 12 bp fragments for C allele. *BglI* restriction enzyme cleaved this same amplicon in 131, 103 and 60 bp fragments for G allele from G74C polymorphism and in 163 and 131 bp fragments for C allele. Restriction conditions followed the manufacturer's instruction.

Amplicons and restriction fragments were analyzed by electrophoresis on polyacrylamide gel (10%) visualized after silver staining. To validate the technique, one heterozygous and one variant homozygous sample for each polymorphism was sequenced in a Sanger-based platform (Applied Biosystems® 3500 Genetic Analyzer) and PCR-RFLP was repeated for at least 5% of total sample obtaining 100% of concordance.

Haplotype analysis

The *TGFB1* haplotypes were inferred based on the genotypes of all study participants using PHASE software version 2.1.1 (Stephens and Scheet 2005; Stephens et al. 2001). Permutation test was also performed in this software to check for haplotype distribution differences among controls and BC subgroups. Haploview 4.2 (Barrett et al. 2005) was used to analyze the linkage disequilibrium (LD) between the *TGFB1* polymorphisms.

Statistical analyses

Binary logistic regression analyses were performed to investigate associations between polymorphisms or haplotype structures and BC adjusted by age (age at diagnosis for patients and age at sample collection for controls) setting case-control status as dependent variable and models of association and age as explanatory variables in forced entry method.

Associations were tested considering genotypic models (heterozygotes or variant homozygotes vs. wild homozygotes); dominant model (heterozygotes and variant homozygotes vs. wild homozygotes) and recessive model (variant homozygotes vs. wild homozygotes and heterozygotes).

Additive model was tested by the Cochran–Armitage test (χ^2 test for trend).

Correlations between polymorphisms or haplotypes structures and clinical parameters were assessed by Kendall's tau-b rank correlation coefficient.

P value adjustments for multiple testing were not applied due to the high dependency between studied polymorphisms (which are inherited as haplotype blocks), between the groups tested (BC subtypes, which are derived from general BC group, were tested always against the same control group) and between clinicopathological parameters analyzed.

All statistical analyses were performed in IBM® SPSS® Statistics 20 software (IBM®, Armonk, New York, USA). All tests were two-tailed with significance level set at 0.05.

Results

Sample characterization

Four BC groups were defined according to ER, PR and HER2 immunohistochemical status: Luminal-HER2$^-$ (L-HER2$^-$; ER/PR$^+$ HER2$^-$), Luminal B-HER2$^+$ (LB-HER2$^+$; ER/PR$^+$ HER2$^+$), HER2 enriched (HER2; ER$^-$ PR$^-$ HER2$^+$) and Triple-negative (TN; ER$^-$ PR$^-$ HER2$^-$). Patient's clinicopathological characteristics divided by subgroups are summarized in Table 1.

Control and BC samples were tested for the Hardy–Weinberg equilibrium and no deviation from expected genotype distributions were found for any genetic variant ($p > 0.05$). Linkage disequilibrium statistics are shown in table S1 (Supplemental information). Among the studied polymorphisms, C-509T and C29T showed the highest linkage disequilibrium ($D' = 0.93$; $r^2 = 0.56$).

The frequencies of the four most common *TGFB1* haplotype structures were compared between the control group and diverse populations around the world using the publicly available data from the 1000 genome project obtained through the web-based application LDlink (Machiela and Chanock 2015). Haplotype frequencies differed significantly ($p < 0.05$ by χ^2 test) from our control group to any population analyzed (data not shown).

We also compared genotype frequencies for each polymorphism in our control group to those from a cohort of elderly healthy subjects from the city of São Paulo, Brazil, whose data is freely available on the Online Archive of Brazilian Mutations (ABraOM: http://abraom.ib.usp.br/). We found no significant differences for G-800A (AbraOM frequencies: GG = 88.7%; GA = 10.8%; AA = 0.5%; $p > 0.05$), C29T (CC = 22.0%; CT = 45.5%; TT = 32.5%; $p > 0.05$) and G74C (GG = 86.8%; GC = 12.2%; CC = 1.0%; $p > 0.05$). C-509T polymorphism did not reach threshold for local depth of coverage in ABraOM analyses and could not be evaluated.

TGFB1 polymorphisms and BC susceptibility

Case-control association studies were performed to assess possible influences of *TGB1* polymorphisms on the susceptibility for BC subgroups. Table 2 shows the genotype distributions and the *p* values for the χ^2 test for trend (additive model). Only one sample (from the L-HER2$^-$ subgroup) could not be amplified for promoter region polymorphisms, and was excluded from analyses involving these polymorphisms. The HER2$^+$ column refers to HER2-positive samples irrespective of ER and PR status (ER$^{+/-}$ PR$^{+/-}$ HER2$^+$).

G74C polymorphism was associated with increased risk for HER2$^+$ cancers in the additive model, while C29T polymorphism was a protective factor. The effects of C29T and G74C were evidenced in HER2-enriched subtype but were not verified in LB-HER2$^+$.

Table 3 summarizes the association analyses. C-509T polymorphism showed a significant positive association for general BC in the dominant model (OR 1.51). This effect was greater in HER2$^+$ (OR 2.16) and in LB-HER2$^+$ tumors (OR 2.28). Otherwise, C29T polymorphism exerted a protective role in a recessive manner in HER$^+$ BCs (OR 0.39). C allele from G74C increased the risk for HER2$^+$ BC in a recessive manner (OR 11.37), but this result should be interpreted carefully given the large confidence interval observed due to the relatively small size of the HER2$^+$ group and the rarity of the G74C C allele, which severely decreases the statistical power of this analysis.

TGFB1 haplotype structures and BC susceptibility

A total of eight possible haplotype structures were represented in the present sample. Table 4 shows the haplotype allele distribution among the studied groups. Individual SNPs are represented sequentially in haplotype structures from G-800A to the G74C locus (left to right).

Although no significant difference was verified between BC subgroups and the control group at 0.05 level of significance, the HER2$^+$ group showed the greatest difference in haplotype distribution compared to the control ($p = 0.1$) (Table 4).

To characterize associations between individual haplotype structures and BC subtypes, age-adjusted logistic regression analyses were performed. Haplotypes with counts below 5% in the control group (GTTG, GCTC and ATCG) were excluded from these analyses. The dominant model was tested for all the haplotypes and the recessive model was also tested for the two most frequent haplotypes (GCTG and GTCG) (Table 5).

Table 1 Clinicopathological features of BC patients

Parameter	L-HER2$^-$ ($n=213$)	LB-HER2$^+$ ($n=42$)	HER2 ($n=24$)	TN ($n=44$)	Control ($n=405$)	
Age (years)						
Median (IQ range)	54 (18)	48 (14)	51 (13.5)	51 (21.5)	55 (15.5)	
Mean (SD)	55.6 (12.6)	50.1 (12.2)	53.0 (14.3)	52.7 (13.8)	53.9 (14.0)	
<40 [*n* (%)]	19 (9.0)	7 (16.7)	3 (12.5)	8 (18.2)	60 (14.8)	
40–49 [*n* (%)]	60 (28.3)	16 (38.1)	7 (29.2)	12 (27.3)	58 (14.3)	
50–59 [*n* (%)]	52 (24.5)	10 (23.8)	8 (33.3)	10 (22.7)	150 (37.0)	
60–69 [*n* (%)]	47 (22.2)	7 (16.7)	4 (16.7)	8 (18.2)	93 (23.0)	
70–79 [*n* (%)]	27 (12.7)	1 (2.4)	1 (4.2)	4 (9.1)	32 (7.9)	
> 80 [*n* (%)]	7 (3.3)	1 (2.4)	1 (4.2)	2 (4.5)	12 (3.0)	
Unknown [*n* (%)]	1	0	0	0	0	
Histological class [*n* (%)]						
IDC	196 (91.6)	40 (95.2)	21 (87.5)	42 (95.4)	–	
ILC	10 (4.7)	0 (0.0)	1 (4.2)	1 (2.3)		
DCIS	5 (2.3)	1 (2.38)	2 (8.4)	0 (0.0)		
LCIS	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)		
Other	2 (0.9)	1 (2.38)	0 (0.0)	1 (2.3)		
Tumor size [*n* (%)]						
0–1.5 cm	30 (14.2)	4 (9.8)	2 (9.1)	5 (11.4)	–	
1.51–3.0 cm	112 (53.1)	18 (43.9)	12 (54.5)	11 (25.0)		
> 3.0 cm	69 (32.7)	19 (46.3)	8 (36.4)	28 (63.3)		
Undocumented	2	1	2	0		
Histopathological grade [*n* (%)]						
I	35 (17.5)	0 (0.0)	0 (0.0)	0 (0.0)	–	
II	101 (50.5)	14 (34.1)	7 (33.3)	6 (14.0)		
III	64 (32.0)	27 (65.9)	14 (66.7)	37 (86.0)		
Unknown	13	1	3	1		
Ki67 [*n* (%)]						
Low	49 (33.1)	4 (16.0)	0 (0.0)	1 (3.0)	–	
Intermediate	74 (50.0)	11 (44.0)	5 (38.5)	8 (24.2)		
High	25 (16.9)	10 (40.0)	8 (61.5)	24 (72.7)		
Unknown	65	17	11	11		
p53 mutation [*n* (%)]						
Positive	36 (23.1)	14 (58.3)	11 (64.7)	22 (59.5)	–	
Negative	120 (76.9)	10 (41.7)	6 (35.3)	15 (40.5)		
Unknown	57	18	7	7		
Lymph node metastasis [*n* (%)]						
Positive	100 (47.8)	18 (46.2)	11 (50.0)	24 (54.4)	–	
Negative	109 (52.2)	21 (53.8)	11 (50.0)	20 (45.5)		
Unknown	4	3	2	0		
Tumor stage [*n* (%)]						
0	6 (3.4)	1 (3.0)	2 (10.0)	0 (0.0)	–	
I	41 (23.5)	3 (9.1)	1 (5.0)	5 (11.9)		
II	79 (44.4)	14 (42.4)	6 (30.0)	18 (42.9)		
III	41 (23.0)	12 (36.4)	9 (45.0)	17 (40.5)		
IV	11 (6.2)	3 (9.1)	2 (10.0)	2 (4.8)		
Unknown	35	9	4	2		

IDC invasive ductal carcinoma, *ILC* invasive lobular carcinoma, *DCIS* ductal carcinoma in situ, *LCIS* lobular carcinoma in situ

Table 2 Genotypes distribution among BC subgroups and additive model testing

Genotype	Subgroups [*n* (%)]						
	Control ($n=405$)	BC ($n=323$)	L-HER2$^-$ ($n=213$)	LB-HER2$^+$ ($n=42$)	HER2 ($n=24$)	HER2$^+$ ($n=66$)	TN ($n=44$)
G-800A							
GG	358 (88.4)	285 (88.5)	190 (89.6)	37 (88.1)	22 (91.7)	59 (89.6)	36 (81.82)
GA	45 (11.1)	36 (11.2)	21 (9.9)	5 (11.9)	2 (8.3)	7 (10.4)	8 (18.18)
AA	2 (0.5)	1 (0.3)	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
p trend	–	0.906	0.661	0.972	0.596	0.739	0.269
C-509T							
CC	177 (43.7)	113 (35.1)	80 (37.7)	12 (28.6)	7 (29.2)	19 (29.8)	14 (31.8)
CT	173 (42.7)	161 (50.0)	101 (47.6)	24 (57.1)	12 (50.0)	36 (53.7)	24 (54.6)
TT	55 (13.6)	48 (14.9)	31 (14.6)	6 (14.3)	5 (20.8)	11 (16.4)	6 (13.6)
p trend	–	0.06	0.233	0.158	0.137	0.051	0.277
C29T							
CC	77 (19.0)	73 (22.6)	49 (23.0)	7 (16.7)	8 (33.3)	15 (22.4)	9 (20.4)
CT	216 (53.3)	177 (54.8)	111 (52.1)	30 (71.4)	13 (54.2)	43 (64.2)	23 (52.3)
TT	112 (27.7)	73 (22.6)	53 (24.9)	5 (11.9)	3 (12.5)	8 (13.4)	12 (27.3)
p trend	–	0.087	0.243	0.215	0.039*	0.03*	0.866
G74C							
GG	352 (86.9)	269 (83.3)	176 (82.6)	35 (83.3)	18 (75.0)	53 (79.1)	40 (90.9)
GC	51 (12.6)	49 (15.2)	35 (16.4)	6 (14.3)	4 (16.7)	10 (16.4)	4 (9.1)
CC	2 (0.5)	5 (1.5)	2 (0.9)	1 (2.4)	2 (8.3)	3 (4.5)	0 (0.0)
p trend	–	0.107	0.138	0.358	0.013*	0.038*	0.421

*$p<0.05$

**$p<0.01$ by χ^2 test for trend

Two haplotypes were significantly associated with general BC: GTCG and GCCG; the former was identified as a risk factor (OR 1.4), and the latter as a protective factor (OR 0.52). The GCCG haplotype was also identified as a protective factor against L-HER2$^-$ tumors (OR 0.49). GCTG was protective against HER2$^+$ BCs both in the recessive (OR 0.35) and dominant model (OR 0.51); but GTCG was identified as a risk haplotype for HER2$^+$ cancers (OR 1.89) (Table 5).

Impact of *TGFB1* variants on BC clinical presentation

The possible impact of the studied polymorphisms and haplotype structures on BC clinical presentation was evaluated. For these analyses, all clinicopathological parameters were categorized as shown in Table 1. Table 6 shows the Tau-b coefficient value for significant correlations. For simplicity, only clinical parameters that were correlated with at least one genetic variant analyzed in any subgroup are shown and nonsignificant Tau-b coefficients have been omitted.

Some *TGFB1* variants had different and even paradoxical effects when L-HER2$^-$ was compared to HER2$^+$ or TN BCs. This is evidenced by the fact that variants indicating better prognosis parameters in L-HER2$^-$, such as C-509T and the GTCG haplotype, correlated negatively with the proliferation index (Ki67), indicated poor prognostic parameters in HER2$^+$ (LN metastasis) and TN BCs (LN metastasis and larger tumor size). G-800A correlated positively with tumor stage in HER2$^+$ samples and with histopathological grade in TN BCs. C29T correlated negatively with lymph node metastasis in HER2$^+$ and TN BCs, and with tumor size in TN cancers. G74C polymorphism indicated early age at diagnosis in HER2$^+$ subgroup and larger tumors in HER2$^+$ and TN patients. Globally, haplotype structures reflected the correlation results shown by individual SNPs represented by them.

LN metastasis is a severe outcome that occurs in later stages of BC development when cells acquire the capability to migrate to draining lymph nodes. Thus, we next checked whether correlations found between *TGFB1* variants and LN metastasis were independent of other clinicopathological parameters. First, we identified that tumor size was associated with LN metastasis in all BC groups (Table S2). Next, we performed logistic regression analyses between each identified risk polymorphism (Table 6) and LN metastasis adjusting by tumor size in HER2$^+$ and TN BCs. These analyses identified the association between C-509T recessive model in HER2$^+$ BCs to be independent of tumor size [factors in the regression model: C-509T (OR 12.83, $p=0.027$); Tumor size ($p=0.04$)], whereas

Table 3 Case–control association analyses in the breast cancer subgroups

Model	Breast cancer subgroups [OR (CI 95%)][a]					
	General BC	L-HER2$^-$	LB-HER2$^+$	HER2	HER2$^+$	TN
G-800A						
GA vs. GG	0.90 (0.56–1.46)	0.77 (0.43–1.35)	1.05 (0.38–2.9)	0.67 (0.15–3.01)	0.89 (0.37–2.12)	1.66 (0.71–3.88)
AA vs. GG	0.85 (0.08–9.5)	1.25 (0.11–14.0)	NA	NA	NA	NA
Dominant	0.90 (0.56–1.45)	0.78 (0.45–1.37)	1.02 (0.37–2.8)	0.65 (0.14–2.89)	0.86 (0.36-2.0)	1.61 (0.69–3.75)
Recessive	0.85 (0.08–9.54)	1.28 (0.11–14.4)	NA	NA	NA	NA
C-509T						
CT vs. CC	1.51* (1.09–2.10)	1.32 (0.91–1.91)	2.28* (1.08–4.80)	1.80 (0.68–4.71)	2.16* (1.16–4.01)	1.83 (0.90–3.69)
TT vs. CC	1.43 (0.90–2.28)	1.26 (0.74–2.13)	1.78 (0.62–5.09)	2.39 (0.72–7.94)	2.29 (0.99–5.35)	1.36 (0.49–3.75)
Dominant	1.49* (1.10–2.04)	1.30 (0.92–1.85)	2.16* (1.05–4.42)	1.94 (0.78–4.82)	2.11* (1.18–3.80)	1.71 (0.87–3.35)
Recessive	1.15 (0.75–1.76)	1.09 (0.67–1.78)	1.10 (0.43–2.79)	1.71 (0.61–4.82)	1.36 (0.66–2.81)	0.96 (0.39–2.42)
C29T						
CT vs. CC	0.84 (0.57–1.24)	0.82 (0.53–1.26)	1.35 (0.56–3.27)	0.52 (0.20–1.33)	0.90 (0.46–1.74)	0.88 (0.39–2.01)
TT vs. CC	0.68 (0.44–1.06)	0.77 (0.47–1.26)	0.49 (0.15–1.61)	0.26* (0.06–0.99)	0.37* (0.15–0.93)	0.90 (0.36–2.26)
Dominant	0.79 (0.54–1.14)	0.80 (0.53–1.22)	1.07 (0.45–2.54)	0.43 (0.18–1.06)	0.70 (0.37–1.34)	0.89 (0.41–1.94)
Recessive	0.77 (0.55–1.09)	0.88 (0.60–1.30)	0.38 (0.14–1.01)	0.40 (0.12–1.37)	0.39* (0.18–0.84)	0.99 (0.49-2.0)
G74C						
GC vs. GG	1.24 (0.80–1.92)	1.38 (0.85–2.24)	1.12 (0.44–2.86)	1.49 (0.48–4.68)	1.22 (0.57–2.59)	0.75 (0.25–2.20)
CC vs. GG	3.16 (0.59–16.8)	1.827 (0.24–13.8)	4.89 (0.36–67.5)	35.34** (3.9-320.6)	11.9* (1.75-81.0)	NA
Dominant	1.31 (0.86–2.01)	1.40 (0.87–2.25)	1.25 (0.52–3.04)	2.26 (0.84–6.05)	1.55 (0.77–3.09)	0.71 (0.24–2.09)
Recessive	3.06 (0.58–16.3)	1.74 (0.23–13.1)	4.53 (0.32–63.8)	33.57* (3.7-302.6)	11.37* (1.65–78.2)	NA

*$p < 0.05$

**$p < 0.01$

[a]OR and CI 95% estimated by binary logistic regression controlling by age

Table 4 Haplotype distribution among control and breast cancer groups

Haplotype structure[a]	Haplotype count [*n* (%)]				
	Control	General BC	L-HER2$^-$	HER2$^+$	TN
GCCG	42 (5.18)	21 (3.26)	14 (3.30)	6 (4.54)	1 (1.14)
GCCC	52 (6.42)	54 (8.38)	38 (8.96)	12 (9.09)	4 (4.54)
GCTG	384 (47.41)	272 (42.24)	187 (44.10)	47 (35.61)	39 (44.32)
GCTC	3 (0.37)	5 (0.78)	1 (0.24)	3 (2.27)	0 (0)
GTCG	273 (33.70)	243 (37.73)	153 (36.08)	55 (41.67)	36 (40.91)
GTTG	7 (0.86)	11 (1.71)	8 (1.89)	3 (2.27)	0 (0)
ACTG	46 (5.68)	35 (5.43)	21 (4.95)	5 (3.79)	8 (9.09)
ATCG	3 (0.37)	3 (0.47)	2 (0.47)	1 (0.76)	0 (0)
Total	810	644	424	132	88
p value	–	0.16	0.71	0.1	0.27

[a]SNP alleles are represented in haplotype structures in the following order: G-800A, C-509T, C29T and G74C

other genetic variants did not show statistical significance in adjusted models.

Discussion

TGFβ is a crucial and paradoxical cytokine in BC pathogenesis: while it regulates cell cycle and apoptosis in early and less aggressive tumors, in advanced carcinomas its expression in tumor milieu is associated with local immunosuppression (Yang et al. 2010), EMT (Parvani et al. 2011), generation of BC stem cells, which are responsible for resistance to treatment and relapses (Asiedu et al. 2011), and the coordination of chemokines and chemokine receptors expression in tumor cells, which are responsible for targeted-organ metastases (Bierie et al. 2009; Pang et al. 2015). For this reason, therapies targeting TGFβ1 signaling in tumor and immunologic cells have been hypothesized, developed and tested in pre-clinical settings (Bai et al. 2014; Bhola et al. 2013).

The polymorphisms included in this study are located on the promoter region and signal peptide sequence of the *TGFB1* gene, and might influence its expression or protein secretion. It was shown that C-509T polymorphism occurs within a consensus binding sequence for the transcription factor Yin Yang 1 (YY1) diminishing its' ligation affinity (Silverman et al. 2004) and that T allele carriers had higher

Table 5 Recessive and dominant association models for haplotype structures

Model	Breast cancer subgroups [OR (CI 95%)][a]			
	General BC	L-HER2$^-$	HER2$^+$	TN
Recessive				
GCTG	0.81 (0.55–1.19) $p=0.279$	0.89 (0.58–1.38) $p=0.61$	0.35 (0.13–0.9) $p=0.029$*	1.34 (0.64–2.80) $p=0.44$
GTCG	1.10 (0.7–1.73) $p=0.689$	1.08 (0.65–1.81) $p=0.764$	1.18 (0.54–2.58) $p=0.679$	0.9 (0.34–2.43) $p=0.84$
Dominant				
GCTG	0.74 (0.53–1.03) $p=0.076$	0.89 (0.61–1.31) $p=0.56$	0.51 (0.29–0.90) $p=0.02$*	0.6 (0.31–1.16) $p=0.131$
GTCG	1.40 (1.03–1.90) $p=0.032$*	1.20 (0.85–1.70) $p=0.299$	1.89 (1.07–3.35) $p=0.028$*	1.8 (0.92–3.52) $p=0.088$
GCCC	1.30 (0.84–2.00) $p=0.243$	1.41 (0.87–2.29) $p=0.166$	1.38 (0.66–2.86) $p=0.392$	0.78 (0.26–2.29) $p=0.64$
ACTG	0.93 (0.57–1.51) $p=0.776$	0.82 (0.46–1.44) $p=0.485$	0.77 (0.31–1.92) $p=0.572$	1.72 (0.74–4.02) $p=0.207$
GCCG	0.52 (0.29–0.94) $p=0.029$*	0.49 (0.24–0.97) $p=0.042$*	0.82 (0.32–2.06) $p=0.666$	0.19 (0.03–1.48) $p=0.113$

*$p<0.05$

**$p<0.01$

[a]OR and CI 95% estimated by binary logistic regression controlling by age

Table 6 Correlation between polymorphisms and clinicopathological parameters

Model by SNP or haplotype	L-HER2$^-$	HER2$^+$					TN		
	Ki67	Age	Size	Hist. Gr.	LN	Stage	Size	Hist. Gr.	LN
G-800A									
Genotypic	–	–	–	–	–	0.25*	–	–	–
Dominant	–	–	–	–	–	–	–	0.19*	–
C–509T									
Genotypic	−0.21**	–	–	–	–	–	–	–	0.29*
Dominant	−0.22**	–	–	–	–	–	0.33*	–	–
Recessive	–	–	–	–	0.34**	–	–	–	–
C29T									
Genotypic	–	–	–	–	–	–	−0.27*	–	−0.29*
Dominant	–	–	–	–	-0.31*	–	–	–	–
G74C									
Genotypic	–	−0.22*	0.27*	–	–	–	0.23*	–	–
Dominant	–	−0.23*	0.28*	–	–	–	0.23*	–	–
Haplotypes									
GCTG Rec	–	–	–	–	–	–	–	–	−0.32*
GCTG Dom	–	–	−0.25*	–	–	−0.24*	–	–	–
GTCG Rec	–	–	–	–	0.38**	–	–	–	–
GTCG Dom	−0.19*	–	–	0.31*	–	–	0.33*	–	–
GCCC Dom	–	−0.24*	–	–	–	–	0.23*	–	–
GCCG Dom	0.18*	–	–	0.21*	–	–	–	–	–
ACTG Dom	–	–	–	–	–	–	–	0.19*	–

*$p<0.05$

**$p<0.01$ estimated by Kendall's Tau-b rank correlation test

TGFβ1 levels compared to CC homozygotes (Grainger et al. 1999).

Regarding C29T polymorphism, C allele-transfected cells had approximately threefold higher secretion activity than cells transfected with T allele (Dunning et al. 2003) and later bioinformatics analyses indicated that this SNP modifies the affinity of the TGFβ1 signal peptide sequence to translocation complexes in endoplasmic reticulum membrane (Susianti et al. 2014).

For G-800A and G74C polymorphisms, there are few data indicating their impact on TGFβ1 production. However, G-800A polymorphism is located on a putative consensus CREB half-site and might reduce affinity for the CREB family of transcription factors (Grainger et al. 1999).

Association studies investigating *TGFB1* variants in BC revealed controversial results in the literature. Evidence for subtype-specific actions of TGFβ1 in BC have been long recognized (Parvani et al. 2011; Wilson et al. 2005), and previous research suggests that low-penetrant variants may have subtype-specific roles (Broeks et al. 2011). However, studies evaluating associations between *TGFB1* polymorphisms and BC have rarely checked for subtype-specific associations. Furthermore, the diverse genetic background observed among studied populations around the globe may also be a source of these conflicting results.

Our control group differed significantly in *TGFB1* haplotype distribution from all the populations presented in the 1000 Genomes Project data, which do not include any Brazilian population. Previous research also showed that the frequency of these haplotypes was different among populations from different countries, even on the same continent and belonging to the same ethnic group (Caucasoid) (Jin et al. 2004). When our control group was compared to a cohort from São Paulo, Brazil, (Naslavsky et al. 2017) no significant difference was found. Furthermore, is it emphasized that our control group was composed by individuals with no documented familial history of BC and no personal history of any cancer, which reasonably may increase the population power to find genetic contributors to BC risk, but do not necessarily reflect the distribution of these haplotypes in general population.

The present study demonstrated a positive association between the C-509T T allele and general BC in the dominant model and a stronger association for $HER2^+$ BCs in the same model. A meta-analysis concluded that T allele form C-509T SNP was modestly protective against BC (Niu et al. 2010); however, to reach this significance, the authors excluded a study comprising three large European populations which demonstrated the T allele to be significantly associated with increased BC risk, which is in accordance with our results (Dunning et al. 2003). Of note, none of the individual studies reported in this meta-analysis showed any protective effect of C-509T.

Regarding C29T polymorphism, a meta-analysis observed a modest increase in BC risk for C allele in the dominant model, particularly in a Caucasoid population (Qiu et al. 2010). Our results demonstrated the T allele as a protective factor for $HER2^+$ tumors in the additive and recessive models, and are somewhat concordant with those from this meta-analysis. Differences among wild genotype defined for analyses occurred because we considered C as the ancestral allele according to the information available in the NCBI SNP bank, while previous literature considers T as the ancestral allele.

There are few available data regarding G74C polymorphism. Jin et al. (2004) reported no association between this polymorphism and BC, whereas Pooja et al. (2013) observed a protective effect for the C allele in north Indian pre-menopausal women. The present study showed an increased susceptibility for $HER2^+$ tumors, especially HER2-enriched, associated with the C allele in recessive and additive models. However, due to the rarity of this allele a larger cohort will be necessary to confirm this association.

Haplotype association analyses revealed that the low producer GCTG haplotype conferred significant protection against $HER2^+$ BC both in the dominant and recessive models, whereas the high-producer GTCG haplotype increased the risk for general and $HER2^+$ BCs in the dominant model. For TN BCs a tendency was observed towards positive association for this same structure (OR 1.8; $p = 0.088$). These results indicate that the association found for this haplotype with general BC might be due to its high prevalence on $HER2^+$ and, possibly, TN groups, that are highly aggressive tumors in which TGFβ1 signaling promotes tumorigenesis (Bhola et al. 2013; Wilson et al. 2005).

On the other hand, the GCCG haplotype showed a protective effect against general BC and L-$HER2^-$ BCs, but had no effect on the susceptibility to $HER2^+$ or TN BCs, suggesting that the effect of this haplotype was restricted to poorly aggressive BC subtypes. These results reinforce the need to study subtype-specific associations for low penetrance genetic variants in BC, as suggested by previous research (Broeks et al. 2011).

Indicating that *TGFB1* variants have important roles in BC prognosis, *TGFB1* C-509T and C29T SNPs were respectively associated to reduced and increased disease free survival of BC patients in a large cohort study in a Chinese population (Shu 2004). In the present study, we tested for possible impacts of *TGFB1* genetic variants on the clinical presentation of BC subgroups.

Our study indicated that in $HER2^+$ and TN BCs TGFβ1 high-producer genotypes (C-509T T allele and GTCG haplotype) positively correlated with LN metastasis, whereas low TGFβ1-producer variants (C29T T allele and GCTG haplotype) showed the opposite trend. TGFβ1-induced EMT promotes the migration of tumor cells to the lymph nodes

by upregulation of CCR7, a mechanism which also depends on the activity of p38 Mitogen-Activated Protein Kinase (MAPK) (Pang et al. 2015). Thus, the observed effect of these variants might be explained by this mechanism, since these tumors display enhanced activation of MAPK pathways (Parvani et al. 2011). Indeed, it has been described for $HER2^+$ BCs that the cross-talk between TGFβ and HER2 signaling pathways lead to a phenomenon called "hyper-EMT", by which a hyperactivation of the MAPK pathway lead to exacerbation of the EMT process, resulting in accelerated tumor progression and resistance to therapy (Chow et al. 2011; Wang 2011).

Conversely, tumor size was negatively correlated with low producer variants (C29T and GCTG) while positively correlated with high-producer alleles (C-509T and GTCG) in $HER2^+$ and TN BCs; while in the L-$HER2^-$ group, high-producer variants negatively influenced proliferation and low producer alleles correlated positively with it. Additionally, high-producer haplotype GTCG correlated with poor differentiation in $HER2^+$ BCs, while the low producer allele GCTG was indicative of lower disease stage in this same group. This scenario is consistent with TGFβ1 exerting cytostatic effects in poorly aggressive ER/PR^+ $HER2^-$ tumors, while promoting growth and EMT in more aggressive cancers and is also supported by a previous report which showed that the high TGFβ1 producer C29T C allele was associated with decreased risk for low stage (0 and I) tumors in a general BC sample, but was overrepresented in advanced stage (III and IV) cases (Shin et al. 2005).

Variants with a less clear functional role (such as G-800A and G74C and their derivate haplotype structures, GCCC and ACTG) were indicative of worst prognosis parameters (higher tumor stage, higher histopathological grade, larger tumors and earlier age of disease onset) in $HER2^+$ and TN BCs. The study of the impact of these variants in TGFβ1 production as well as observational studies with larger cohorts of BC patients are necessary to elucidate the possible role of these variants.

It is also important to note that increased susceptibility variants for $HER2^+$ BCs were also positively correlated with worst prognosis in this group, while the protective variants suggested better prognosis for these tumors, indicating that TGFβ1 may be important for both their initiation (probably favoring growth of cells expressing HER2 in a heterogeneous tissue) and progression. Otherwise, in L-$HER2^-$ BCs the GCCG haplotype was protective, but positively correlated with Ki67 staining in established tumors, indicating that as these tumors evolve, cells may change their response to TGFβ1.

In conclusion, the present study indicated that *TGFB1* allelic variants seem to act in a subtype-specific manner in BC, and that their roles are consistent with TGFβ1 roles in this neoplasia (Figure S1). These results, along with previous data in the literature, give further evidence of TGFβ1 relevance in breast neoplasia, and indicate this cytokine and its genetic variants as promising candidates for susceptibility and prognosis markers in breast cancer.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Supplemental information
Journal of Cancer Research and Clinical Oncology

Transforming growth factor beta 1 (TGFβ1) polymorphisms and haplotype structures have dual roles in breast cancer pathogenesis

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Table S1 - Linkage disequilibrium analyses.

Polymorphisms	D'	LOD	r^2
G-800A x C-509T	0.705	3.32	0.019
G-800A x C29T	0.76	5.75	0.034
G-800A x G74C	0.991	0.79	0.005
C-509T x C29T	0.927	126.79	0.56
C-509T x G74C	1.0	15.5	0.051
C29T x G74C	0.812	9.91	0.062

Table S2 - Tau-b rank correlation coefficient between LN metastasis and indicated clinicopathological parameters among BC subgroups.

Parameter	General BC	L-HER2$^-$	HER2$^+$	TN
Age	-0.03 (p = 0.52)	-0.01 (p = 0.90)	-0.11 (p = 0.36)	-0.03 (p = 0.85)
Tumor size	0.26 (p < 0.001)	0.21 (p = 0.001)	0.33 (p = 0.005)	0.38 (p = 0.004)
Hist. grade	0.06 (p = 0.27)	0.09 (p = 0.16)	0.08 (p = 0.53)	-0.11 (p = 0.48)
Ki67	0.13 (p = 0.043)	0.20 (p = 0.01)	-0.15 (p = 0.34)	0.04 (p = 0.80)
p53	0.064 (p = 0.34)	0.l2 (p = 0.13)	-0.09 (p = 0.58)	-0.16 (p = 0.30)

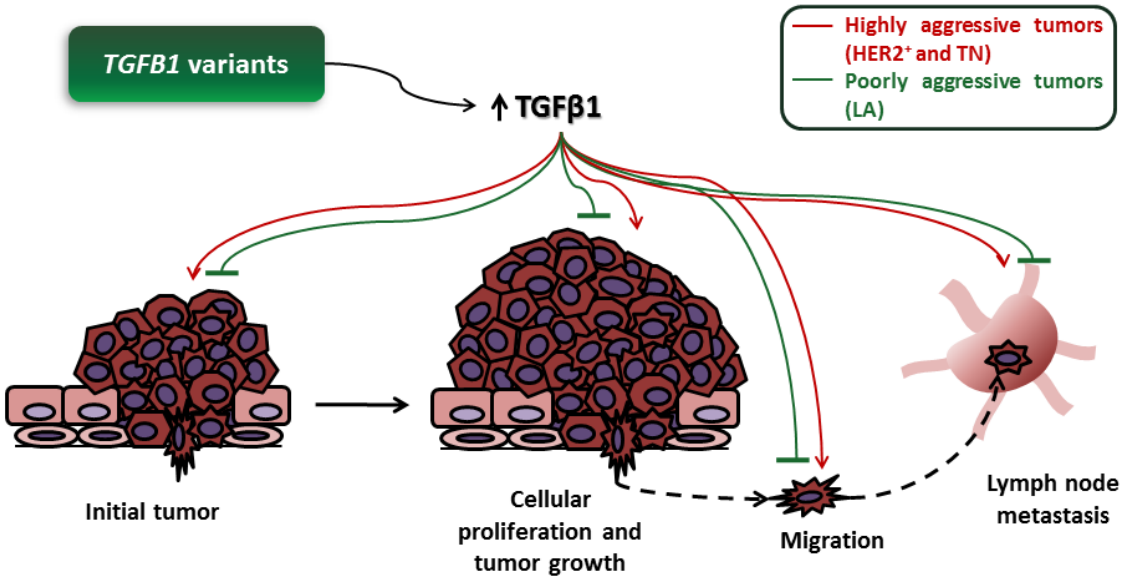

Figure S1. Subtype-specific actions of *TGFB1* variants involved in TGFβ1 production in BC pathogenesis. The dual role of TGFβ1 in breast cancer has been proposed. In the initial stage or weakly aggressive disease, TGFβ1 can induce cell cycle arrest and apoptosis. However, in more advanced disease and highly invasive microenvironment this cytokine induces cell growth, invasiveness and motility promoting tumor progression. *TGFB1* genetic variants related to TGFβ1 production seems to act in the same manner during BC pathogenesis.

3.3 ARTIGO 3: "TRANSFORMING GROWTH FACTOR BETA RECEPTOR II (TGFBR2) PROMOTER REGION POLYMORPHISM IN BRAZILIAN BREAST CANCER PATIENTS: ASSOCIATION WITH SUSCEPTIBILITY, CLINICOPATHOLOGICAL FEATURES, AND INTERACTION WITH TGFB1 HAPLOTYPES"

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EPIDEMIOLOGY

Transforming growth factor beta receptor II (*TGFBR2*) promoter region polymorphism in Brazilian breast cancer patients: association with susceptibility, clinicopathological features, and interaction with *TGFB1* haplotypes

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Abstract

Purpose Transforming growth factor beta (TGFβ) has paradoxical effects in breast cancer (BC), inhibiting initial tumors while promoting aggressive ones. A polymorphism on *TGFBR2* promoter region (G-875A, rs3087465) increases TGFβ type II receptor expression and is protective against cancer. Previously, we have shown that *TGFB1* variants have subtype-specific roles in BC. This work sought to investigate the association between *TGFBR2* and susceptibility and clinicopathological features in BC subgroups.

Methods *TGFBR2* G-875A was analyzed through PCR-RFLP in 388 BC patients and 405 neoplasia-free women. Case–control analyses as well as interaction with *TGFB1* haplotypes previously associated with BC were tested through age-adjusted logistic regression. Correlations between G-875A and clinicopathological parameters were assessed through Kendall's Tau-b test. All statistical tests were two-tailed ($\alpha = 0.05$).

Results *TGFBR2* G-875A was protective against BC in additive, genotypic, and dominant models. In subgroup-stratified analyses, these effects were greater in hormonal receptor-positive and luminal-A tumors, but were not significant in other subgroups. Logistic models including *TGFB1* variants showed that in luminal-A tumors, G-875A retained its significance while *TGFB1* haplotype showed a trend towards significance; otherwise, in $HER2^+$ tumors *TGFB1* variants remained significant while *TGFBR2* showed a trend for association. There was no interaction between these genes. In correlation analyses, G-875A positively correlated with histopathological grade in total sample, and a trend towards significance was observed in triple-negative BCs.

Conclusion These results indicate that G-875A is a protective factor against BC, especially from luminal-A subtype, but may promote anaplasia in established tumors, consistent with TGFβ signaling roles in BC.

Keywords Transforming growth factor beta · TGFBR2 · Polymorphism · Breast neoplasm · Disease susceptibility

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10549-019-05370-1) contains supplementary material, which is available to authorized users.

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Introduction

Transforming growth factor beta (TGFβ) subfamily of cytokines comprises three TGFβ isoforms: TGFβ1, which is the most abundant and widely studied one, TGFβ2, and TGFβ3. TGFβs are expressed in specific spatial and temporal patterns during embryogenesis and display pleiotropic functions acting in many physiological and pathological conditions coordinating cell differentiation, proliferation, and apoptosis [1].

After secretion and activation in the extracellular milieu, interaction of TGFβs with proteoglycans on the cell membrane (referred to as TGFβRIII) promotes TGFβ binding to the serine/threonine kinase receptor TGFβ receptor 2 (TGFβRII) and then to TGFβ receptor 1 (ALK5 or TGFβRI), activating both classical (SMAD-mediated) and alternative pathways (including the Ras-MAPK, PI3K/AKT/mTOR, and Rho GTPase cascades) [2].

TGFβ signaling has opposing effects in carcinogenesis, acting as a tumor suppressor in poorly aggressive tumors while promoting epithelial to mesenchymal transition (EMT) and stimulating tumor growth and metastases in highly aggressive cancers [3–6].

In breast cancer (BC), these effects are clear among different subtypes, with luminal-A (LA) tumors showing cytostatic and apoptotic responses to TGFβ, while aggressive subtypes, such as those overexpressing the human epidermal growth factor receptor 2 (HER2) and those from triple-negative (TN) subgroup, show enhanced growth and metastatic potential [5, 7, 8]. Recently, we have shown that functional single-nucleotide polymorphisms (SNPs) in TGFβ1 gene (*TGFB1*) display subtype-specific associations with BC susceptibility and clinicopathological features that are consistent with TGFβ effects in these subtypes [9].

TGFβRII gene (*TGFBR2*) is located on chromosome 3 (3p22 *locus*), and a SNP on its promoter region (G-875A, rs3087465) was shown to increase TGFβRII expression in epithelial cells [10] and has been studied in several cancer types, such as prostate [11], head and neck [10], lung [12], gastric [13, 14], esophageal [15], and BC [16, 17]. Importantly, it was associated with decreased susceptibility to cancer in a pan-cancer meta-analysis [18]. In BC, this polymorphism was associated with decreased susceptibility for estrogen and/or progesterone receptor-positive (ER/PR^+) and HER2-negative ($HER2^-$) tumors [16], suggesting that it may also have subtype-specific roles in BC.

Therefore, in the present study, we aimed to evaluate the possible association of *TGFBR2* G-875A SNP with BC susceptibility and clinical presentation and its potential dependence and interaction with *TGFB1* haplotype structures considering BC molecular subgroups.

Methods

Sample characterization

The present sampling was described previously [9, 19]. Biological material [blood samples, surgical excision tissues, or formalin-fixed-paraffin-embedded (FFPE) tissues] was collected from 388 unselected and unrelated women with BC from Londrina Cancer Hospital (Londrina, Parana, Brazil). For control group, blood samples were collected from 405 unrelated women with no self-reported personal history of neoplasia, no familial history of BC, and no mammary alterations proven by a recent mammographic exam (performed within the past 2 years from collection time). The invitations, questionnaire applications, and sample collections were carried out during routine medical exams at primary health care unities in the city of Londrina and at the Clinical Hospital from Londrina State University.

Mean and median age were 53.9 (standard deviation: 14) and 55 (interquartile range: 15.5) years, respectively, in control group (Table S1), and 54.4 (standard deviation: 12.7) and 53 (interquartile range: 19) for whole BC group.

Clinicopathological features retrieved from patients' medical records included age at diagnosis, tumor size, histopathological grade, lymph node metastasis status, disease stage (according to the UICC criteria), and immunohistochemistry (IHC) score for p53, Ki67, estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), all assessed according to the ASCO standard protocols [20, 21]. Samples with dubious HER2 IHC staining were submitted to fluorescent in situ hybridization (FISH) analysis to check for HER2 genetic amplification. Table 1 shows the clinicopathological features for all BC patients. Some features were missed from patients' medical records ("NA" lines), and these patients were not considered in statistical analyses involving these parameters. For disease stage, patients without complete information on tumor size, lymph node and distant metastasis were not considered.

Patients were classified as hormonal-receptor-positive (HR^+, ER/PR^+) and $HER2^+$ and into intrinsic subtypes: luminal-A (LA; ER/PR^+ $HER2^-$); luminal-B $HER2^+$ (LB; ER/PR^+HER2^+); HER2-enriched (HER2; $ER^-PR^-HER2^+$); and triple negative (TN; $ER^-PR^-HER2^-$). Table S1 shows the patients' clinicopathological features according to BC subtype. As expected, $HER2^+$ and TN tumors exhibited characteristics of more aggressive tumors as evidenced by higher proportion of patients with larger tumor sizes, higher proliferation indexes, histopathological grade, p53 mutations, lymph node metastasis, and higher disease stages (Table S1).

Table 1 Patients' clinicopathological features at diagnosis time

Parameter	Category	Frequency [*n* (%)]
Age (years)	< 40	43 (11.1)
	40–49	113 (29.2)
	50–59	100 (25.8)
	60–69	74 (19.1)
	70–79	47 (12.1)
	≥ 80	10 (2.6)
	NA	1
ER	Positive	267 (71.4)
	Negative	107 (28.6)
	NA	14
PR	Positive	197 (52.7)
	Negative	177 (47.3)
	NA	14
HER2	Positive	66 (18.5)
	Negative	290 (81.5)
	NA	32
Tumor size (cm)	< 1.5	94 (24.8)
	1.51–3.0	177 (46.7)
	> 3.0	108 (28.5)
	NA	9
Histopathological grade	I	43 (11.9)
	II	147 (40.8)
	III	170 (47.2)
	NA	28
Ki67	Low	59 (23.0)
	Intermediate	110 (42.8)
	High	88 (34.2)
	NA	131
p53	Positive	109 (40.1)
	Negative	163 (59.9)
	NA	116
Lymph node metastasis	Positive	183 (50.8)
	Negative	177 (49.2)
	NA	28
Stage	0	11 (3.7)
	I	58 (19.3)
	II	130 (43.2)
	III	83 (27.6)
	IV	19 (6.3)
	NA	87

The high miscegenation observed in Brazilian population hampers reliable individuals' classification into ethnic groups, even through genetic markers [22–24]. However, both BC and control groups were served by the Brazilian public health system (SUS) and were collected in Londrina city, which is located in southern Brazil, a region that shows a high degree of European inheritance (Caucasoid ethnicity) [23, 25–27].

DNA extraction

For blood samples, DNA was extracted using Biopur Mini Spin kit (Biometrix Diagnóstica®, Curitiba, Parana, Brazil). Excised tumor tissues were mechanically dissociated and the DNA was obtained through salting-out method [28]. From FFPE tissues, DNA was extracted as previously described [29] or using the innuPREP DNA Mini Anlytik Jena AG kit (Jena, Germany).

DNA samples were quantified in a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Wilmington, DE, EUA) at 260 nm wavelength and the absorbance ratio at 260 nm and 280 nm was calculated to assess protein contamination.

TGFBR2 rs3087465 genotyping

TGFBR2 rs3087465 genotyping was performed through polymerase chain reaction (PCR) followed by restriction fragment polymorphism (RFLP) analysis according to Zhang et al. [16] with modifications. A 152-base-pair (bp) fragment flanking the SNP was amplified through PCR using the following oligonucleotides: 5′-GGAATGTCTTGGGCAAATCT-3′ and 5′-ACCTGAATGCTTGTGCTTTTATT-3′. PCR was performed using 2.5 µL of 10×PCR buffer, 0.1 mM of dNTP, 0.15 µM of each primer, 1.5 mM of $MgCl_2$, 1 U of Taq DNA polymerase, all from Invitrogen™ (Carlsbad, CA, USA), approximately 3 ng/µL of target DNA and ultrapure water to complete 25 µL.

PCR amplicons were subjected to enzymatic restriction using *HpyCH4III* endonuclease (New England Biolabs®, Ipswich, USA), which cleaves the amplicons from A allele generating 93-bp and 59-bp fragments, but not those from G allele. Therefore, after cleavage, wild homozygous (GG) individuals hold just the 152-bp amplicons, heterozygous (GA) individuals present 152-, 93-, and 59-bp fragments, and variant homozygous (AA) present 93- and 59-bp fragments. A variant homozygous individual was used as a control for enzymatic restriction in all analyses. Fragments were visualized through electrophoresis in 10% polyacrylamide gels stained with silver nitrate.

To confirm the accuracy of the genotyping method, one individual for each genotype was sequenced in a 3500 Genetic Analyzer® (Applied Biosystems) using the BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Austin, TX, USA) and at least 5% of total sample was repeated for PCR–RFLP analysis, obtaining 100% of concordance between results.

Statistical analyses

Chi-squared (χ^2) and Fisher's exact tests were applied to assess differences in genotype and allele frequencies

between groups. Chi-squared tests for trend were applied to assess the additive model of association.

Odds ratios (OR) and 95% confidence intervals (95% CI) were estimated through age-adjusted binary logistic regressions. Genotypic (heterozygotes or variant homozygotes vs. wild homozygotes), dominant (heterozygotes and variant homozygotes vs. wild homozygotes), and recessive (variant homozygotes vs. heterozygotes and wild homozygotes) models were tested.

Age-adjusted logistic regression models were also applied to test the independence of association for *TGFBR2* and *TGFB1* haplotypes comprising rs1800468, rs1800469, rs1800470, and rs1800471 SNPs which were previously shown to be associated with BC subtypes in the present sample [9]. In interaction analyses, logistic models included age, relevant *TGFBR2* and *TGFB1* models of inheritance as individual factors, and the interaction terms between them.

Correlations between *TGFBR2* polymorphism and clinicopathological features were tested using the Kendal's Tau-b rank correlation test.

All statistical analyses were performed in IBM® SPSS® Statistics 20 software (IBM®, Armonk, New York, USA). All tests were two-tailed with significance level set at 0.05.

Results

TGFBR2 rs3087465 and breast cancer susceptibility

A strong statistical difference ($p < 0.001$) was observed in both genotype and allele frequencies in BC group compared to control group (Table 2). Also, a strong significance was noted in the chi-squared test for trend comparing BC and control group ($p < 0.001$), suggesting that variant allele may act additively in BC susceptibility. In subgroup-stratified analyses, these effects were greater in HR^+ and LA subtypes, but were not noted in other BC subgroups (Table 2).

Table 3 shows that G-875A was negatively associated with BC in genotypic and dominant models. In subtype-stratified analyses, the effects of GA genotype and A allele

Table 3 Association analyses between TGFBR2 polymorphism and BC subtypes

BC subgroup	*TGFBR2* G-875A association models [OR (95% CI)][†]			
	GA	AA	Dominant	Recessive
General BC ($n=388$)	0.61** (0.45–0.82)	0.49** (0.29–0.82)	0.59*** (0.44–0.78)	0.62 (0.38–1.02)
HR^+ ($n=270$)	0.52*** (0.38–0.73)	0.53* (0.30–0.92)	0.52*** (0.38–0.71)	0.71 (0.42–1.22)
$HER2^+$ ($n=66$)	0.74 (0.43–1.27)	0.33[#] (0.10–1.13)	0.67 (0.39–1.13)	0.38 (0.12–1.27)
LA ($n=215$)	0.50*** (0.35–0.71)	0.48* (0.30–0.98)	0.50*** (0.36–0.71)	0.74 (0.42–1.32)
LB ($n=42$)	0.74 (0.38–1.42)	0.35 (0.08–1.54)	0.66 (0.35–1.26)	0.41 (0.10–1.74)
HER2 ($n=24$)	0.77 (0.33–1.79)	0.30 (0.04–2.40)	0.68 (0.30–1.55)	0.35 (0.05–2.64)
TN ($n=76$)	0.76 (0.46–1.26)	0.39[#] (0.13–1.14)	0.69 (0.42–1.13)	0.44 (0.16–1.28)

[†]Odds ratio (OR) and 95% confidence interval (95% CI) obtained through age-adjusted logistic regression

[#]$0.05 < p < 0.1$

***$p < 0.001$

**$p < 0.01$

*$p < 0.05$

Table 2 Comparison between genotypes and allele frequencies between control and BC groups

TGFBR2 G-875A	Control (405)	BC (388)	HR^+ (270)	$HER2^+$ (66)	LA (215)	LB (42)	HER2 (24)	TN (76)
Genotype [*n* (%)]								
GG	165 (40.7)	210 (54.1)	154 (57.0)	33 (50.0)	125 (58.1)	21 (50.0)	12 (50.0)	38 (50.0)
GA	195 (48.1)	150 (38.7)	94 (34.8)	30 (45.5)	72 (33.5)	19 (45.2)	11 (45.8)	34 (44.7)
AA	45 (11.1)	28 (7.2)	22 (8.1)	3 (4.5)	18 (8.4)	2 (4.8)	1 (4.2)	4 (5.3)
p value[†]	–	0.0006	0.0002	0.1617	0.0002	0.3150	0.4688	0.1649
p-trend[‡]	–	0.0002	0.0002	0.0661	0.0003	0.1395	0.2376	0.0627
Allele frequency [*n* (%)]								
G	525 (64.8)	570 (73.4)	402 (74.4)	96 (72.7)	322 (74.9)	61 (72.6)	35 (72.9)	110 (72.4)
A	285 (35.2)	206 (26.6)	138 (25.6)	36 (27.3)	108 (25.1)	23 (27.4)	13 (27.1)	42 (27.6)
p value[§]	–	0.0002	0.0002	0.0919	0.0003	0.1843	0.2783	0.0765

[†]Obtained in chi-squared (χ^2) test

[‡]Obtained in chi-squared test for trend (Cochran–Armitage)

[§]Obtained in Fisher's exact test

in dominance were stronger in HR^+ and LA subgroups, while AA genotype was associated with HR^+ and LA subgroups but with less statistical confidence than in general BC (Table 3). A tendency towards association ($0.05 < p < 0.1$) was noted for AA genotype in $HER2^+$ and TN subgroups (Table 3).

TGFBR2 rs3087465 interactions with *TGFB1* haplotypes in breast cancer susceptibility

To investigate the relationship between *TGFBR2* G-875A and *TGFB1* haplotypes shown to be associated with BC using previous data from a cohort of the present sample (encompassing all the 405 controls and 323 of the BC patients) [9], models of inheritance for each gene in which at

Table 4 Dependence and interaction analyses between *TGFBR2* G-875A and *TGFB1* haplotypes

Subgroup (*n*)	Group of factors (*TGFBR2*/*TGFB1*)	Models [OR (95% CI)][†]	
		1	2
LA (210)	Group 1		
	TGFBR2 GA (genotypic)	0.51*** (0.36–0.73)	0.51*** (0.35–0.74)
	TGFBR2 AA (genotypic)	0.58[#] (0.32–1.06)	0.61 (0.23–1.15)
	TGFB1 GCCG haplotype (dominant)	0.56[#] (0.28–1.09)	0.62 (0.23–1.69)
	TGFBR2 GA by *TGFB1* GCCG (dominant)	–	0.88 (0.20–3.93)
	TGFBR2 AA by *TGFB1* GCCG (dominant)	–	0.71 (0.10–4.82)
	Group 2		
	TGFBR2 (dominant)	0.52*** (0.37–0.73)	5.53*** (0.37–0.75)
	TGFB1 GCCG haplotype (dominant)	0.56[#] (0.29–1.11)	0.62 (0.23–1.69)
	TGFBR2 dominant by *TGFB1* GCCG (dominant)	–	0.84 (0.22–3.26)
$HER2^+$ (66)	Group 1		
	TGFBR2 AA (genotypic)	0.35[#] (0.10–1.18)	0.47 (0.05–4.10)
	TGFB1 GCTG haplotype (dominant)	0.64 (0.37–1.12)	0.68 (0.31–1.49)
	TGFBR2 AA by *TGFB1* GCTG (dominant)	–	0.65 (0.05–9.10)
	Group 2		
	TGFBR2 AA (genotypic)	0.31[#] (0.09–1.08)	–
	TGFB1 GCTG haplotype (recessive)	0.38* (0.16–0.92)	–
	TGFBR2 AA by *TGFB1* GCTG (recessive)	–	–
	Group 3		
	TGFBR2 AA (genotypic)	0.33[#] (0.10–1.12)	–
	TGFB1 GTCG haplotype (dominant)	1.51 (0.87–2.60)	–
	TGFBR2 AA by *TGFB1* GTCG (dominant)	–	–

[†]Odds ratio (OR) and 95% confidence interval (95% CI) obtained through age-adjusted logistic regression

[#]$0.05 < p < 0.1$

***$p < 0.001$

*$p < 0.05$

least one of them showed a significant association ($p < 0.05$) and the other showed at least a trend ($0.05 < p < 0.1$) towards a significant association were set as dependent variables in age-adjusted logistic models to test their dependency (Table 4, Model 1 column); interactions factors between *TGFBR2* and *TGFB1* variants were added to these logistic models to test their interaction (Table 4, Model 2 column).

Table 5 Correlation between *TGFBR2* polymorphism and clinicopathological features

BC subtype	Parameter	Models for *TGFBR2* [Tau-b (*p* value)]		
		Additive	Dominant	Recessive
General BC	Age	−0.053 (0.176)	−0.052 (0.216)	−0.033 (0.362)
	Tumor size	−0.002 (0.956)	−0.003 (0.944)	0.004 (0.931)
	Hist. grade	0.098 (0.041*)	0.125 (0.012*)	−0.045 (0.331)
	Ki67	0.043 (0.450)	0.064 (0.275)	−0.060 (0.290)
	p53	−0.049 (0.397)	−0.028 (0.638)	−0.106 (0.056)
	LN	0.052 (0.309)	0.052 (0.324)	0.028 (0.591)
	Stage	0.022 (0.664)	0.018 (0.733)	0.027 (0.600)
LA	Age	−0.039 (0.468)	−0.026 (0.642)	−0.066 (0.200)
	Tumor size	−0.068 (0.214)	−0.075 (0.187)	−0.011 (0.832)
	Hist. grade	0.054 (0.375)	0.069 (0.293)	−0.021 (0.680)
	Ki67	0.074 (0.327)	0.094 (0.230)	−0.031 (0.696)
	p53	−0.048 (0.520)	−0.042 (0.598)	−0.054 (0.446)
	LN	0.016 (0.811)	0.002 (0.981)	0.065 (0.343)
	Stage	−0.017 (0.798)	−0.034 (0.621)	0.046 (0.501)
LB	Age	−0.052 (0.674)	−0.053 (0.687)	−0.023 (0.841)
	Tumor size	−0.047 (0.716)	−0.047 (0.730)	−0.021 (0.834)
	Hist. grade	0.078 (0.605)	0.094 (0.548)	−0.060 (0.706)
	Ki67	−0.050 (0.774)	−0.085 (0.650)	0.100 (0.543)
	p53	−0.129 (0.504)	−0.131 (0.509)	−0.060 (0.771)
	LN	0.118 (0.448)	0.129 (0.417)	0.006 (0.970)
	Stage	0.217 (0.154)	0.202 (0.197)	0.158 (0.305)
HER2	Age	−0.211 (0.159)	−0.244 (0.132)	0.051 (0.501)
	Tumor size	0.018 (0.930)	0.061 (0.763)	−0.118 (0.340)
	Hist. grade	0.222 (0.275)	0.204 (0.350)	0.158 (0.306)
	Ki67	−0.300 (0.239)	−0.300 (0.239)	NA
	p53	0.044 (0.858)	0.044 (0.858)	NA
	LN	0.038 (0.863)	0.101 (0.651)	−0.229 (0.292)
	Stage	−0.088 (0.640)	−0.046 (0.806)	−0.230 (0.295)
$HER2^+$	Age	−0.111 (0.248)	−0.124 (0.225)	0.006 (0.938)
	Tumor size	−0.038 (0.720)	−0.025 (0.817)	−0.059 (0.456)
	Hist. grade	0.127 (0.300)	0.134 (0.290)	0.025 (0.833)
	Ki67	−0.084 (0.565)	−0.102 (0.501)	0.039 (0.777)
	p53	−0.067 (0.658)	−0.061 (0.693)	−0.055 (0.736)
	LN	0.092 (0.469)	0.120 (0.354)	−0.073 (0.571)
	Stage	0.076 (0.533)	0.087 (0.471)	−0.038 (0.783)
TN	Age	−0.099 (0.271)	−0.094 (0.325)	−0.063 (0.357)
	Tumor size	0.159 (0.085#)	0.156 (0.103)	0.084 (0.417)
	Hist. grade	0.190 (0.104)	0.215 (0.063#)	−0.016 (0.895)
	Ki67	−0.133 (0.274)	−0.115 (0.355)	−0.138 (0.319)
	p53	−0.181 (0.132)	−0.166 (0.170)	−0.137 (0.331)
	LN	0.125 (0.302)	0.153 (0.210)	−0.079 (0.522)
	Stage	0.168 (0.207)	0.178 (0.203)	0.027 (0.815)

#$0.05 < p < 0.1$

*$p < 0.05$

Only LA and HER2$^+$ subgroups met the above-mentioned criteria.

In LA subgroup, *TGFBR2* retained its significance in heterozygosis (Table 4, Model 1, Group 1 of factors; $p < 0.001$) and in dominant model (Table 4, Model 1, Group 2; $p < 0.001$), while *TGFB1* GCCG haplotype was not significant, but showed a trend towards a protective association (Table 4, Model 1; $p = 0.1$ for both).

Otherwise, in HER2$^+$ subgroup, just the *TGFB1* GCTG haplotype in recessive model was significant when tested along with *TGFBR2* AA genotype (Table 4, Model 1, Group 2; $p = 0.031$). In all models (Table 4, Model 1, Groups 1, 2 and 3), *TGFBR2* showed a trend for association ($0.05 < p < 0.1$) as when it was tested in univariate analysis (Table 2).

There was no interaction between *TGFBR2* and *TGFB1* in any subtype tested (Table 4, Model 2 column).

TGFBR2 rs3087465 and clinicopathological parameters

For correlation analyses, clinicopathological parameters were categorized as in Table 1, except for age and tumor size, which were tested as continuous variables. Positive correlations between histopathological grade and G-875A polymorphism in additive and dominant models were observed in general BC sample (Table 5; Tau-b = 0.098, $p = 0.04$ and Tau-b = 0.125, $p = 0.01$, respectively). In TN subgroup, trends towards a positive correlation were observed between G-875A additive model and tumor size (Tau-b = 0.159, $p = 0.08$) and between dominant model and histopathological grade (Table 5; Tau-b = 0.215, $p = 0.06$).

Discussion

The paradoxical roles of TGFβ signaling in cancer have been extensively documented. In BC, these effects are evident in different molecular subtypes and disease stages, with highly aggressive subtypes and advanced tumors showing enhanced growth and invasive potential and less aggressive subtypes and initial tumors displaying cytostatic and apoptotic responses to TGFβ [5–8, 30, 31]. Importantly, these effects were reflected in functional *TGFB1* polymorphisms, with high production variants being associated with risk for aggressive subtypes of BC and correlating with worst prognosis parameters in these tumors, while they were associated with protection and better prognosis parameters in less aggressive subtypes [9, 32].

Alterations in TGFβ signaling components were found in several cancers, and include point mutations, deletions, and loss of expression [33]. Among these components, *TGFBR2* is often mutated or inactivated [33, 34], including in BC [35]. In the cBioPortal [36, 37] database (http://www.cbioportal.org/), genetic alterations on the main components of the classical TGFβ pathway, including ligands (TGFβ1, TGFβ2, and TGFβ3), receptors (TGFβRI, TGFβRII, and TGFβRIII), intracellular mediators (SMAD2, SMAD3, and SMAD4) and inhibitors (SMAD7, SMURF1, and SMURF2) are found in approximately 27% of total non-redundant primary tissue samples ($n = 1617$). Alterations in *TGFBR2* are observed in 1.6% of these tumors (Figure S1).

These are mainly composed of amplification or deep deletion both in TGFβ signaling effectors and inhibitors, reflecting the multifaceted nature of TGFβ signaling on BC, where both gain or loss of function may be selected during carcinogenic process [6]. Indeed, it has been shown that loss of TGFβ signaling enhances breast carcinogenesis, acting mainly in the initiation phase. Otherwise, enhanced TGFβ signaling stimulates EMT process and the generation of a basal-like phenotype in BC cells [6, 38] and promotes the expression of chemokine receptors like CXCR4 [39, 40], coordinating targeted metastases to bone-marrow and liver, and of CCR7, seeding lymph node metastases [41].

The complete abrogation of TGFβ signaling in BC cells also stimulates metastasis and disease progression [42]. This apparent contradiction was solved by the finding that TGFβ abrogation enhances the secretion of chemokines (namely, CCL9, CXCL1, and CXCL9) that attract myeloid-derived suppressor cells (MDSC), which promote BC metastasis, angiogenesis, and immunosuppression through matrix remodeling and cytokine secretion [39, 43]. These models were corroborated by studies with clinical samples showing that a TGFβ signaling signature correlated with enhanced metastatic potential in ER$^-$ cancers [44], while TGFβ-deficient signatures correlated with metastasis in ER$^+$ tumors [6, 39] and suggest that TGFβ signaling can either inhibit or promote BC metastasis in different contexts involving different chemokine networks [6].

In the present study, we showed that rs3087465 may be protective against BC, with higher statistical confidence for HR$^+$ and LA tumors (Tables 2 and 3). These results are in accordance with those from Zhang et al. [16], which showed that this polymorphism was associated with protection against mammary carcinoma with ER$^+$, PR$^+$, and ER/PR$^+$ profiles. However, trends for a protective association in HER2$^+$ and TN tumors observed in the present study might suggest that this polymorphism may also be protective against these subtypes. This issue should be solved by further studies with larger samples enriched for these subtypes, as the present sample may be underpowered to detect significant associations for these low-prevalent BC subtypes. Indeed, this polymorphism was studied in several cancer types previously [10–17], being globally associated

with decreased susceptibility for cancer [18], which suggest its role as a pan-cancer protective marker.

G-875A was shown to increase *TGFBR2* promoter region activity in reporter assays with human keratinocytes from HaCaT lineage [10]. Previous research using AliBaba2.1 transcription factor binding site prediction software [45] suggested that A allele could bind to TATA-box binding protein (TBP), CCAAT-enhancer binding protein (C/EBP), glucocorticoid receptor alpha (GR-α), and progesterone receptor (PR), whereas G allele may bind to C/EBT, GR-α, PR, and Oct1 [46]. Using the PROMO 3.0.2 [47] software, available at ALGGEN server (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3), we observed the putative binding of PR (both α and β) on G and A alleles (with higher dissimilarity score for G allele), and of TBP specifically on A allele. However, neither C/EBP nor GR-α was predicted to bind this *locus*. Additionally, X-box binding protein 1 (XBP-1) was shown to have a putative binding site only in G allele.

The Roadmap Epigenomic Locus Mapping Consortium and ENCODE project data [48] available through the HaploReg [49] database (https://pubs.broadinstitute.org/mammals/haploreg) indicate that this *locus* associates with promoter and enhancer histone markers and DNAse peaks in several tissues and cells, including breast, such as myoepithelial cells, which are suggested as originating cells for TN BCs [50] and binds to the HEY1 transcription factor in HepG2 hepatocytes. Furthermore, HaploReg data show that G-875A is linkage disequilibrium with other polymorphisms altering the consensus binding sites for several transcription factors in *TGFBR2* promoter region, which may also deregulate TGFβRII expression and might be involved in BC susceptibility. These data indicate that G-875A may increase *TGFBR2* expression via multiple mechanisms, making the results found herein consistent with TGFβRII acting as a tumor suppressor in BC initiation [34, 51].

To genetically characterize our population, we compared G-875A allelic frequency from our control group to populations around the world using data from 1000 Genomes project [52, 53], available through the LDlink application (https://ldlink.nci.nih.gov/?tab=ldhap) [54]. Allele frequencies differed from all but Puerto Rician, Colombian, Peruvian, Vietnamese, and Pakistani populations. Overall, the frequency of variant allele A in our control group was lower than in South Asian ($p < 0.001$) and African populations ($p < 0.001$), in which this was the most prevalent allele instead of G, but was higher than in Mexican ($p = 0.016$), East Asian ($p < 0.001$), European ($p < 0.001$), and pooled American Admixed ($p = 0.011$) populations. These differences might be attributable to the unique genetic background of Brazilian population, which derives mainly from the recent miscegenation between Amerindian, European, and African populations [22–24, 27].

Highlighting the importance of *TGFBR2* in BC, a genome-wide association study (GWAS) identified rs12493607, occurring at *TGFBR2* intron 2, as a risk factor for BC [55], especially from $HER2^+$ subgroup [56], and studies focusing on TGFβ pathway, identified rs4522809, also on *TGFBR2* intron 2 [57], and rs1078985, on *TGFBR2* intron 3 [58], as protective factors. Importantly, rs3087465 was not included for analysis in any of these GWAS and there is no evidence for linkage disequilibrium among any pair of these four *TGFBR2 loci* in any population (Table S2), with all theoretically possible sixteen combinations among them being observed with great variability among worldwide populations (Table S3). Therefore, these data suggest that their association with BC susceptibility is independent from each other.

Of note, as with rs3087465, the other variants have also shown potential to regulate *TGFBR2* expression according to HaploReg data [49]: rs12493607 is associated with promoter and enhancer histone markers and alters the consensus motif for Myeloid Zinc Finger 1 (MZF1) transcription factor, and has been shown to be a quantitative trait locus (QTL) for *TGFBR2* in blood cells; rs4522809 associates with enhancer histone markers and DNAse peaks and alters the binding motif for Heat Shock Factor (HSF) and KRAB-associated protein 1 (KAP1); and rs1078985 associates with DNAse peaks and alters the consensus sequence for Maf and Myb transcription factors, and has been also shown to be a QTL for *TGFBR2* in blood cells.

Altogether, these results suggest that *TGFBR2* polymorphisms might be involved in the initiation of breast carcinogenesis, probably by altering the expression of TGFβRII in breast cells. Future association studies investigating the haplotype structures composed by these four polymorphisms may be of interest to fully elucidate their role in BC and in TGFβRII expression.

In regression models including *TGFBR2* and *TGFB1* haplotype structures, we noted that in LA subtype just *TGFBR2* was significant, while in $HER2^+$ BC models only *TGFB1* GCTG in recessive model retained its significance. There was no effect modification between them when they were tested as interaction terms. This may indicate that *TGFBR2* has dominant effects on LA tumors, while *TGFB1* may act mainly in $HER2^+$ tumors, where low-producer *TGFB1* variants were protective [9].

Differences in *TGFB1* and *TGFBR2* variants in governing tumor initiation may be attributable to differences in their action pattern during carcinogenesis: while increased expression of *TGFBR2* in pre-cancerous cells may hamper their proliferation, once a subpopulation of cells develop genomic instability and acquire HER2 amplification,

TGFβ1 may favor their outgrowth over other cells in a heterogeneous lesion [8, 31, 59].

TGFβRII may also have subtype-specific prognostic relevance in BC, since higher TGFβRII expression indicated decreased overall survival in BC, with greater effect in ER^- patients [60], whereas in ER^+ patients treated with Tamoxifen, low-TGFβRII expression predicted shorter disease-free survival [61].

Importantly, these effects may be regulated genetically, since rs1367610, a G > C polymorphism occurring at *TGFBR2* intron 4, was associated with decreased overall survival in ER^- patients treated with chemotherapy in two independent large genome-wide studies [62, 63]. This polymorphism was shown to be a QTL affecting the binding site for several transcription factors and being associated with several histone enhancer and promoter markers in HaploReg database [49]. Furthermore, there is also no linkage disequilibrium between this SNP and any of the SNPs shown affect BC susceptibility in previous studies (Tables S2 and S3) [62], suggesting that different *TGFBR2* variants may control BC initiation and progression.

Our results also shown that G-875A in additive and dominant models indicates higher histopathological grade in BC (Table 5). In subtype-stratified analyses, a trend towards a positive correlation was observed just in TN tumors in dominant model; also in TN BCs, a trend towards a positive correlation was noted for tumor size in additive model.

These data might be consistent with TGFβRII governing EMT, a process by which cells lose epithelial characteristics and acquire a mesenchymal phenotype that is associated with tissue anaplasia and higher histopathological grade [64]. Indeed, TGFβ signaling is a strong EMT inducer in BC, particularly in subtypes with high activation of MAP kinase pathways like TN BC [41, 65, 66]. Of note, it has been shown that two micro-RNAs that are decreased in BC tissue, miR-153 and miR-655, directly target *TGFBR2* mRNA, and the overexpression of these miRNAs in TN BC cell line MDA-MB-231 decreases TGFβRII expression and hampers EMT, inhibiting cancer progression, which is reversed by transient TGFβRII expression [67, 68], pointing *TGFBR2* as an important gene governing EMT and invasiveness in TN cancers.

In conclusion, *TGFBR2* gene is a promisor marker for BC susceptibility and prognosis with possible different effects in BC subgroups, having a great effect on the susceptibility for LA BCs. Further association and mechanistic studies investigating haplotype structures composed by the five *TGFBR2* SNPs associated with BC susceptibility (rs3087465, rs4522809, rs12493607, and rs1078985) and prognosis (rs1367610) in cohorts with larger sample size for rare subtypes may fully elucidate *TGFBR2* role in BC and reveal a valuable marker for this disease.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Ethics Committee for Research Involving Human Subjects from Londrina State University - CEP/UEL 189/2013—CAAE 17123113400005231) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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Supplementary information

Transforming growth factor beta receptor II (*TGFBR2*) promoter region polymorphism in Brazilian breast cancer patients: association with susceptibility, clinicopathological features and interaction with *TGFB1* haplotypes

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Table S1. Clinicopathological features per BC groups.

Parameter	LA	LB	HER2	TN	Control
Age (years)					
Median (IQ range)	55 (18.5)	48 (13.0)	51 (13.5)	53 (21.8)	55 (15.5)
Mean (SD)	55.8 (12.6)	50.0 (12.0)	53.0 (14.3)	54.4 (13.8)	53.9 (14.0)
<40 [n(%)]	19 (8.9)	7 (16.3)	3 (12.5)	11 (14.5)	60 (14.8)
40-49 [n(%)]	60 (28.2)	17 (39.5)	7 (29.2)	19 (25.0)	58 (14.3)
50-59 [n(%)]	52 (24.4)	10 (23.3)	8 (33.3)	19 (25.0)	150 (37.0)
60-69 [n(%)]	47 (22.1)	7 (16.3)	4 (16.7)	11 (14.5)	93 (23.0)
70-79 [n(%)]	28 (13.1)	1 (2.3)	1 (4.2)	14 (18.4)	32 (7.9)
>80 [n(%)]	7 (3.3)	1 (2.3)	1 (4.2)	2 (2.6)	12 (3.0)
Unknown [n(%)]	1	0	0	0	0
Tumor size					
Median (IQ range)	2.0 (1.5)	2.5 (1.28)	2.2 (2.2)	3.0 (2.7)	
Mean (SD)	2.62 (1.62)	3.01 (2.02)	2.85 (1.51)	3.49 (2.32)	
0-1.5 cm [n (%)]	30 (14.2)	4 (9.6)	2 (9.1)	11 (14.5)	
1.51-3.0 cm [n (%)]	112 (52.8)	19 (45.2)	12 (54.5)	22 (28.9)	
>3.0 cm [n (%)]	70 (33.0)	19 (45.2)	8 (36.4)	43 (56.6)	
Undocumented	2	1	2	1	
Histopathological grade [n (%)]					
I	35 (17.4)	1 (2.4)	0 (0.0)	0 (0.0)	
II	102 (50.7)	14 (33.3)	7 (33.3)	16 (22.2)	
III	64 (31.8)	27 (64.3)	14 (66.7)	56 (77.8)	
Unknown	13	1	3	4	
Ki67 [n (%)]					
Low	50 (33.6)	4 (15.4)	0 (0.0)	4 (6.6)	
Intermediate	74 (49.7)	12 (46.2)	5 (38.5)	16 (26.2)	
High	25 (16.8)	10 (38.5)	8 (61.5)	41 (67.2)	
Unknown	65	17	11	15	
p53 mutation [n (%)]					
Positive	36 (22.9)	15 (60.0)	11 (64.7)	42 (63.6)	
Negative	121 (77.1)	10 (40.0)	6 (35.3)	24 (36.4)	
Unknown	57	18	7	10	
Lymph node metastasis [n (%)]					
Positive	100 (47.8)	19 (47.5)	11 (50.0)	35 (53.0)	
Negative	109 (52.2)	21 (52.5)	11 (50.0)	31 (47.0)	
Unknown	5	3	2	10	
Tumor stage [n (%)]					
0	6 (3.4)	1 (2.9)	2 (10 .0)	0 (0.0)	
I	41 (23.0)	3 (8.8)	1 (5.0)	5 (11.9)	
II	79 (44.4)	15 (44.1)	6 (30.0)	18 (42.9)	
III	41 (23.0)	12 (35.3)	9 (45.0)	17 (40.5)	
IV	11 (6.2)	3 (8.8)	2 (10.0)	2 (4.8)	
Unknown	36	9	4	34	

Table S2. Linkage disequilibrium between *TGFBR2* SNPs associated with BC susceptibility and prognosis.

Location	SNP	Linkage disequilibrium [r^2 (D')] ‡				
		rs3087465	**rs4522809**	**rs12493607**	**rs1078985**	**rs1367610**
Promoter	**rs3087465**	1.0 (1.0)	-	-	-	-
Intron 2	**rs4522809**	0.0 (0.01)	1.0 (1.0)	-	-	-
Intron 2	**rs12493607**	0.048 (0.35)	0.198 (0.68)	1.0 (1.0)	-	-
Intron 3	**rs1078985**	0.003 (0.09)	0.0 (0.04)	0.016 (0.32)	1.0 (1.0)	-
Intron 4	**rs1367610**	0.0 (0.087)	0.008 (0.43)	0.001 (0.1)	0.016 (1.0)	1.0 (1.0)

‡ Data retrieved from the web-based application LDlink (https://ldlink.nci.nih.gov/?tab=ldhap)

Table S3. Haplotype structures of *TGFBR2* SNPs associated with BC per population.

Haplotype†	1000 Genomes populations [Allele count (%)]‡					
	AFR	**AMR**	**EUR**	**EAS**	**SAS**	**Total**
G G G A G	154 (11.65)	**228 (32.85)**	**278 (27.63)**	104 (10.32)	166 (16.97)	**930 (18.57)**
G A C A G	58 (4.39)	66 (9.51)	101 (10.04)	**466 (46.23)**	**174 (17.79)**	865 (17.27)
A G G A G	**401 (30.33)**	54 (7.78)	37 (3.68)	21 (2.08)	62 (6.34)	575 (11.48)
A A G A G	340 (25.72)	45 (6.48)	21 (2.09)	7 (0.69)	51 (5.21)	464 (9.27)
A A C A G	64 (4.84)	44 (6.34)	28 (2.78)	110 (10.91)	72 (7.36)	318 (6.35)
G A G A G	95 (7.19)	81 (11.67)	69 (6.86)	27 (2.68)	31 (3.17)	303 (6.05)
A A G G G	59 (4.46)	25 (3.6)	77 (7.65)	19 (1.88)	73 (7.46)	253 (5.05)
A G G G G	87 (6.58)	7 (1.01)	15 (1.49)	21 (2.08)	101 (10.33)	231 (4.61)
G A C G G	5 (0.38)	28 (4.03)	120 (11.93)	26 (2.58)	38 (38.9)	217 (4.33)
G G G G G	11 (0.83)	30 (4.32)	83 (8.25)	49 (4.86)	37 (37.8)	210 (4.19)
G A G G G	32 (2.42)	12 (1.73)	19 (1.89)	46 (4.56)	29 (2.97)	138 (2.76)
A G C A G	3 (0.23)	3 (0.43)	7 (0.7)	59 (5.85)	36 (36.8)	108 (2.16)
G G C A G	3 (0.23)	12 (1.73)	11 (1.09)	33 (3.27)	31 (3.17)	90 (1.8)
A A G A C	2 (0.15)	9 (1.3)	28 (2.78)	1 (0.1)	36 (3.68)	76 (1.52)
G A C A C	1 (0.08)	18 (2.59)	39 (3.88)	11 (1.09)	3 (0.31)	72 (1.44)
G A G A C	0 (0.0)	5 (0.72)	19 (1.89)	0 (0.0)	14 (1.43)	40 (0.8)
G G G A C	1 (0.08)	7 (1.01)	19 (1.89)	1 (0.1)	6 (0.61)	34 (0.68)
G G C A C	0 (0.0)	5 (0.72)	8 (0.8)	0 (0.0)	6 (0.61)	19 (0.38)
G G C G G	0 (0.0)	1 (0.14)	11 (1.09)	4 (0.4)	3 (0.31)	19 (0.38)
A A C G G	3 (0.23)	6 (0.86)	4 (0.4)	1 (0.1)	1 (0.1)	15 (0.3)
A G C A C	0 (0.0)	4 (0.58)	4 (0.4)	0 (0.0)	2 (0.2)	10 (0.2)
A A C A C	1 (0.08)	4 (0.58)	1 (0.1)	2 (0.2)	1 (0.1)	9 (0.18)
A G C G G	0 (0.0)	0 (0.0)	5 (0.5)	0 (0.0)	2 (0.2)	7 (0.14)
A G G A C	0 (0.0)	0 (0.0)	2 (0.2)	0 (0.0)	3 (0.31)	5 (0.1)
Total	**1322**	**694**	**1008**	**1006**	**978**	**5008**

† Individual SNP alleles are represented in haplotype structures as follows: rs3087465 (G>A), rs4522809 (A>G), rs12493607 (G>C), rs1078985 (A>G) and rs1367610 (G>C).

‡ Haplotype frequencies were retrieved from the web-based application LDlink (https://ldlink.nci.nih.gov/?tab=ldhap). AFR: African; AMR: Ad Mixed American; EUR: European; EAS: East Asian; SAS: South Asian. Most frequent haplotype structures in each group are in bold.

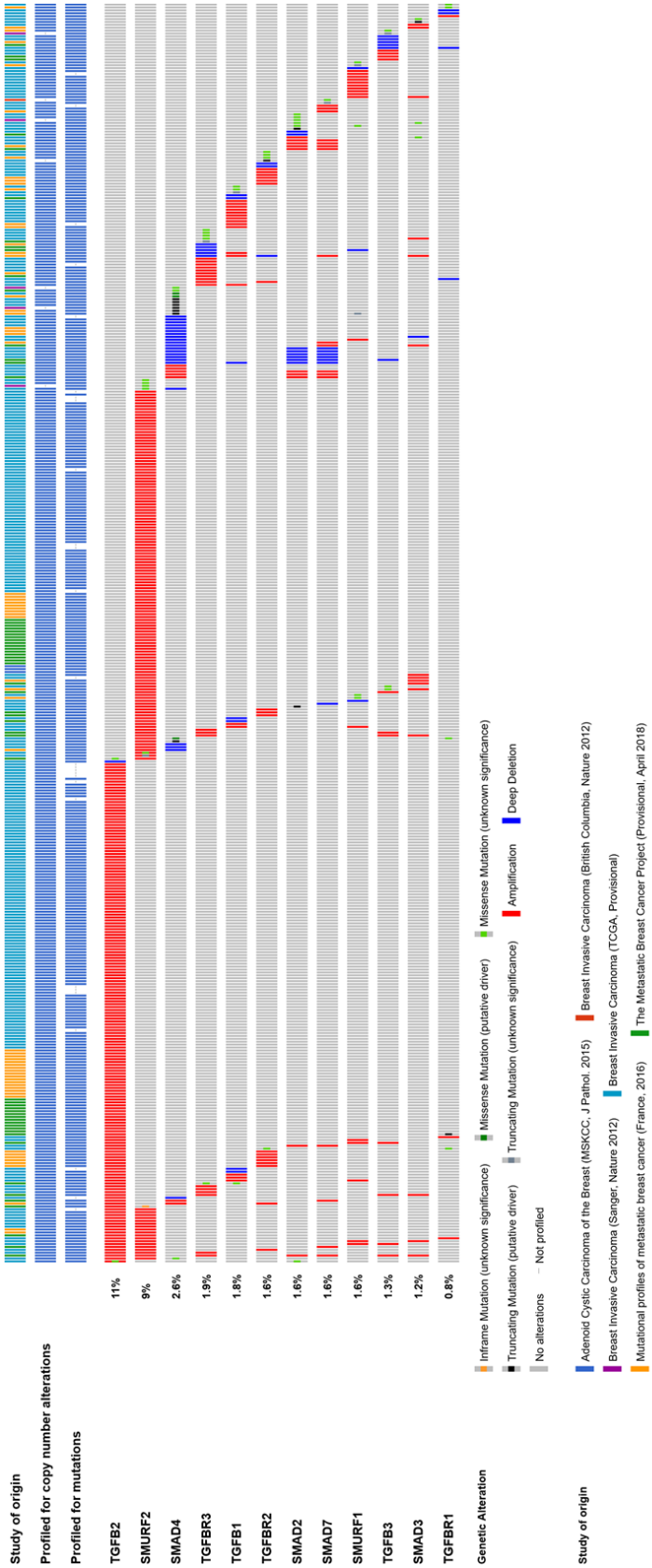

Figure S1. Oncoplot showing genetic alterations in TGFβ classical pathway main components. Oncoplot was generated through the cBioPortal database (http://www.cbioportal.org/) selecting for studies with primary samples (directly derived from patients) and with non-redundant samples.

3.4 ARTIGO 4: "TRANSFORMING GROWTH FACTOR BETA 1 (TGFB1) PLASMATIC LEVELS IN BREAST CANCER PATIENTS AND HEALTHY WOMEN: ASSOCIATION WITH PATIENTS' CHARACTERISTIC AND TGFB1 HAPLOTYPES"

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Transforming growth factor beta 1 (TGFβ1) plasmatic levels in breast cancer and neoplasia-free women: Association with patients' characteristics and *TGFB1* haplotypes

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ABSTRACT

Transforming growth factor beta 1 (TGFβ1) is a pleiotropic cytokine that acts in a context-dependent manner. In breast cancer (BC) this cytokine exerts subtype- and stage-specific roles, inhibiting poorly aggressive tumors while enhances the invasive potential of highly aggressive cancers. Single-nucleotide polymorphisms (SNPs) affecting TGFβ1 production largely reflect this pattern of association, but studies investigating systemic TGFβ1 levels in BC patients and their association with clinical features or SNPs produced conflicting conclusions. Therefore, the present work investigated plasmatic TGFβ1 levels through enzyme linked immunosorbent assay (ELISA) in 341 individuals previously genotyped for four *TGFB1* SNPs [G-800A (rs1800468), C-509T (rs1800469), T29C (rs1800470) and G74C (rs1800471)], encompassing 184 neoplasia-free women with clinical information regarding health status, 113 treatment-free pre-surgery BC patients and 44 treated BC patients. Results have shown that TGFβ1 levels varied greatly in function of health status in neoplasia-free women, and disease-free individuals had higher TGFβ1 levels than both treatment-free or treated BC patients. There was no correlation between TGFβ1 with clinicopathological features in treatment-free BC general group, but it was negatively correlated with tumor size in luminal-B-HER2$^+$ patients and with histopathological grade in triple-negative group. Also, *TGFB1* ACTG haplotype (from G-800A to G74C) was associated with decreased TGFβ1 levels compared to the reference GCTG haplotype, and regression analyses showed that this association was independent of age, health status or BC diagnosis. In conclusion, several factors may influence TGFβ1 levels, and ACTG haplotype seems to be an important factor regulating TGFβ1 production.

1. Introduction

Breast cancer (BC) is the most frequently diagnosed cancer and the leading cause of cancer death in women for most countries worldwide, showing an increasing trend of incidence that may be associated with global population growth and aging [1]. BC clinical presentation is heterogenous, encompassing at least four well-defined subgroups with differing etiology and clinical evolution characterized in clinical routine [2–4] and presenting patient-to-patient variability in disease outcomes, even when considering a single subtype, which complicate its clinical management [5].

Genetic polymorphisms are known to contribute at least in part to this inter-patient variability in disease risk and prognosis [6], while tumor stroma and stromal-derived cytokines and growth factors are reported to play a role in disease progression; in this way, polymorphisms in stromal-related genes might play a role in BC risk and evolution, and may exert subtype-specific effects in this disease [7].

Transforming growth factor β (TGFβ) superfamily of growth factors include several members involved in many embryogenic, physiologic and pathologic processes. Within this large superfamily, the TGFβ

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subfamily comprises TGFβ1, TGFβ2 and TGFβ3 isoforms, which are pleiotropic cytokines acting through the same set of transmembrane receptors to promote cell- and context-dependent effects. Among these, TGFβ1 is the most abundant form, being expressed at variable levels in all human tissues and acting on virtually all human cell types [8].

TGFβs are secreted in an inactive form, linked to a large latent complex, which attaches to the extracellular matrix [9–11], where inactive TGFβ remains bonded until its release by matrix proteases, acidification or oxidative stress in extracellular *milieu* [12]. After releasing, TGFβ acts on nearby cells through binding to the serine/threonine kinase TGFβ receptors type 2 and type 1 (TGFβRII and TGFβRI, respectively), that classically activate SMAD2 and 3 transcription factors (TFs), which act in concert with cell-specific TFs. Alternatively, pathways such as the Ras-MAPK, PI3K/Akt/mTOR and small Rho GTPases are activated [13].

Hence, depending on the pathways and transcription factors active on the target cell, different responses are produced by TGFβ. This effect is largely illustrated in BC, where TGFβ mainly inhibits proliferation and promotes apoptosis of tumor cells from initial or poorly aggressive subtypes, such as luminal-A, while enhances epithelial-to-mesenchymal transition (EMT) and promotes immune tolerance and disease progression in advanced tumors and aggressive subtypes, such as those overexpressing HER2 and from triple-negative (TN) subgroup [14–16].

Despite these largely characterized roles of TGFβ1 in BC, studies investigating systemic or tissue TGFβ1 in BC patients have produced controversial results [17–20]. Furthermore, TGFβ1 systemic levels have been shown to be regulated genetically [21], and several single nucleotide polymorphisms (SNPs) in components of TGFβ pathway potentially altering their expression, largely reflects the context-specific effects of TGFβ1 in BC [22–25]. Also, many SNPs in *TGFB1* gene have shown potential to modulate TGFβ1 production in different contexts, but associations between these individual SNPs and circulating TGFβ1 have been controversial and the study of their haplotype structures is necessary to fully understand their contribution to TGFβ1 production [26].

Therefore, the present study aimed to investigate TGFβ1 plasmatic levels in Brazilian BC patients and neoplasia-free women as well as its association with BC characteristics, such as molecular subtypes and clinicopathological features, and with *TGFB1* promoter region [G-800A (rs1800468) and C-509T (rs1800469)] and signal-peptide [T29C (rs1800470) and G74C (rs1800471)] SNPs and their haplotype structures.

2. Methods

2.1. Sample collection and characterization

Patients were selected based on their clinical features and their *TGFB1* genotypes for rs1800468 (G-800A), rs1800469 (C-509T), rs1800470 (T29C) and rs1800471 (G74C) from a previous case-control study by our group [24]. Peripheral blood samples were collected in vacuum tubes containing EDTA and plasma was separated from blood cells through centrifugation at 1200g for 7 min, aliquoted and stored in 1.5 mL microtubes at −80 °C until use.

For control group, 184 patients were selected, consisting of individuals with no personal history of neoplasia, no familial history of BC, with a mean age of 52 years [standard deviation (sd) = 14] and a median age of 53 years [interquartile range (IQR) = 18]. Of these, 63 patients declared not having any infectious, allergic or inflammatory manifestation, nor any chronic inflammatory or autoimmune disease, and were not under pharmacologic treatment for any condition on collection time; for this group, the mean age was 58 years (sd = 10) and the median age was 56 (IQR = 11). Another 33 patients declared to have age-related diseases, such as osteoporosis (4), type-2 diabetes (18), hypertension (2), arthrosis (7) and gastritis (2); the mean age for this cohort was 63 years (sd = 9) and the median age was 64 years (IQR = 10). For the remaining 88 patients, there was no information regarding current health status; these patients had a mean age of 43 years (sd = 14) and a median age of 44 years (IQR = 22).

Table 1
Breast cancer patients' clinicopathological features.

Parameter[a]	Classification	Breast cancer groups [n(%)] Treatment-free (n = 113)	Treated (n = 44)
Age	< 40	15 (13.3)	6 (13.6)
	40–49	26 (23.0)	15 (34.1)
	50–59	32 (28.3)	13 (29.5)
	60–69	25 (22.1)	8 (18.2)
	70–79	11 (9.7)	2 (4.5)
	> 80	4 (3.6)	0 (0.0)
	Mean (sd)	55 (13)	50 (11)
	Median (IQR)	51 (15)	51 (15)
ER	Positive	88 (77.9)	26 (59.1)
	Negative	25 (22.1)	18 (40.9)
PR	Positive	54 (47.8)	23 (52.3)
	Negative	59 (52.2)	21 (47.7)
HER2	Positive	18 (15.9)	12 (27.3)
	Negative	95 (84.1)	32 (72.7)
Subtype	Luminal-A	79 (69.9)	20 (45.5)
	Luminal-B ($HER2^+$)	11 (9.7)	6 (13.6)
	HER2-enriched	7 (6.2)	6 (13.6)
	Triple-negative	16 (14.2)	12 (27.3)
Tumor size	< 1.5	28 (24.8)	12 (28.6)
	1.51–3.0	45 (39.8)	14 (33.3)
	> 3.0	40 (35.4)	16 (38.1)
	Missed	0	2
	Mean (sd)	2.9 (2.1)	3.1 (2.4)
	Median (IQR)	2.3 (1.8)	2.3 (2.5)
Hist.grade	I	11 (10.1)	4 (10.3)
	II	51 (46.8)	14 (35.9)
	III	47 (43.1)	21 (53.8)
	Missed	4	5
Ki67	Low	26 (23.2)	1 (7.7)
	Intermediate	54 (48.2)	5 (38.5)
	High	32 (28.6)	7 (53.8)
	Missed	1	31
p53	Positive	40 (36.7)	7 (63.6)
	Negative	69 (63.3)	4 (36.4)
	Missed	4	33
Lymph node metastasis	Positive	52 (46.4)	27 (62.8)
	Negative	60 (53.6)	16 (37.2)
	Missed	1	1
Stage	0	7 (6.2)	0 (0.0)
	I	24 (21.2)	8 (18.2)
	II	46 (40.7)	15 (34.1)
	III	28 (24.8)	20 (45.5)
	IV	8 (7.1)	1 (2.3)

[a] ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

BC group consisted of 157 patients divided into two arms: a treatment-free arm, consisting of neoadjuvant-treatment-free patients who had blood samples collected immediately before the tumor resection surgery (n = 113); and an "under-treatment" arm, which consisted of patients that had already undergone tumor resection surgery and were under adjuvant therapy (n = 44) during blood collection time.

Immunostaining scores for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), measured according to the American Society of Clinical Oncology (ASCO) protocols [27,28], were used to classify patients into the intrinsic BC subtypes: Luminal-A (LA; ER/PR^+HER2^-), Luminal-B-$HER2^+$ (LB; ER/PR^+HER2^+), HER2-enriched (HER2; $ER^-PR^-HER2^+$) and triple negative (TN; $ER^-PR^-HER2^-$). Clinicopathological features were retrieved from the medical records of patients available at Londrina Cancer Hospital and are shown in Table 1.

The frequencies of the *TGFB1* SNPs genotypes and haplotypes were similar among control individuals, treatment-free and treatment-

Table 2
TGFB1 SNP genotypes and haplotypes frequencies among study groups.

TGFB1 Genotype	Control [n (%)]		Breast cancer [n (%)]	
	Total	No disease	Treat.-free	Treated
G-800A				
G/G	150 (81.5)	55 (87.3)	101 (89.4)	35 (79.5)
G/A	32 (17.4)	7 (11.1)	11 (9.7)	9 (20.5)
A/A	2 (1.1)	1 (1.6)	1 (0.9)	0 (0.0)
C-509T				
C/C	99 (53.8)	29 (46.0)	51 (45.1)	16 (36.4)
C/T	55 (29.9)	26 (41.3)	46 (40.7)	21 (47.7)
T/T	30 (16.3)	8 (12.7)	16 (14.2)	7 (15.9)
T29C				
T/T	54 (29.3)	19 (30.2)	36 (31.9)	10 (22.7)
C/T	89 (48.4)	32 (50.8)	58 (51.3)	25 (56.8)
C/C	41 (22.3)	12 (19.0)	19 (16.8)	9 (20.5)
G74C				
G/G	144 (78.3)	52 (82.5)	96 (85.0)	39 (88.6)
G/C	38 (20.6)	11 (17.5)	15 (13.3)	4 (9.1)
C/C	2 (1.1)	0 (0.0)	2 (1.7)	1 (2.3)
Haplotypes				
GCTG/GCTG	32 (17.4)	13 (20.6)	26 (23.0)	6 (13.6)
GCTG/Other	93 (50.5)	34 (54.0)	59 (52.2)	22 (50.0)
GTCG/GTCG	25 (13.6)	8 (12.7)	14 (12.4)	7 (15.9)
GTCG/Other	59 (32.1)	26 (41.3)	42 (37.2)	21 (47.7)
GCCC/GCCC	2 (1.1)	0 (0.0)	1 (0.9)	1 (2.3)
GCCC/Other	36 (19.6)	10 (15.9)	15 (13.3)	2 (4.5)
ACTG/ACTG	2 (1.1)	1 (1.6)	0 (0.0)	0 (0.0)
ACTG/Other	30 (16.3)	7 (11.1)	12 (10.6)	8 (18.2)
GCCG/GCCG	0 (0.0)	0 (0.0)	1 (0.9)	0 (0.0)
GCCG/Other	20 (10.9)	4 (6.3)	5 (4.4)	4 (9.1)
GTTG/GTTG	1 (0.5)	0 (0.0)	2 (1.8)	0 (0.0)
GTTG/Other	2 (1.1)	0 (0.0)	2 (1.8)	0 (0.0)
GCTC/GCTC	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
GCTC/Other	2 (1.1)	1 (1.6)	2 (1.8)	2 (4.5)
ATCG/ATCG	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
ATCG/Other	2 (1.1)	0 (0.0)	1 (0.9)	1 (2.3)

receiving BC patients (Table 2). More information about collection procedures and sample characteristics can be found in previous publications [23,24,29]. All procedures were approved by the Ethics Committee for research involving human beings from Londrina State University (CEP/UEL 189/2013 – CAE 17123113400005231) and all participants signed a free-informed consent form prior to the biological material collection.

2.2. TGFβ1 quantification

TGFβ1 was quantified in patients' plasma through Enzyme-Linked Immunosorbent Assay (ELISA) using the BD OptEIA™ Human TGFβ1 ELISA set (Cat. No. 559119, BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. For each assay a calibration curve (ranging from 0.125 to 8 ng/mL) was prepared by serial dilution of recombinant TGFβ1, supplied with the kit, in assay diluent [10% fetal bovine serum (Gibco®, Grand Island, NY, USA) in phosphate buffered saline (PBS, Laborclin®, Pinhais, PR, Brazil)]. Both calibration curves and samples were prepared in duplicate. Plasma samples were diluted (1:5) in PBS, acidified by hydrochloric acid (1 M) and neutralized by sodium hydroxide (1 M) prior to the assay, according to the manufacturer's instructions to quantify total (latent and activated) TGFβ1. Duplicates showing coefficients of variation higher than 20% were not considered. Samples outside the calibration curve limits were repeated using a different dilution. Plates were washed with 0.05% Tween-20 in PBS between each step using an automated ELx50™ Microplate Strip Washer (BioTek Instruments Inc., Winooski, VT, USA) following the manufacturer's instructions and the absorbance at 450 nm was read in an ELx800™ Microplate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA). Data was processed using the Gen5™ software (BioTek Instruments Inc., Winooski, VT, USA).

2.3. Statistical analyses

Since the data did not have normal distribution as assessed through Shapiro-Wilk test, non-parametric tests were applied in all statistical analyses. Mann-Whitney *U* test was applied for comparisons involving only two groups. For comparison between three or more groups, the Kruskal-Wallis test was applied, followed by the Dunn's post-test.

To test for correlations between TGFβ1 and clinicopathological features or *TGFB1* genetic variants, Kendall's rank correlation tests were performed. SNPs were coded according to the following models: genotypic (wild homozygotes = 0, heterozygotes = 1, variant homozygotes = 2), dominant (wild homozygotes = 0, heterozygotes and variant homozygotes = 1) and recessive (wild homozygotes and heterozygotes = 0, variant homozygotes = 1). For haplotypes, models were constructed to test the effects of each haplotype versus the remaining pool of haplotypes (genotypic model: one copy of tested haplotype = 1, two copies of tested haplotype = 2, other = 0) or considering the more frequent haplotype (GCTG) as reference (genotypic model: GCTG/GCTG individuals = 0, GCTG/tested haplotype = 1, two copies of tested haplotype = 2); dominant and recessive models were also derived for haplotypes in each of these situations.

To test the combined effect of multiple variables in TGFβ1 plasmatic levels, binary logistic regression analyses were performed. In these analyses, TGFβ1 was dichotomized according to the median TGFβ1 plasmatic levels of the sample set being analyzed (bellow or equal to the median vs above the median) and fixed as the dependent variable, and relevant parameters were set as explanatory variables.

The statistical tests were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) and IBM® SPSS® Statistics 20 (IBM®, Armonk, New York, USA) software for windows operational system. All statistical analyses were two-tailed and had a 5% significance level. Graphs are represented as dot plots where control-group individuals are represented by empty geometric forms with grey borders while BC patients are represented by black filled geometric forms; bars represent the median.

3. Results

3.1. TGFβ1 levels in breast cancer patients and controls

TGFβ1 levels in control group (median = 6.63 ng/mL; n = 184) varied significantly among individuals who declared not having any disease (median = 10.92 ng/mL; n = 63) and individuals having age-related diseases (median = 3.42 ng/mL; n = 33; $p < 0.001$) or individuals without health status information (median = 5.64 ng/mL; n = 88; $p < 0.01$; Fig. 1A). Therefore, subsequent analyses were made considering patients with no self-declared disease as the reference control group.

TGFβ1 levels were significantly higher in disease-free control women in comparison to treatment-free (median = 5.43 ng/mL; n = 113; $p < 0.01$) or treatment-receiving BC patients (median = 4.44 ng/mL; n = 44; $p < 0.01$; Fig. 2A). Considering only non-treated BC samples, luminal-A subtype showed lower TGFβ1 plasmatic levels compared to control (Fig. 2B), while other subtypes did not reach significance. No significant difference was evidenced in TGFβ1 levels among different BC subtypes.

3.2. TGFβ1 levels and breast cancer clinicopathological features

Correlations were tested between TGFβ1 plasmatic levels and clinicopathological features at diagnosis in treatment-free BC patients (Table 3). No significant correlation was observed in general BC group

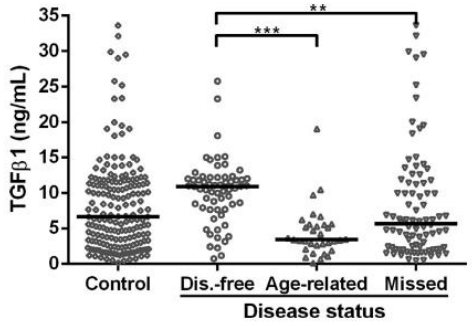

Fig. 1. TGFβ1 levels in control group. TGFβ1 levels were significantly higher in individuals declaring to have no disease at diagnosis compared to individuals with age-related diseases and with no information regarding disease status at collection time.

(Table 3). However, in HER2-positive BCs TGFβ1 levels were negatively correlated with tumor size (Table 3, HER2$^+$, Tau-b = −0.33; $p < 0.05$), and this correlation was stronger for luminal-B HER2$^+$ BCs (Table 3, LB, Tau-b = −0.367; $p < 0.05$), but was not observed in HER2-enriched subtype (Table 3, HER2, Tau-b = −0.15, $p > 0.05$). Also, a negative correlation with histopathological grade (HG) was observed in triple-negative subtype (Table 3, TN, Tau-c = −0.5, $p < 0.05$). However, these results should be considered with caution due to the small sample size in these subgroups. Of note, TGFβ1 levels were also compared among the groups defined by categoric clinicopathological features (e.g.: for lymph node metastasis, positive group vs negative group) within each subtype using Kruskal-Wallis and Mann-Whitney tests besides correlation tests, but no significant difference was found.

Furthermore, to avoid the potential confounding effects of genetic variants, correlations between TGFβ1 levels and clinicopathological parameters were tested for general BC considering only homozygous individuals for GCTG (n = 26) or GTCG (n = 14) haplotypes, and no significant difference was found in these analyses.

3.3. TGFβ1 levels and TGFB1 genetic variants

Initially, correlations between TGFβ1 levels and *TGFB1* SNPs or haplotype structures in treatment-free BC group and in disease-free controls were tested. No correlation was found in BC group and no single SNP was associated with differential TGFβ1 plasmatic levels neither in BC nor in control group (Supplementary Fig. 1). Otherwise, significant negative correlations in the control group were evidenced for ACTG haplotype in comparison to the reference GCTG haplotype in genotypic (Tau-c = -0.417; $p = 0.008$; n = 18) and dominant (Tau-c = −0.531; $p = 0.008$; n = 18) models.

Indeed, TGFβ1 levels were lower in ACTG/GCTG and ACTG/ACTG individuals than in GCTG/GCTG individuals (Supplementary Fig. 2 and Fig. 3A and B, n = 18). Strikingly, this difference was evident, and gradually stronger, considering also individuals with age-related disease (Fig. 3C and D, n = 28) and the total control group (Fig. 3D and E, n = 51) in the analyses. No significant difference in TGFβ1 plasmatic levels was found for other haplotypes either in BC or control group in any model tested (Supplementary Fig. 2).

3.4. Multivariate analysis of factors associated with TGFβ1 levels in control and BC patients

Factors associated with TGFβ1 levels in previous analyses were tested in multivariate logistic regression models to check for their individual contribution to TGFβ1 plasmatic levels (Table 4).

In control group, ACTG haplotype in dominant model remained significantly associated with decreased TGFβ1 levels in comparison to the reference GCTG haplotype even when adjusted by age and disease status, which were also significant factors in this multivariate model (Table 4, "Control" group).

The association of ACTG with TGFβ1 levels considering the general sample (both control and BC patients) was also tested, and it was also capable of predicting decreased cytokine levels when adjusted by cancer status (control, treatment-free BC or treated BC) and age in this model (Table 4, "General" group).

4. Discussion

In the current work, we have found that TGFβ1 levels were higher in neoplasia-free women with no reported disease than in neoplasia-free women with age-associated disease, women without health information and BC patients, either treatment-free or under-treatment. For BC patients we found no difference among subtypes, and weak correlations, which were not confirmed in other tests, were found between TGFβ1 and tumor size in Luminal-B-HER2$^+$ patients and with HG in TN subtype. Furthermore, ACTG haplotype was an independent predictor of lower TGFβ1 plasmatic levels in comparison to GCTG (prevalent) haplotype.

TGFβ1 is an extremely pleiotropic cytokine acting in many physiological and pathological processes in a cell- and context-dependent manner regulating cell survival, proliferation and differentiation [30]. These effects are clear among different BC subtypes, with less aggressive Luminal-A subtype tumors showing mainly cytostatic and apoptotic responses to TGFβ while highly proliferative and invasive subtypes, such as those overexpressing HER2 and from TN subgroups,

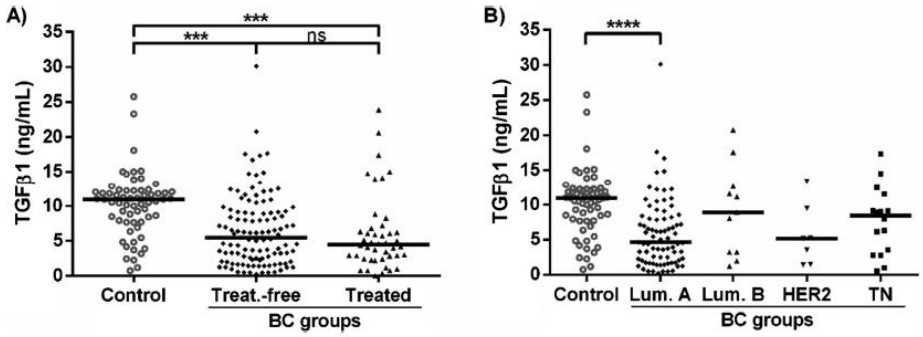

Fig. 2. Comparison of TGFβ1 plasmatic levels among disease-free controls and BC patients. (A) Disease-free controls had higher TGFβ1 levels than treatment-free ($p < 0.001$) or under-treatment patients ($p < 0.001$). (B) TGFβ1 levels were higher in disease-free controls than in luminal-A (Lum. A) patients ($p < 0.0001$), but did not vary among BC subgroups in treatment-free BC group.

Table 3
Correlation between plasmatic TGFβ1 and clinicopathological features in BC subgroups.

Feature[a]	BC groups[b] (correlation coefficient [Tau])						
	BC (n = 113)	HR^+ (n = 90)	$HER2^+$ (n = 18)	Lum. A (n = 79)	Lum. B (n = 11)	HER2 (n = 7)	TN (n = 16)
Age	0.013	−0.007	0.158	−0.039	0.294	−0.195	0.185
Size	−0.04	−0.045	−0.330*	0.003	−0.367*	−0.150	−0.154
HG	−0.003	0.008	−0.157	0.026	−0.223	0.163	−0.500*
Ki67	0.125	0.155	0.058	0.125	0.298	−0.327	−0.270
p53	0.083	0.071	0.266	0.000	0.296	0.327	−0.094
LNM	0.158	0.169	−0.049	0.148	−0.066	0.000	0.016
Stage	0.009	0.019	−0.067	0.036	−0.154	0.061	−0.167

[a] HG: Histopathological grade; LNM: Lymph node metastasis.
[b] BC: Breast cancer (general group); $HER2^+$: HER2-positive samples (irrespective of HR); HER2: HER2-enriched subtype; HR^+: Hormonal-receptor-positive samples; LA: Luminal-A; LB: Luminal-B; TN: Triple-negative.
* $p < 0.05$.

show enhanced metastatic potential [16,31–33]. Also, different chemokine networks are triggered by overactivation [33,34] or abrogation [35,36] of TGFβ in different tumors, coordinating BC metastasis to target organs, including lymph node, bone marrow, lung, brain and liver.

Despite these well documented effects of TGFβ1 in BC, studies analyzing systemic TGFβ1 levels in this disease produced contradictory results: while an initial work have found no difference between BC patients and neoplastic-free individuals [17] other studies reported that BC patients have increased circulating TGFβ1 [18,20,37,38]. Further, while some works suggested that TGFβ1 would decrease after primary tumor removal [37,38], other failed to identify this effect [20].

Reasons for these controversies are difficult to grasp, but may include issues regarding analytical protocols, encompassing the material type (serum or plasma), processing and analysis, as well as sample heterogeneity, including differences in proportions of patients with differing tumor subtypes, clinical characteristics or disease stage within the sample, as well as the composition of control group. The current study showed increased TGFβ1 plasmatic levels in disease-free controls in comparison to treatment-free or under-treatment (post-surgery) BC patients; however, TGFβ1 levels did not vary between these two BC groups. Also, in non-neoplastic individuals, health status may influence TGFβ1 plasmatic levels, since individuals with no self-declared disease had significantly higher TGFβ1 than individuals with age-related diseases and individuals without disease-status information.

Regarding tumor subtypes, a previous work has shown that ER^-PR^- patients had higher TGFβ1 than ER/PR^+ BCs [19], while another work have shown that TN patients, which are also ER^-PR^-, had decreased TGFβ1 when compared to general BC group [20]. In the current study we found no significant difference in TGFβ1 plasmatic levels among BC subtypes; however, the sample size for rare subtypes both in our work and in previous studies is small, and might be underpowered to detect any difference in systemic TGFβ1 among these BC subtypes.

Controversial results have been also produced by studies correlating TGFβ1 levels with tumor stage and clinicopathological features: while some studies reported increased TGFβ1 in advanced or metastatic BCs [18,39,40], other works did not confirmed these findings [17,20]. Given the subtype-specific effects of TGFβ1 in BC [16,41], it is reasonable to expect that these correlations may also be influenced by the proportion of BC subtypes in studied samples, and subtype-stratified analyses would provide more clinically relevant information for these data.

In this context, the present work did not show any association between TGFβ1 levels and disease stage, lymph node metastasis or any other clinicopathological feature tested in general treatment-free BC group; otherwise, negative correlations were observed between TGFβ1 levels and tumor size in $HER2^+$ BCs, especially from luminal-B subtype, and histopathological grade in TN cancers; however, due to the small sample size in BC subgroups other than the more frequent hormonal receptor-positive and luminal-A subtype, these results should be viewed with caution, and their significance await confirmation from further studies.

Another source of variation might arise from the genetic background of populations being analyzed in different studies as well as in case and control groups within the same study, once TGFβ1 levels have been shown to be regulated genetically [21] and many SNPs with varying frequencies among populations worldwide have shown potential to regulate TGFβ1 production at different levels (transcription, translation and secretion) and alter susceptibility for many diseases, including BC [26].

A previous work by our group investigated the association between *TGFB1* promoter region (G-800A and C-509T) and signal peptide (T29C and G74C) SNPs and their haplotype structures with the susceptibility and clinical presentation of different BC subtypes, identifying subtype-specific associations reflecting the documented TGFβ1 roles on BC [24]. Therefore, in the present work we selected sub-cohorts of controls and BC patients from that study to investigate the possible influences of these genetic variants on TGFβ1 plasmatic levels.

Notably, consulting the ENCODE and The Roadmap Epigenomic Locus Mapping Consortium data [42] available through HaploReg v4.1 databases [43], all the four SNPs have shown potential as transcriptional regulators, since they all have expression quantitative trait loci (eQTL) hits for *TGFB1* or nearby genes, are associated with promoter and enhancer histone markers and with DNAse peaks in multiple tissues and are predicted to alter the binding motifs for multiple transcription factors: G-800A alters the binding motifs for ATF3 and SP1; C-509T alters the HNF4 and NKX2 binding sites; T29C alters the binding sites for ASCL2, BHLHE40, CTCF, MYF, NRSF, PBX3, RAD21 and SIN3AK20; and G74C alters the AP1, AP2, BCL, E2F, ELF1, ETS, HEY1, SRF, SIN3AK20, YY1 and p300 binding sequences.

Furthermore, these SNPs have also shown potential to regulate TGFβ1 in different contexts: G-800A A allele was associated with decreased TGFβ1 secretion from *in vitro*-stimulated peripheral blood mononuclear cells (PBMCs) [44]; C-509T was firstly described as a genetic factor explaining 8.2% of additive genetic variance of TGFβ1 systemic levels in a twin study [21] and *in vitro* assays showed differential binding of TFs between the two alleles and differential expression through reporter assays [45–48]; for T29C, *in vitro* assays with transfected constructs showed that C-allele-transfected HeLa cells secreted almost 3-fold more TGFβ1 than T-allele-transfected cultures [49], and differential endoplasmic reticulum transport efficiency was suggested [50]; finally, for G74C SNP, stimulated PBMCs from GC individuals secreted significantly less TGFβ1 than those from GG individuals in culture [51]. Other *TGFB1* SNPs beyond those represented here were also shown to potentially impact TGFβ1 production [52,53].

Despite the proven roles for these SNPs in TGFβ1 under controlled conditions, studies investigating their association with TGFβ1 expression systemically or in tissue biopsies have produced controversial

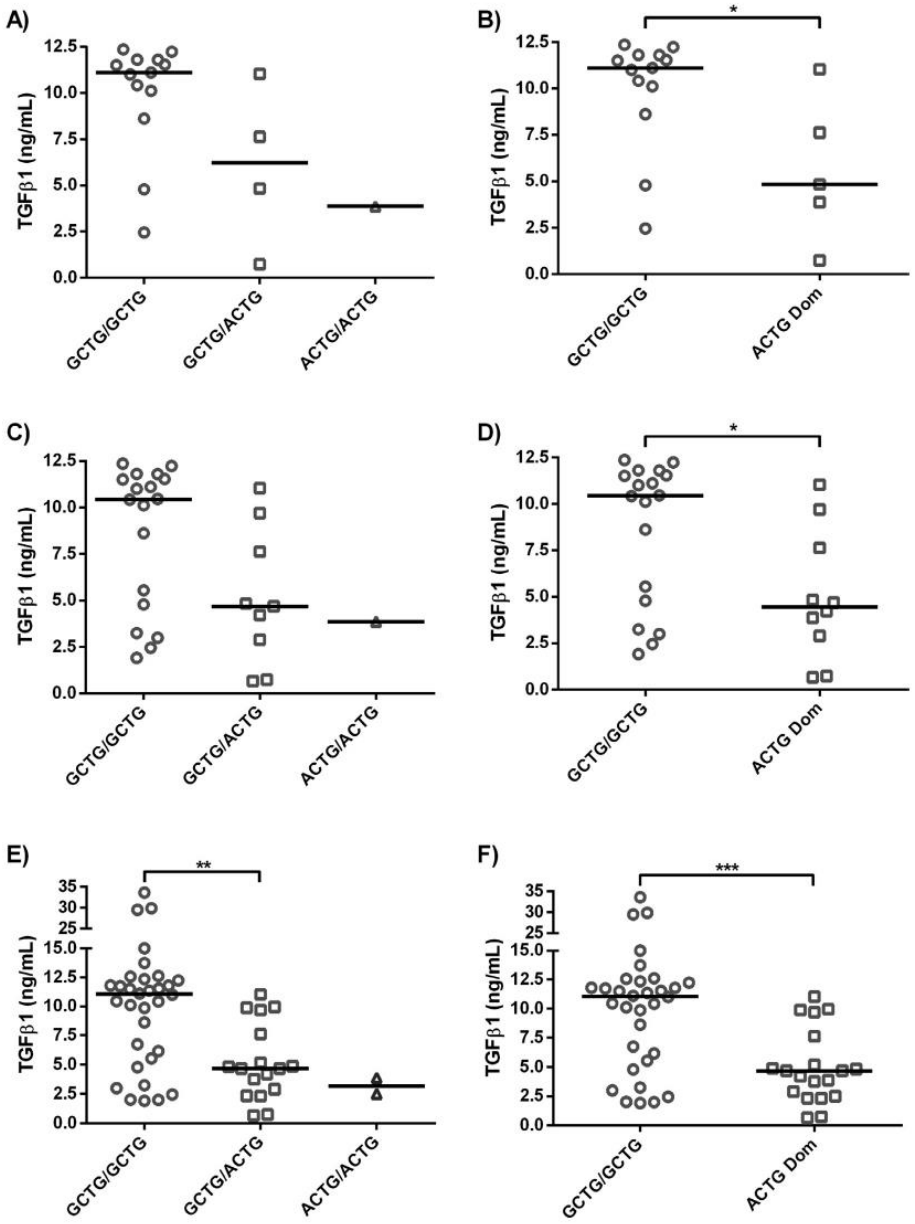

Fig. 3. ACTG haplotype is associated with decreased TGFβ1 levels compared to GCTG haplotype. TGFβ1 levels for ACTG haplotype were compared against GCTG haplotype in genotypic (A, C and E) and dominant (B, D and F) models in control individuals without disease (A and B), individuals without disease plus individuals with age-associated diseases (C and D) and the whole control group (E and F). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

results, with some of them confirming their predicted influence on TGFβ1 production [47,54–57] while others failed to observe these effects [34,58–63]. Divergences might arise from the small effect exerted by each of these SNPs on TGFβ1 production alone, which despite prominent under controlled conditions, are difficult to identify under natural contexts; furthermore, the combination of SNPs in haplotype blocks might mask their individual roles [26]. Further, since each SNP site bonds to different sets of TFs, tissue- and context-specific roles on the regulation of TGFβ1 production are expected for each of them.

Indeed, we did not find any significant correlation between TGFβ1 levels and any SNP individually, neither in the control nor in the BC group. However, ACTG haplotype was associated with decreased TGFβ1 levels compared to the reference GCTG allele in healthy controls both in genotypic and dominant models. Also, considering individuals with age-related diseases and without disease information in these analyses did not change these results. Strikingly, logistic regression models adjusted by age and disease status showed that ACTG was independently associated with TGFβ1 values below the median on the GCTG background. Furthermore, in general sample, it was also associated with decreased TGFβ1 levels independently of BC diagnosis.

Table 4
Multivariate logistic regression analyses for factors associated with TGFβ1 levels.

Group	Factors in the model	Exp(B)[a]	95%CI[b]	*p*-value
Control (n = 51)	Model 1			
	Age	0.95	0.906–0.999	0.044
	Disease status			
	No disease	Reference	–	–
	Age-related/missed	0.145	0.028–0.747	0.021
	ACTG vs GCGT (Dominant)			
	GCTG/GCTG	Reference	–	–
	GCTG/ACTG + ACTG/ACTG	0.155	0.039–0.622	0.009
General (n = 90)	Model 2			
	Age	0.983	0.953–1.014	0.277
	Control vs BC			
	Control	Reference	–	–
	Treatment-free BC	0.702	0.268–1.843	0.473
	Treated BC	0.076	0.008–0.706	0.023
	ACTG vs GCGT (Dominant)			
	GCTG/GCTG	Reference	–	–
	GCTG/ACTG + ACTG/ACTG	0.302	0.109–0.837	0.021

[a] Exp(B): Exponentiation of B factor (Odds ratio);
[b] CI: Confidence interval.

These data point ACTG haplotype as an independent factor influencing TGFβ1 levels in general population. Since this haplotype differ from the reference GCTG only by the G-800A allele, these results are in accordance with previous research showing decreased TGFβ1 secretion from stimulated PBMCs from G-800A A allele carriers with no detected effect of other SNPs, such as C-509T, T29C and G74C, in the same settings [44]. Notwithstanding, our results highlight the need to control for other variants in haplotype structures when investigating genotype-phenotype relationships under natural and uncontrolled conditions.

5. Conclusion

In conclusion, TGFβ1 levels are highly variable even in apparently homogeneous populations, which might be attributable to multiple factors in human subjects. Genetic polymorphisms play a major role in this phenomenon, and multiple SNPs may act in concert regulating TGFβ1 production, making it necessary to consider their joint actions in haplotype structures when analyzing their effects. Our results highlight ACTG haplotype as a regulator of TGFβ1, and provide new insights to the comprehension of the complex regulation of TGFβ1 production and about the genetic architecture of the many physiological and pathological processes in which this pleiotropic cytokine takes part.

CRediT authorship contribution statement

Glauco Akelinghton Freire Vitiello: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Marla Karine Amarante:** Methodology, Supervision, Writing - review & editing. **Julie Massayo Maeda Oda:** Conceptualization, Methodology, Investigation. **Bruna Karina Banin Hirata:** Investigation. **Carlos Eduardo Coral Oliveira:** Conceptualization, Methodology, Investigation, Resources, Supervision, Writing - review & editing. **Clodoaldo Zago Campos:** Investigation, Resources. **Karen Brajao Oliveira:** Resources, Supervision. **Roberta Losi Guembarovski:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Maria Angelica Ehara Watanabe:** Conceptualization, Supervision, Writing - review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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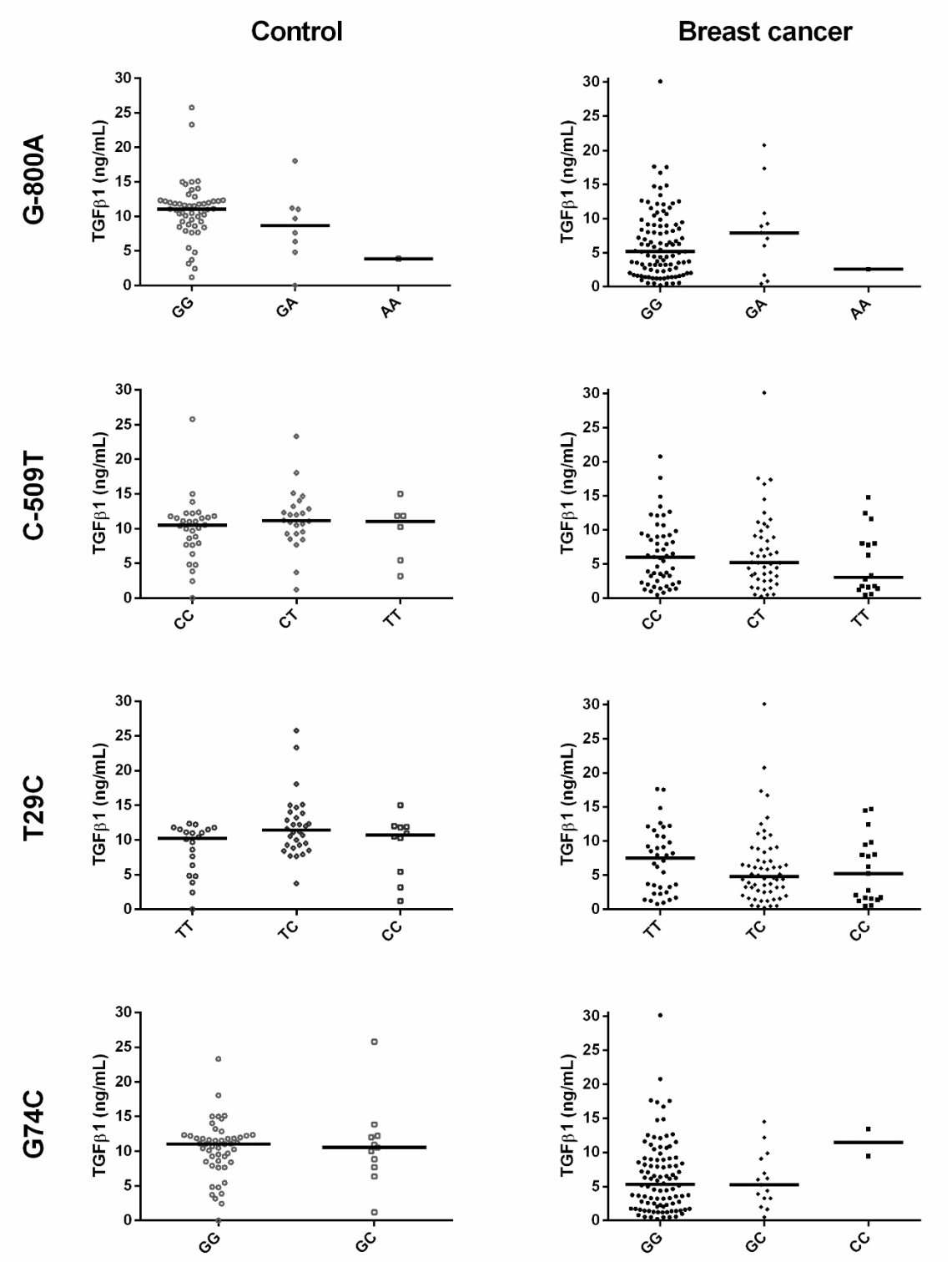
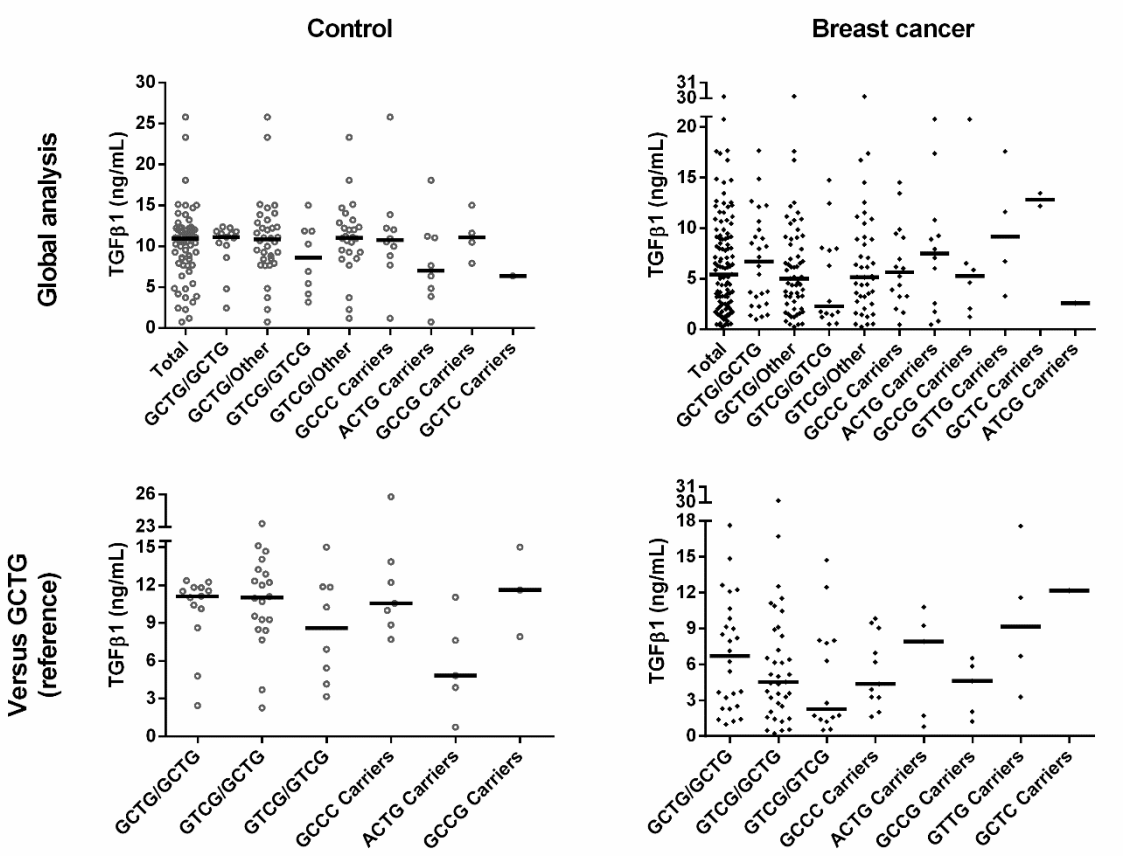
Supplementary material

Transforming growth factor beta 1 (TGFβ1) plasmatic levels in breast cancer and neoplasia-free women: association with patients' characteristics and *TGFB1* haplotypes

Vitiello, G.A.F.; Amarante, M.K.; Oda, J.M.M.; Banin-Hirata, B.K.; Oliveira, C.E.C.; Campos, C.Z.; Oliveira, K.B.; Losi-Guembarovski, R.; Watanabe, M.A.E.



Supplementary figure 1. TGFβ1 plasmatic levels per *TGFB1* SNPs genotypes in disease-free control women (left panel) and treatment-free breast cancer patients (right panel). No significant difference in TGFβ1 levels was observed for any individual SNP.



Supplementary figure 2. TGFβ1 plasmatic levels according to *TGFB1* haplotypes in disease-free women (left panel) and treatment-free breast cancer patients (right panel). Statistical analyses were conducted considering the effect of each haplotype against the total sample (upper charts) or fixing the most frequent GCTG haplotype as the reference (lower panel).

3.5 ARTIGO 5: "TRANSFORMING GROWTH FACTOR BETA 1 (TGFB1) PATHWAY COMPONENTS IN BREAST CANCER TISSUE FROM AGGRESSIVE SUBTYPES: CORRELATION WITH SINGLE NUCLEOTIDE POLYMORPHISMS, PLASMATIC TGFB1 AND CLINICOPATHOLOGICAL FEATURES"

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Abstract
Transforming growth factor β signaling exerts context-specific effects in breast cancer (BC) pathogenesis, acting as a tumor suppressor in less aggressive subtypes and in initial tumors, but enhancing the invasive potential of highly aggressive BCs. Single nucleotide polymorphisms (SNPs) in TGFβ-signaling components where shown to play a role in the genetic control of their expression and in BC susceptibility and clinical presentation. Despite this, studies investigating the association between the protein expression of TGFβ-signaling molecules and BC prognosis rarely considered disease subtypes and SNPs. Therefore, the present study investigated the expression of TGFβ1, TGFβRII and p-SMAD2/3 through immunohistochemistry in primary tumor tissue from 34 patients with luminal-B (LB), HER2-enriched (HER2) and triple negative (TN) BC subtypes genotyped for *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) and *TGFBR2* (rs3087465) SNPs. A strong positive correlation was observed between the expression of these markers in tumor tissue in pairwise analyses, and an inverse correlation was observed between intratumor and plasmatic TGFβ1 levels in TN BCs. However, no correlation was found between them and the analyzed SNPs. Otherwise, in LB tumors, p-SMAD2/3 was associated with older age at diagnosis and inversely correlated with p53 mutation and lymph-node metastasis, while tumor-size negatively correlated with TGFβ1 and TGFβRII in this same subgroup. Also, in wild-type p53 tumors, tumor size and Ki67 negatively correlated with both TGFβ1, TGFβRII and p-SMAD2/3. Therefore, these results are consistent with TGFβ-signaling acting as a tumor suppressor in less aggressive BC subgroups, such as those in LB subtype and without p53 mutations.

Keywords: Transforming growth factor beta; Breast neoplasm; Immunohistochemistry; Biomarkers; Prognosis.

Introduction

Breast cancer (BC) is a heterogeneous neoplastic disease comprising at least four clinically-relevant subtypes identified by pathologic assessment of key markers [1-3]. Currently, BC is responsible for approximately a quarter of cancer cases in women and for 15% of cancer-related death in this population worldwide [4]. Moreover, great patient-to-patient variability in disease evolution is observed even within well-defined molecular subtypes [5].

Several factors are known to play a role in BC progression, and among them intratumor growth factors and cytokines seems to play a special role controlling both tumor-cell-intrinsic programs, such as apoptosis, survival, proliferation and differentiation, as well as stromal-related processes, such as angiogenesis, extracellular matrix remodeling and anti-tumor immune responses, which together can facilitate BC evolution and metastasis [6].

Transforming growth factor beta β (TGFβ) is a family of growth factors with pleiotropic activities regulating cell survival, proliferation, apoptosis and differentiation in cell- and context- dependent manners. Within these, TGFβ1, TGFβ2 and TGFβ3 constitute the TGFβ subfamily of cytokines, of which TGFβ1 is the mostly abundant and widely expressed throughout human tissues. All the three isoforms are secreted as inactive large latent complexes which remain attached to the extracellular matrix until they are activated by diverse stimuli such as acidification, oxidative stress and metalloproteinases [7].

The three isoforms also elicit similar signaling pathways acting through the same set of transmembrane receptors: TGFβRIII is represented by proteoglycans (endoglin and betaglycan) and functions to facilitate the binding of TGFβ ligands to the ligand-specific serine-threonine kinase receptor TGFβRII, which then recruits, phosphorylates and activates the other TGFβ serine-threonine kinase receptor, TGFβRI. These activated receptors them phosphorylate and activate cytoplasmic SMAD2 and SMAD3 transcription factors (TFs), which complexes to SMAD4 and translocate to the nucleus to interact with other TFs and act as co-activators or co-repressors of TGFβ target genes [7]. Alternatively, other pathways are directly activated by TGFβ signaling, such as the RAS/MAPK, PI3K/AKT/mTOR and Rho-GTPase [8].

The complexity of TGFβ signaling leads to paradoxical effects in cancer: while in normal epithelial cells and in initial tumors it induces of apoptosis and cell-cycle arrest, thus exerting antitumor effects, in more aggressive neoplasms it can act as a carcinogenic promoter by inducing cell migration and epithelial-to-mesenchymal transition (EMT), by promoting angiogenesis and by inhibiting anti-tumor immunity, collectively enhancing the metastatic potential [9-12]. In BC these effects are clear among different disease subgroups and stages, where tumor suppressor effects are observed mainly in luminal BCs and in initial tumors, while pro-tumor effects take place mainly in $HER2^{+}$ and triple negative subtypes [11, 13, 14] and in p53-mutated tumors [15].

Over the last years, our group have investigated single nucleotide polymorphisms (SNPs) in *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) [16] and *TGFBR2* (rs3087465) [17] genes in BC susceptibility and clinical presentation, showing that these variations hold subtype-specific effects. Also, it was shown that *TGFB1* haplotypes composed

by these SNPs can impact the cytokine plasmatic levels [18]. However, the relationship between these polymorphisms, systemic TGFβ1 and TGFβ signaling in BC tissue have not been evaluated.

Furthermore, studies investigating intratumor protein expression of TGFβ pathway components and correlating these markers with BC clinical presentation and with prognosis produced contradictory conclusions which may be reminiscent of context-specific effects of TGFβ1 acting in different BC subgroups, since the effects of these markers in BC subtypes was poorly characterized by these previous works [19-26].

Therefore, this study sought to analyze intratumor expression of TGFβ1, TGFβRII and activated (Ser423/425-phosphorylated) SMAD2/3 (p-SMAD2/3) through immunohistochemistry in a cohort of patients with aggressive BC subtypes (Luminal-B-HER2$^+$, HER2-enriched and triple negative) with data available regarding at-diagnosis clinicopathological data, *TGFB1* and *TGFBR2* SNPs and plasmatic TGFβ1 levels to investigate the relationship between these variables and the possible effects of these markers within each subtype and in subgroups defined by p53 mutation.

Methods

Sample selection

For the current study 34 formalin-fixed, paraffin embedded (FFPE) tissues from equivalent number of patients diagnosed for aggressive BC subtypes (Luminal-B-HER2$^+$, HER2-enriched and triple negative) with data available regarding *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) and *TGFBR2* (rs3087465) SNPs from previous studies [16, 17] were collected. Twenty-one of these patients also had at-diagnosis plasmatic TGFβ1 levels measured in a previous study [18]. Clinicopathological features for patients involved in this study are shown in Table S1 (Supplementary information), while information regarding their genotypes for *TGFB1* and *TGFBR2* are shown in Table S2.

Clinicopathological data were retrieved from patients' medical records available at Londrina Cancer Hospital. Pathological assessments were performed according to the American Society of Clinical Oncology (ASCO) protocols [27, 28]. Immunostaining for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were assessed to classify patients into the following BC intrinsic subtypes: Luminal-B (LB; ER/PR$^+$HER2$^+$), HER2-enriched (HER2; ER$^-$PR$^-$HER2$^+$) and triple-negative (TN; ER$^-$PR$^-$HER2$^-$). Disease staging was based on the pathologic TNM score, according to the Union for International Cancer Control (UICC) criteria.

Other clinicopathological data included: age at diagnosis, pathologic tumor size, histopathologic grade, pathologic nodal status, proliferation index (Ki67) and p53 immunostaining, which is used as a classical indirect indicator for missense p53 mutations [29] and associates with worse disease prognosis [30, 31].

The entire research protocol was approved by Londrina State University ethics committee for research involving human subjects (CAAE 73557317.0.0000.5231) and written informed consent was signed by patients prior to biological material collection.

Immunohistochemistry

For immunohistochemistry staining, FFPE BC tumor tissue sections at 4 μm were dewaxed, hydrated and heat-treated in 1 mM EDTA buffer for antigenic unmasking on a pressure cooker at 95.8°C for 20 min. Sections were incubated overnight at room temperature with goat anti-human TGFβ1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-146, 1:100), goat anti-human TGFβRII (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-400, 1:100) and goat anti-human Ser423/425-phosphorylated-SMAD2/3, specific for (p-SMAD2/3; Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-11769, 1:100), followed by secondary antibody polymer conjugation (ImmunoDetector HRP/DAB, BioSB, Santa Barbara, CA, USA; catalog BSB0005, lot. 0005GFI18) and by color development with diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA). A methodical negative control went through the first step of the procedure by incubation with the vehicle instead of the primary antibody.

Histological slides were analyzed under the optic microscope to identify areas that best represented TGFβ1, TGFβRII and p-SMAD2/3 immunostaining, where brownish color was considered to be evidence of a positive expression. From each sample, 3 photomicrographs of 800 × 600 pixels were obtained from 400x magnification field, using Amscope cam (FMA050), adapted in the optic microscope. Digitally acquired images were analyzed in the ImageJ 1.44 software for Windows (Java image software in public domain: http://rsb.info.nih.gov/ij/), using the threshold tool with color-based selection for positive staining. Routines for image analysis were defined in ImageJ macro language and performed on RGB images without further treatment. The number of pixels in the selected color range was divided by the total number of pixels in each field. Results were expressed by the relation between the positive area fraction per total area fraction as the percentage (%) of TGFβ1, TGFβRII and p-SMAD2/3 staining.

All pathological procedures, from tissue staining to image acquisition and analyses, were performed by an experienced pathologist (J.C.) who was blind regarding patients'

identification, BC subtype, clinicopathological features and genotypes for *TGFB1* and *TGFBR2* SNPs.

Online data repositories

To complement our data on the expression of TGFβ-signaling components in BC tissue, online data-banks and analysis resources were used: The Human Protein Atlas (https://www.proteinatlas.org/) was used to evaluate the pattern of TGFβ1 immunostaining in BC tissue, while the GEPIA2 databank and analysis resource (http://gepia2.cancer-pku.cn/), which makes data from The Cancer Genome Atlas (TCGA) available, was used to investigate correlations between TGFβ1 components at mRNA level. Also, TIMER analysis resource (https://cistrome.shinyapps.io/timer/) [32] was used to check the relative expression of TGFβ1-pathway components in different cancer types and BC subtypes.

Statistical analyses

All statistical analyses were performed using IBM® SPSS® Statistics 22.0 (IBM®, Armonk, New York, USA) or GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA) software. All tests were two-tailed and the significance level adopted was of 5%.

Non-parametric statistics were applied in all tests since the data did not have normal distribution through Shapiro-Wilk test. The absolute values for staining intensity were used and Mann-Whitney U test was applied for comparison between two groups while comparison between three groups were made through Kruskall-Wallys test followed by Dunn's post-test.

Pairwise correlations were tested through Kendall's rank correlation tests through the cross-tables SPSS subprogram. In these analyses, Tau-b coefficient was adopted when two continuous variables were being tested and the corrected Tau-c coefficient was reported for correlations between a continuous variable and a categorical ordinal variable. Also, for subgroup-stratified correlations correction for multiple tests were applied according to the Benjamini-Hochberg method [33] and *q*-values were reported.

Results

Expression of TGFβ1, TGFβR2 and p-SMAD2/3 in breast cancer tissue

TGFβ1 and TGFβRII expression were predominantly cytoplasmic and/or membranous, while p-SMAD2/3 had cytoplasmic staining (Fig 1). Data from The Human Protein Atlas have also shown cytoplasmic/membranous staining for TGFβ1 in human BC tissue (Figure S1). TGFβRII and p-SMADs were not evaluated in The Human Protein Atlas

project.

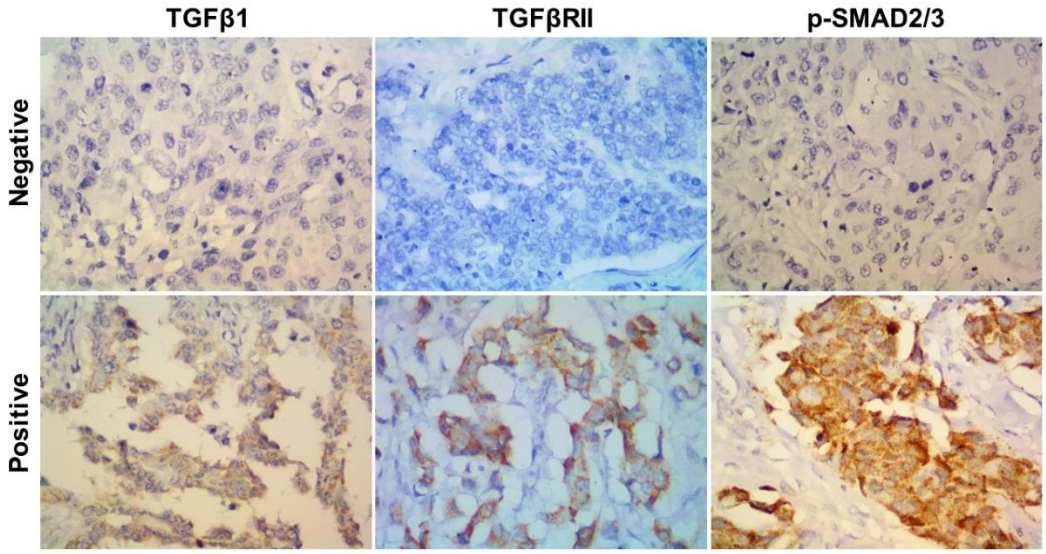

Figure 1. Representative photomicrographs showing BC tumor sections with negative and positive staining for TGFβ1, TGFβRII and p-SMAD2/3. 400X magnification.

Interestingly, TGFβ1 and TGFβRII immunostainings had a bimodal distribution that was consistent among different subtypes, with the average value (approximately 6.25% for both) dividing the sample into low (bellow the mean) and high (above the mean) expression groups (Figure 2A and B). For p-SMAD2/3, otherwise, data distribution assumed a continuous behavior for LB and TN subgroups, but was bimodal for HER2 BCs (Fig 2C).

LB BCs tended to have increased staining for all markers while TN cancers had the lowest staining in our sample (Fig 2), however no significant differences were noted when comparing different BC subtypes. This was also corroborated by the TCGA data, analyzed through the TIMER online analysis resource, showing higher mRNA expression for *TGFB1* and *TGFBR2* genes in Luminal BCs and decreased expression in Basal (TN) BCs (Figure S2).

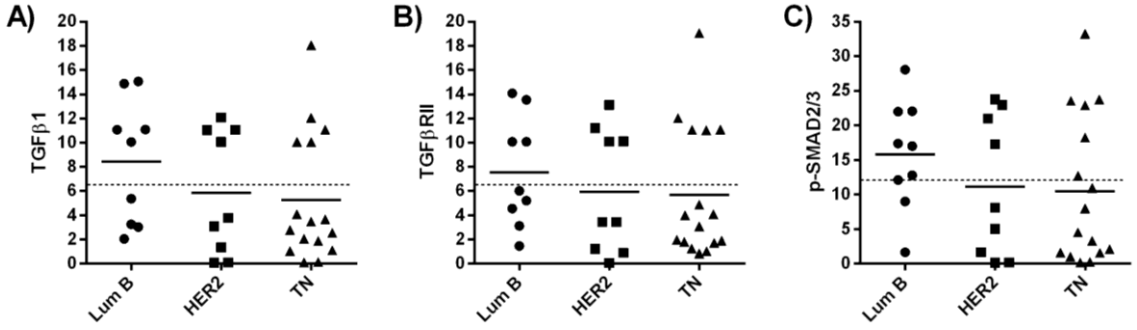

Figure 2. Immunostaining scores for TGFβ1 (A), TGFβRII (B) and p-SMAD2/3 (B) among BC molecular subtypes. Data is represented as the mean percentage of positive area in 3 fields analyzed per slide. Black lines represent the mean for each subtype. Dashed lines represent the mean for TGFβ1 (**A**; 6.253), TGFβRII (**B**; 6.294) and p-SMAD2/3 (**C**; 12.056) considering all BC samples.

Also, there was a strong correlation between the staining intensity for the three markers (Fig. 3) which was consistent among BC subtypes (Table S3). Extremely significant correlations ($p < 0.0001$) were also observed between the expression of *TGFB1*, *TGFBR2* and *SMAD7* genes at mRNA level using the TCGA data available through GEPIA2 analysis resource (Figure S3). SMAD7 was used in these analyses as a reporter gene for TGFβ activation of SMAD2/3, since this gene is directly activated as a negative feedback loop for this signaling pathway.

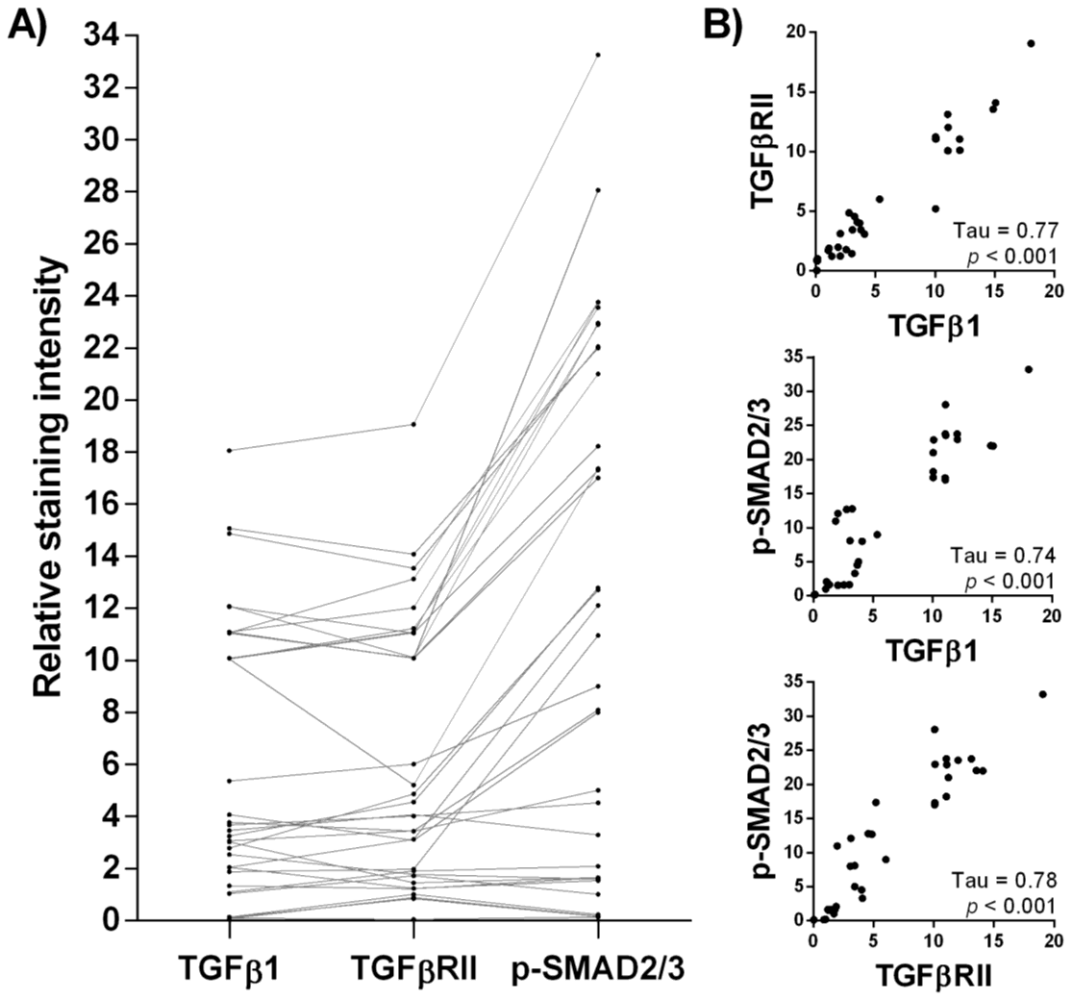

Figure 3. Correlation between TGFβ1, TGFβRII and p-SMAD2/3 in breast cancer tissue. **(A)** Distribution of data regarding TGFβ1, TGFβRII and p-SMAD2/3 immunostaining in BC tissue. Lines connect data from the same patients. **(B)** Correlation between TGFβ1 and TGFβRII, TGFβ1 and p-SMAD2/3 and between TGFβRII and p-SMAD2/3. Tau-b correlation coefficient and *p*-values are shown within each graph.

Intratumor TGFβ-signaling is not correlated to plasma TGFβ1 nor with *TGFB1* and *TGFBR2* genetic polymorphisms

For 21 of the samples (6 from LB, 7 from HER2-enriched and 8 from TN subgroups), data regarding TGFβ1 plasmatic levels at diagnosis were available from a previous research by our group. Also, all patients were genotyped for *TGFB1* rs1800468, rs1800469, rs1800470 and rs1800471 and for *TGFBR2* rs3087465 SNPs in previous studies. This allowed us to test the correlation between these variables and intratumor TGFβ1, TGFβRII and activated p-SMAD2/3.

No correlation was found between intratumor TGFβ1 staining and systemic TGFβ1 levels in general BC sample (Table 1). However, in TN subtype, but not in LB or HER2-enriched subtypes, there was a significant negative correlation between plasmatic TGFβ1 and both intratumor TGFβ1 (Table 1; Tau-b = -0.643; $p = 0.026$) and p-SMAD2/3 staining (Table 1; Tau-b = -0.571; $p = 0.048$).

Table 1. Correlation between plasmatic TGFβ1 and intratumor TGFβ components.

Breast cancer groups	Correlation with plasmatic TGFβ1 [Tau-b (*p*)]		
	TGFβ1	TGFβRII	p-SMAD2/3
General BC (n = 21)	-0.196 (0.215)	-0.171 (0.277)	-0.276 (0.08)
Luminal-B (n = 6)	0.067 (0.851)	0.200 (0.573)	-0.333 (0.348)
HER2-enriched (n = 7)	-0.333 (0.293)	-0.333 (0.293)	-0.333 (0.293)
Triple-negative (n = 8)	-0.643 (0.026)*	-0.500 (0.083)	-0.571 (0.048)*

*Significant correlation ($p < 0.05$).

Regarding *TGFB1* and *TGFBR2* SNPs, no significant correlation was found for intratumor TGFβ1, TGFβRII or p-SMAD2/3 staining, either in the general BC group (Table 2) or in subtype-stratified analyses (data not shown).

Table 2. Correlation between *TGFB1* and *TGFBR2* SNPs TGFβ1 components staining.

Variant	Model[a]	Correlation with genetic variant [Tau-c (*p*)]		
		TGFβ1	TGFβRII	p-SMAD2/3
***TGFBR2* G-875A (rs3087465)**	**Additive**	-0.127 (0.456)	-0.138 (0.399)	-0.151 (0.327)
	Dominant	-0.183 (0.357)	-0.211 (0.279)	-0.194 (0.321)
	Recessive	0.021 (0.877)	0.042 (0.733)	-0.028 (0.787)
***TGFB1* G-800A (rs1800468)**	**Dominant**	0.066 (0.549)	0.107 (0.301)	0.031 (0.684)
***TGFB1* C-509T (rs1800469)**	**Additive**	0031 (0.820)	0.005 (0.971)	0.013 (0.927)
	Dominant	-0.028 (0.802)	-0.007 (0.955)	-0.010 (0.930)
	Recessive	0.055 (0.765)	0.014 (0.940)	0.035 (0.851)
***TGFB1* T29C (rs1800470)**	**Additive**	-0.096 (0517)	-0.153 (0.300)	-0.093 (0.513)
	Dominant	-0.121 (0.289)	-0.183 (0.121)	-0.128 (0.231)
	Recessive	-0.066 (0.737)	-0.093 (0.627)	-0.028 (0.886)
***TGFB1* G25C (rs1800471)**	**Additive**	-0.026 (0.792)	-0.067 (0.513)	-0.057 (0.552)
	Dominant	-0.048 (0.727)	-0.104 (0.480)	-0.090 (0.514)
	Recessive	0.055 (0.334)	0.021 (0.714)	0.000 (1.000)
***TGFB1* GCTG haplotype**	**Additive**	-0.075 (0.596)	-0.039 (0.787)	0.000 (1.000)
	Dominant	-0.104 (0.606)	-0.090 (0.651)	-0.038 (0.849)
	Recessive	0.003 (0.959)	0.059 (0.459)	0.073 (0.364)
***TGFB1* GTCG haplotype**	**Additive**	-0.096 (0.477)	-0.127 (0.359)	-0.062 (0.662)
	Dominant	-0.145 (0.300)	-0.131 (0.372)	-0.066 (0.637)
	Recessive	-0.045 (0.790)	-0.087 (0.602)	-0.031 (0.861)

[a] Additive model: wild homozygotes = 0, heterozygotes = 1; variant homozygotes = 2; Dominant model: wild homozygotes = 0, heterozygotes and variant homozygotes = 1; Recessive model: wild homozygotes and heterozygotes = 0, variant homozygotes = 1

Correlation between clinicopathological parameters and TGFβ-signaling components expression

Correlations between clinicopathological parameters and intratumor staining for TGFβ1, TGFβRII and p-SMAD2/3 were also tested. No significant relationship was observed between these variables and any clinicopathological parameters in general sample or in HER2-enriched and TN subtypes (Table 3).

Otherwise, in LB subtype, p-SMAD2/3 was positively correlated with age at diagnosis (Tau-b = 0.551; p = 0.004; q = 0.084) and negatively correlated with p53 status (Tau-c = -0.813; p = 0.001; q = 0.042) and with the presence of lymph-node metastasis (LNM; Tau-c = -0.691; p = 0.007; q = 0.118), while tumor size was negatively correlated with TGFβ1 (Tau-b = -0.444; p = 0.004; q = 0.084) and TGFβRII (Tau-b = -0.592; p = 0.0001; q = 0.042) (Table 3).

Table 3. Correlation between clinicopathological parameters at diagnosis and intratumor staining for TGFβ1 components according to evaluated breast cancer subtypes.

Subtype	Parameter	Correlation with clinicopathological feature [Tau (*p*)]		
		TGFβ1	**TGFβRII**	**p-SMAD2/3**
General BC	**Age**	-0.014 (0.879)	-0.056 (0.585)	0.038 (0.735)
	Tumor size	-0.206 (0.133)	-0.173 (0.243)	-0.146 (0.302)
	Hist. grade	0.189 (0.139)	-0.099 (0.458)	-0.078 (0.549)
	Ki67	-0.116 (0.438)	-0.082 (0.556)	-0.085 (0.557)
	p53	0.020 (0.924)	0.051 (0.804)	-0.012 (0.955)
	LNM	-0.031 (0.876)	-0.078 (0.585)	-0.068 (0.641)
	Stage	-0.072 (0.644)	-0.018 (0.907)	-0.042 (0.771)
Luminal-B	**Age**	0.377 (0.085)	0.353 (0.102)	0.551 (0.004)*
	Tumor size	-0.444 (0.004)*	-0.592 (0.001)*	-0.167 (0.571)
	Hist. grade	0.185 (0.596)	0.148 (0.602)	0.407 (0.135)
	Ki67	-0.333 (0.194)	-0.296 (0.141)	-0.111 (0.453)
	p53	-0.563 (0.109)	-0.563 (0.063)	-0.813 (0.001)*
	LNM	-0.593 (0.052)	-0.593 (0.052)	-0.691 (0.007)*
	Stage	-0.243 (0.489)	-0.296 (0.427)	-0.259 (0.303)
HER2-enr.	**Age**	-0.085 (0.746)	-0.141 (0.483)	-0.085 (0.658)
	Tumor size	-0.148 (0.700)	-0.074 (0.806)	-0.074 (0.806)
	Hist. grade	-0.296 (0.396)	-0.099 (0.773)	-0.198 (0.559)
	Ki67	0.296 (0.423)	0.198 (0.648)	0.198 (0.648)
	p53	0.000 (1.000)	0.000 (1.000)	0.000 (1.000)
	LNM	0.000 (1.000)	-0.198 (0.608)	-0.099 (0.800)
	Stage	-0.165 (0.573)	0.033 (0.921)	-0.033 (0.919)
Triple-neg.	**Age**	-0.192 (0.098)	-0.226 (0.104)	-0.226 (0.081)
	Tumor size	0.154 (0.492)	0.120 (0.620)	0.068 (0.771)
	Hist. grade	-0.016 (0.955)	0.102 (0.643)	0.047 (0.865)
	Ki67	0.059 (0.805)	0.000 (1.000)	0.000 (1.000)
	p53	0.284 (0.303)	0.356 (0.177)	0.284 (0.303)
	LNM	0.141 (0.629)	0.047 (0.870)	0.047 (0.871)
	Stage	0.199 (0.377)	0.199 (0.321)	0.129 (0.541)

*Significant correlation ($p < 0.05$).

Guided by previous research indicating that p53 mutation status was an important factor switching TGFβ-signaling from a tumor suppressor to a tumor promoter [15], correlations between TGFβ components and clinicopathological data stratifying patients by p53 status assessed through immunohistochemistry, as previously described [29], was assessed

(Table 4).

In p53-negative, or wild-type (WT) group, all TGFβ-signaling components negatively correlated both with tumor-size (Table 4; TGFβ1: Tau-b = -0.49, p = 0.018, q = 0.137; TGFβRII: Tau-b = -0.5, p = 0.019, q = 0.137; p-SMAD2/3: Tau-b = -0.431, p = 0.036, q = 0.216) and with Ki67 (TGFβ1: Tau-c = -0.568, p = 0.000, q = 0.018; TGFβRII: Tau-c = -0.479, p = 0.007; p-SMAD2/3: Tau-c = -0.462, p = 0.004, q = 0.072), while no significant correlation was observed in $p53^+$ group (Table 4).

Table 4. Correlation between clinicopathological parameters at diagnosis and intratumor staining for TGFβ1 components according to p53 status.

p53 status	Parameter	Correlation with clinicopathological feature [Tau (*p*)]		
		TGFβ1	**TGFβRII**	**p-SMAD2/3**
$p53^-$ (WT)	**Age**	0.144 (0.357)	0.104 (0.522)	0.116 (0.473)
	Tumor size	-0.490 (0.018)*	-0.500 (0.019)*	-0.431 (0.036)*
	Hist. grade	-0.024 (0.933)	0.071 (0.784)	0.095 (0.712)
	Ki67	-0.568 (0.000)*	-0.479 (0.007)*	-0.462 (0.004)*
	LNM	-0.237 (0.485)	-0.189 (0.568)	-0.284 (0.400)
	Stage	0.053 (0.864)	0.160 (0.586)	0.094 (0.686)
$p53^+$ (mut.)	**Age**	-0.183 (0.209)	-0.160 (0.342)	-0.190 (0.233)
	Tumor size	-0.087 (0.637)	0.033 (0.868)	-0.040 (0.840)
	Hist. grade	-0.199 (0.253)	-0.100 (0.576)	-0.191 (0.269)
	Ki67	0.332 (0.194)	0.399 (0.117)	0.355 (0.167)
	LNM	0.199 (0.444)	0.111 (0.687)	0.111 (0.677)
	Stage	-0.216 (0.288)	-0.116 (0.584)	-0.191 (0.336)

*Significant correlation ($p < 0.05$).

Discussion

The paradoxical effects of TGFβ signaling in breast morphogenesis and carcinogenesis has been extensively investigated in cell culture and animal models, and confirmed in clinical samples: while it is a potent cell cycle suppressor and apoptosis inducer in normal epithelial cells and in early or poorly aggressive neoplasia, it can induce EMT and immunotolerance in advanced tumors or more aggressive BC subtypes [11, 13-15].

Previous research have shown that genetic polymorphisms in *TGFB1* and *TGFBR2* genes potentially altering their expression showed subtype-specific associations with susceptibility and clinical presentation in BC, which were consistent with TGFβ1 biological effects [16, 17]. Also, it was shown that a rare *TGFB1* haplotype was associated with plasmatic TGFβ1 levels [18]. However, there was no study investigating the relationship between BC tissue expression of TGFβ signaling components, *TGFB1* and *TGFBR2* SNPs and systemic TGFβ1 on the literature.

In the current study TGFβ1, TGFβRII and p-SMAD2/3 were assessed in BC tumor cells through immunohistochemistry, and cytoplasmic staining was noted for all of them, which was corroborated by data from the human protein atlas and by previous research [22-24]. A high correlation between these markers in BC tissue was also shown, which was also consistent with gene-expression data from TCGA and with previous studies using immunohistochemistry [21, 23, 26], suggesting that TGFβ1 may exert paracrine and autocrine effects in BC cells activating classical SMAD-mediated pathway.

A previous study in prostate cancer has shown concordance between plasmatic and intratumor TGFβ1 staining [34]. However, our data have not shown any correlation between them in general BC group, and a surprising negative correlation was observed in TN subgroup. To our knowledge, this is the first study investigating the relationship between plasmatic and intratumor TGFβ signaling in BC, and our results indicate that plasmatic TGFβ1 may not reflect tumor *milieu*, posing important insights for future research on this field. Of note, virtually all human tissues can produce TGFβ1, and this might mask the tumor-produced TGFβ1 in peripheral blood.

Also, *TGFB1* and *TGFBR2* SNPs were not correlated with the protein expression of TGFβ1 components, despite all of them were shown to play a role in genetic control of TGFβ1 production by previous research [35-41]. It is possible that the subtle effects exerted by each of them individually, despite significant in well-controlled conditions such as cell culture experiments and twin-studies, may not be evident in complex and heterogeneous conditions, such as BC tumor tissue. Unfortunately, our sample size was too small to investigate the effects of rare SNPs and haplotype structures which previously associated with TGFβ1 plasmatic levels [18].

Previous works have also produced controversial results regarding correlations between the expression of TGFβ components and clinicopathological features [19-26] or BC prognosis [19, 23, 26], and these effects might be attributable to the context-specific effects of TGFβ in BC. Indeed, despite some of these studies investigated the differential TGFβ effects in ER^+ or ER^-, few of them considered more specific BC subtypes.

The current research has shown no correlation between any clinicopathological feature and TGFβ signaling components in the general BC group. However, in subtype stratified analysis, TGFβ components were associated with better prognosis parameters in LB subgroup, as evidenced by p-SMAD2/3 staining intensity being positively correlated with age at diagnosis and negatively correlated with p53 mutation and LNM, and by tumor size being negatively correlated with both TGFβ1 and TGFβRII expression.

Regarding the age at diagnosis, previous work has also shown that intracellular TGFβ1 was associated with older age at disease onset, while extracellular-TGFβ1, TGFβRII and p-SMAD2 were associated with early age of onset in BC, independently of ER-status [21]. Another study has found that TGFβRII but not p-SMAD2 was associated with younger age at diagnosis [25], while others failed to observe any association between TGFβ signaling components and patients' age [19, 20], however none of them investigated specifically LB BCs. Of note, in our study a trend for an inverse correlation was also noted between p-SMAD2/3 and age in the TN BC group (Tau-c = -0.226; p = 0.08) suggesting that p-SMAD2/3 might have subtype specific associations with age in BC.

Previous studies has also shown that TGFβ1 [20], p-SMAD2 [21] and TGFβRI [19] immunostainings were positively associated with LNM specifically in ER^- and TN BCs. Also, in ER^- cancers, TGFβRII staining was associated with larger tumor size [21]. The current study, otherwise, found opposite trends in LB tumors. Of note, ER^- and TN cancers have increased invasive potential compared to ER^+ (luminal) BCs. Therefore, these data are consistent with the biological effects of TGFβ1 in promoting aggressive cancer while retaining tumor suppressor effects in less aggressive BCs [10-13, 16].

This model is consistent with studies demonstrating that a gene-expression signature for TGFβ signaling indicated enhanced metastatic potential in ER^- BCs [42], whereas a TGFβ deficient signature correlated with metastasis in ER^+ tumors [12, 43]. This is also corroborated by immunohistochemistry analyses showing that low TGFβ1 staining predicts longer disease-free survival (DFS) in TN BC [20] and high TGFβRII predicts shorter DFS in ER^- cancers [19], while p-SMAD2/3 staining was associated with increased DFS in ER^+ group [23].

Of note, TGFβ was shown to mediate the action of anti-estrogen therapy in ER^+ BCs, promoting apoptosis in tamoxifen-treated cells [44]. Mechanistically, ER and TGFβ signaling were shown to crosstalk in breast carcinogenesis [45] and ERα was shown to physically interact and inhibit p-SMAD2/3 signaling by promoting their degradation, which blocks TGFβ-induced EMT and migration [46-48]. On the other hand, TGFβ signaling seems necessary to counteract ERα-induced proliferation of breast cells [49]. Therefore, in this model, the co-activation of ERα and TGFβ signaling in BC is associated with better prognosis by maintaining luminal-differentiation through ERα on mammary cells while inhibiting ER-mediated proliferation, though TGFβ cytostatic effects.

Furthermore, p53 was shown to be an important factor mediating the switching of TGFβ signaling from a tumor suppressor to a tumor promoter. Mechanistically it was shown that SMAD proteins physically interact with MAPK-phosphorylated p53 and mediate EMT in

morphogenesis [50], and that in cancers with p53 mutations and Ras/MAPK activation a protein-complex is formed between MAPK-phosphorylated mutated-p53, SMADs and p63, whose tumor suppressor functions are blocked, leading to EMT and enhanced invasiveness [15].

Despite this, previous researches investigating the TGFβ-signaling in cancer tissue have not taken p53 mutation status into account. Here, we used p53 immunostaining as an indirect measure of p53 mutation, as previously described [29-31], and showed that in $p53^-$ (wild-type) group, TGFβ-signaling was associated with decreased tumor-size and proliferation, while in $p53^+$ (mutated) BCs, no significant correlation was observed. These data might indicate that TGFβ1 exerts tumor-suppressive effects in the WT-p53 group, but not in cancers harboring p53 mutation, consistent with the above-mentioned model.

Importantly, despite p53 immunostaining and mutation-status has been associated with aggressive BC phenotypes, its' prognostic role in BC has been debatable [51], as it did not show sufficient evidence to support recommendation for its use in clinical practice routine [52]. However, the results reported herein and by previous data [15] might support a role for TGFβ-signaling in conferring a clinical significance for p53 immunostaining in BC.

Therefore, the present study suggests that TGFβ signaling exert tumor-suppressive effects in luminal-B and WT-p53 BCs, consistent with context-specific roles of TGFβ in cancer. Further prospective studies with larger samples are encouraged to confirm these findings and might reveal promisor prognostic and therapeutic biomarkers for these BC subtypes.

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Supplementary information

Transforming growth factor beta 1 (TGFβ1) pathway components in breast cancer tissue from aggressive subtypes: correlation with single nucleotide polymorphisms, plasmatic TGFβ1 and clinicopathological features

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Table S1. Patients' clinicopathological features.

Parameter	General BC (n = 34)	LB-HER2$^+$ (n = 9)	HER2 (n = 9)	TN (n = 16)
Age				
Median (IQ range)	56 (18)	57 (21)	51 (13)	57 (22)
Mean (SD)	56 (12)	57 (11)	49 (10)	59 (14)
<40 [n(%)]	3 (8.8)	0 (0.0)	2 (22.2)	1 (6.3)
40-49 [n(%)]	9 (26.5)	3 (33.3)	2 (22.2)	4 (25.0)
50-59 [n(%)]	9 (26.5)	2 (22.2)	3 (33.3)	4 (25.0)
60-69 [n(%)]	8 (23.5)	3 (33.3)	2 (22.2)	3 (18.8)
70-79 [n(%)]	3 (8.8)	1 (11.1)	0 (0.0)	2 (12.5)
>80 [n(%)]	2 (5.9)	0 (0.0)	0 (0.0)	2 (12.5)
Tumor size [n (%)]				
Median (IQ range)	3.0 (2.3)	2.4 (0.7)	2.5 (1.6)	4.0 (1.6)
Mean (SD)	3.4 (1.6)	2.4 (0.9)	3.0 (1.1)	4.2 (1.8)
< 1.5 cm	2 (5.9)	2 (22.2)	0 (0.0)	0 (0.0)
1.51-3.0 cm	13 (38.2)	5 (55.6)	5 (55.6)	3 (18.8)
> 3.0 cm	19 (55.9)	2 (22.2)	4 (44.4)	13 (81.2)
Hist. Grade [n (%)]				
I	1 (2.9)	1 (11.1)	0 (0.0)	0 (0.0)
II	10 (29.4)	5 (55.6)	3 (33.3)	2 (12.5)
III	23 (67.6)	3 (33.3)	6 (66.7)	14 (87.5)
Ki67 [n (%)]				
Low	1 (3.0)	1 (11.1)	0 (0.0)	0 (0.0)
Intermediate	15 (45.5)	7 (77.8)	4 (44.4)	4 (26.7)
High	17 (51.5)	1 (11.1)	5 (55.6)	11 (73.3)
Unknown	1	0	0	1
p53 [n (%)]				
Negative	13 (40.6)	5 (62.5)	2 (22.2)	6 (40.0)
Positive	19 (59.4)	3 (37.5)	7 (77.8)	9 (60.0)
Unknown	2	1	0	1
Lymph node metastasis [n (%)]				
Negative	15 (44.1)	3 (33.3)	5 (55.6)	7 (43.8)
Positive	19 (55.9)	6 (66.7)	4 (44.4)	9 (56.3)
Disease Stage [n (%)]				
0 (CDIS)	2 (5.9)	1 (11.1)	1 (11.1)	0 (0.0)
I	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
II	19 (55.9)	5 (55.6)	5 (55.6)	9 (56.3)
III	11 (32.4)	3 (33.3)	2 (22.2)	6 (37.5)
IV	2 (5.9)	0 (0.0)	1 (11.1)	1 (6.3)

Table S2. Genotypes for *TGFB1* and *TGFBR2* of BC patients.

SNP	General BC (n = 34)	Luminal-B (n = 9)	HER2-enr (n = 9)	TN (n = 16)
TGFBR2 G-875A				
GG	17 (50.0)	4 (44.4)	2 (22.2)	11 (68.8)
GA	15 (44.1)	4 (44.4)	7 (77.8)	4 (25.0)
AA	2 (5.9)	1 (11.1)	0 (0.0)	1 (6.3)
TGFB1 G-800A				
GG	31 (91.2)	8 (88.9)	9 (100.0)	14 (87.5)
GA	3 (8.8)	1 (11.1)	0 (0.0)	2 (12.5)
AA	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)
TGFB1 C-509T				
CC	6 (17.6)	2 (22.2)	3 (33.3)	1 (6.3)
CT	20 (58.8)	5 (55.6)	4 (44.4)	11 (68.8)
TT	8 (23.5)	2 (22.2)	2 (22.2)	5 (25.0)
TGFB1 T29C				
TT	5 (14.7)	3 (33.3)	1 (11.1)	1 (6.3)
TC	18 (52.9)	5 (55.6)	4 (44.4)	9 (56.3)
CC	11 (32.4)	1 (11.1)	4 (44.4)	6 (37.5)
TGFB1 G25C				
GG	28 (82.4)	8 (88.9)	6 (66.7)	14 (87.5)
GC	4 (11.8)	1 (11.1)	1 (11.1)	2 (12.5)
CC	2 (5.9)	0 (0.0)	2 (22.2)	0 (0.0)

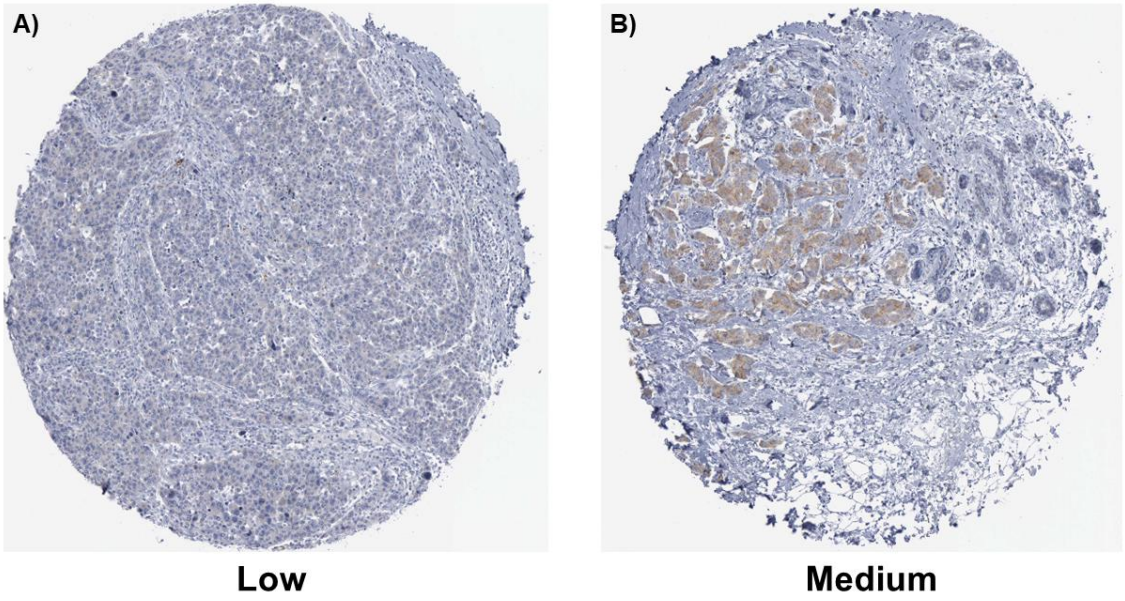

Figure S1. Expression of TGFβ1 in human breast cancer tissue in The Human Protein Atlas project. A) A representative sample showing low cytoplasmic staining for TGFβ1. **B)** A representative sample showing medium cytoplasmic TGFβ1 staining specifically in tumor cells. Photomicrographs retrieved from The Human Protein Atlas databank and analysis resource (https://www.proteinatlas.org/ENSG00000105329-TGFB1/pathology/breast+cancer#).

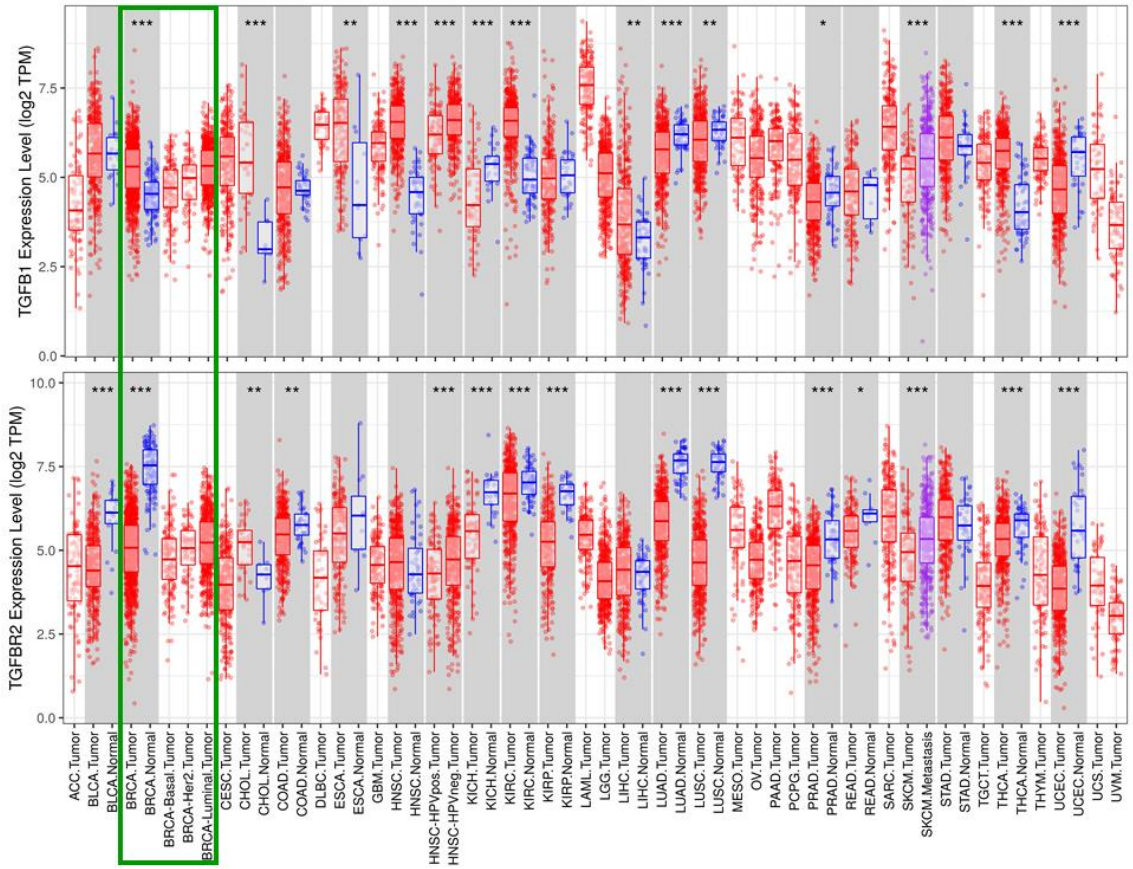

Figure S2. Expression of *TGFB1* and *TGFBR2* genes among cancers in TCGA database. Shown are the expression of *TGFB1* (upper panel) and *TGFBR2* (lower panel) genes in all cancer types included in TCGA project. Breast cancer data is highlighted inside the green box (BRCA). Both genes had higher expression in Luminal BCs and lower expression in TN cancers, although not significant. Graphs generated using the TIMER analysis resource for TCGA data (https://cistrome.shinyapps.io/timer/).

Table S3. Correlation between TGFβ1 components among breast cancer subtypes.

Correlation	Lum. B (n =9)	HER2 (n = 9)	TN (n = 16)
TGFβ1 x TGFβRII	0.873 (0.001)	0.722 (0.007)	0.767 (<0.001)
TGFβ1 x p-SMAD2/3	0.611 (0.022)	0.778 (0.004)	0.833 (<0.001)
TGFβR2 x p-SMAD2/3	0.592 (0.028)	0.944 (<0.001)	0.833 (<0.001)

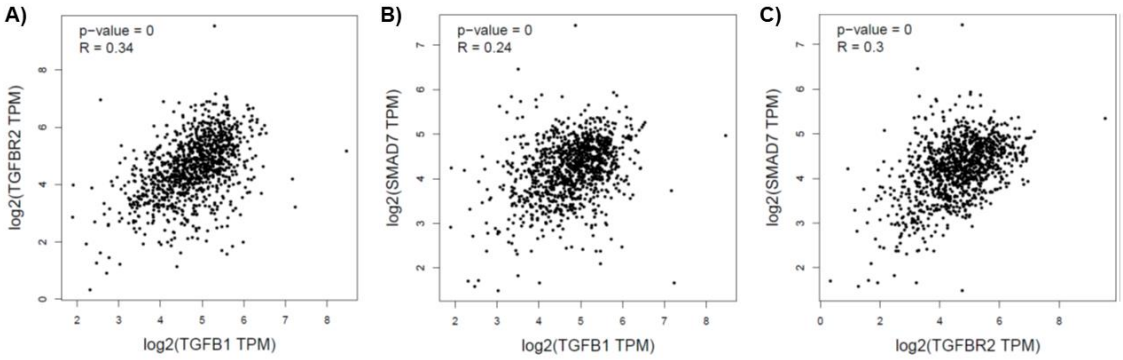

Figure S3. Correlation between *TGFB1*, *TGFBR2* and *SMAD7* genes in breast cancer samples from TCGA project. Extremely significant correlations were evidenced between the expression of *TGFB1* and *TGFBR2* **(A)**, *TGFB1* and *SMAD7* **(B)** and between *TGFBR2* and *SMAD7* **(C)** genes in tumor tissue samples from the breast cancer cohort of TCGA project. *SMAD7* was evaluated as a reporter gene for the activation of classical TGFβ pathway in these analyses. Graphs and statistics generated using the GEPIA2 analysis resource (http://gepia2.cancer-pku.cn/#index).

3.6 Artigo 6: "Germline APOBEC3B deletion influences clinicopathological parameters in luminal-A breast cancer: evidences from a southern Brazilian cohort"

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ORIGINAL ARTICLE – CANCER RESEARCH

Germline *APOBEC3B* deletion influences clinicopathological parameters in luminal-A breast cancer: evidences from a southern Brazilian cohort

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Abstract

Purpose APOBEC3A and APOBEC3B cytidine deaminases have been implicated in the pathogenesis of multiple cancers, including breast cancer (BC). A germline deletion linking *APOBEC3A* and *APOBEC3B loci* (*A3A/B*) has been associated with higher APOBEC-mediated mutational burden, but its association with BC risk have been controversial. Therefore, this study investigated the association between *A3A/B* and BC susceptibility and clinical presentation in a Brazilian cohort.

Methods *A3A/B* deletion was evaluated through allele-specific PCR in 341 BC patients and 397 women without familial or personal history of neoplasia from Brazil and associations with susceptibility to BC subtypes were tested through age-adjusted logistic models while correlations with clinicopathological parameters were tested using Kendall's tests.

Results No association was found between *A3A/B* and BC susceptibility; however, in Luminal-A BCs, it was positively correlated with tumor size (Tau-c = 0.125) and Ki67 (Tau-c = 0.116) and negatively correlated with lymph node metastasis (LNM) (Tau-c = − 0.162). The negative association between *A3A/B* with LNM in Luminal-A BCs remained significant even after adjusting for tumor size and Ki67 in logistic models (OR = 0.22; $p = 0.008$).

Conclusion These results show that although *A3A/B* may not modify BC susceptibility in Brazilian population, it may affect clinicopathological features in BC subtypes, promoting tumor cell proliferation while being negatively associated with LNM in Luminal-A BCs.

Keywords APOBEC deaminases · Breast neoplasm · Susceptibility · Prognosis · Copy-number variations

Introduction

The apolipoprotein-B mRNA-editing enzyme catalytic polypeptide-like (APOBEC) superfamily includes 11 genes in humans, encompassing the activation-induced deaminase gene (*AID*) and *APOBEC1* which are mapped to the chromosome 12, the *APOBEC2*, in chromosome 6, the *APOBEC3* cluster (*APOBEC3A, B, C, D, F, G* and *H*), in chromosome 22 and *APOBEC4*, located in chromosome 1. Except for *APOBEC2* and *APOBEC4*, which are poorly characterized and probably derived from pseudogenes, all APOBEC genes encode functional cytidine deaminase enzymes, participating in several physiologic and immunological processes: AID is involved in the hypermutation of IgGs in B lymphocytes, APOBEC1 is necessary to generate the short isoform of apolipoprotein-B in the liver and APOBEC3 family members are mainly involved in innate immunity against viruses and retroelements by mutating single-stranded DNA (ssDNA) intermediates (Burns et al. 2015).

By their intrinsic capability of generating mutations, these enzymes have been implicated in cancer pathogenesis (Henderson and Fenton 2015); early evidences have shown

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that APOBEC1 overexpression could give rise to hepatocellular carcinoma in mice (Yamanaka et al. 1995) and that AID could contribute to the development of lymphoma (Okazaki et al. 2003; Ramiro et al. 2004). More recently, a mutational signature characterized by cytosine (C) to thymine (T) or guanine (G) mutations in TC dinucleotides (where C is the mutated cytosine) occurring in spatially delimited regions in the genome of tumor cells and associating with double-stranded breaks was identified in breast cancers (BCs), and was suggested to be generated by APOBEC3-family enzymes (Nik-Zainal et al. 2012; Stephens et al. 2005).

Subsequent research making use of multi-omics data from The Cancer Genome Atlas (TCGA) project confirmed the role of APOBEC3 as a mutational source in BC and in multiple other cancers (Roberts et al. 2013) and suggested APOBEC3B as the main candidate mutagen among APOBEC3 family members (Burns et al. 2013a; b). However, this was in contrast with the finding that a 29.5 kilobase (kb) germline copy-number variation (CNV) deleting all the *APOBEC3B* coding region and linking its 3′ untranslated region (3′-UTR) to *APOBEC3A* coding exons (*A3A/B*) was associated with increased BC risk and with increased putative APOBEC-driven mutational burden (Nik-Zainal et al. 2014).

Subsequently, it was shown that signatures from APOBEC3A (enriched for YTCA sites) and APOBEC3B (enriched for RTCA sites) were distinguishable in tumor genomes, and that APOBEC3A-induced mutations were enriched in clinical samples (Chan et al. 2015). It was also shown that the linking of *APOBEC3B* 3′-UTR to *APOBEC3A* coding exons stabilizes APOBEC3A expression through differential binding of micro-RNAs (miRs) (Revathidevi et al. 2016), and increases APOBEC3A-driven mutagenesis (Caval et al. 2014). Notably, APOBEC3A was shown to translocate to the nucleus and to be a more potent mutagen in vitro (Burns et al. 2013a; Caval et al. 2014; Sommer et al. 2013).

Several studies have analyzed the influence of *A3A/B* germline deletion in BC risk and prognosis in populations from Asian and Caucasoid ethnicity, producing contradictory results, which suggests that the association of this germline deletion with BC may be population-specific (Klonowska et al. 2017).

Of note, the frequency of this CNV varies greatly among populations worldwide, but its frequency in Brazilian populations is only described for two small cohorts of native indigenous people (Kidd et al. 2007), but not for the general Brazilian population. Furthermore, the association between *A3A/B* with BC risk was never evaluated in the context of a highly admixed population before. Therefore, the present study sought to investigate for the first time the potential influence of this CNV in the susceptibility and clinical presentation of BC subtypes from a Brazilian cohort.

Materials and methods

Human subjects

The sample of the present retrospective study was characterized in previous publications (Vitiello et al. 2019,2018a; b). For current analyses, a cohort of 341 unselected and unrelated BC patients and 397 unrelated women with no personal history of any neoplasia, no familial history of BC and no evidence of mammary alteration proven by recent (within the past 2 years from collection time) mammograph exam were enrolled. The research protocol was approved by Londrina State University Ethics Committee for Research Involving Human Subjects (CEP/UEL 189/2013—CAAE 17123113400005231) and all volunteer donors signed a free-informed consent prior to the biological material collection.

From BC group, surgically excised tumor tissue and/or peripheral blood samples were collected on surgery or during routine medical exams at Londrina Cancer Hospital (Londrina, PR, Brazil). At-diagnosis clinicopathological data were retrieved from patients medical records available at Londrina Cancer Hospital and included: age at diagnosis, pathologic tumor size, histopathological grade, lymph node metastasis (LNM), cellular proliferation index (Ki67), p53 mutation, immunostaining for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), which were used to classify patients into intrinsic subtypes and were assessed according to the American Society of Clinical Oncology (ASCO) standard protocols, and disease stage, determined according to the TMN-system recomended by the Union for International Cancer Control (UICC) criteria.

Hormonal receptors (HR, namely ER and PR) and HER2 statuses were used as single or combined markers to stratify patients into subgroups or molecular subtypes as follows: Hormonal receptor-positive (HR^+: $ER/PR^+HER2^{\pm}$); HER2-positive ($HER2^+$: $ER^{\pm}PR^{\pm}HER2^+$); Luminal-A (LA: ER/PR^+HER2^-); Luminal-B (LB: ER/PR^+HER2^+); HER2-enriched (HER2: $ER^-PR^-HER2^+$) and triple negative (TN: $ER^-PR^-HER2^-$). Table 1 shows the clinicopathological features for patients in general sample and for each BC subtype.

For control group, invitation, questionnaire and peripheral blood sample collections were carried out during routine medical exams at Londrina State University Clinical Hospital or at primary health care unities in the region of Londrina (Paraná, Brazil). The mean and median ages were similar between control (mean = 54 years old; standard deviation = 13.95; median = 55 years old, interquartile range = 14.5) and BC group (Table 1).

The high miscegenation observed in Brazilian population hampers ethnic classification in a reliable way even

Table 1 Clinicopathological features of breast cancer patients

Parameter	Breast cancer subgroups				
	Total ($n=341$)	LA ($n=207$)	LB ($n=40$)	HER2 ($n=22$)	TN ($n=42$)
Age (years)					
Median (IQR)	52 (18)	54.5 (17)	48 (12)	51 (14.0)	51 (22)
Mean (SD)	54.4 (12.6)	55.8 (12.6)	50.0 (10.6)	53.3 (14.9)	52.7 (14.0)
<40 [n(%)]	36 (10.6)	18 (8.7)	5 (12.5)	3 (13.6)	8 (19.0)
40–49 [n(%)]	101 (29.7)	57 (27.7)	17 (42.5)	6 (27.3)	11 (26.2)
50–59 [n(%)]	88 (25.9)	53 (25.7)	10 (25.0)	7 (31.8)	9 (21.4)
60–69 [n(%)]	70 (20.6)	45 (21.8)	7 (17.5)	4 (18.2)	8 (19.0)
70–79 [n(%)]	35 (10.3)	26 (12.6)	1 (2.5)	1 (4.5)	4 (9.5)
≥ 80 [n(%)]	10 (2.9)	7 (3.4)	0 (0)	1 (4.5)	2 (4.8)
NA	1	1	0	0	0
Tumor size (cm)					
Median (IQR)	2.4 (1.9)	2.0 (1.5)	2.5 (1.0)	2.2 (2.6)	3.5 (3.0)
Mean (SD)	3.0 (2.5)	2.6 (1.6)	2.9 (2.0)	2.8 (1.6)	4.3 (3.6)
0–1.5 [n (%)]	81 (24.4)	57 (27.8)	6 (15.8)	3 (15.0)	7 (16.7)
1.51–3.0 [n (%)]	156 (47.0)	101 (49.3)	23 (60.5)	11 (55.0)	11 (26.2)
>3.0 [n (%)]	95 (28.6)	47 (22.9)	9 (23.7)	6 (30.0)	24 (57.1)
NA	9	2	2	2	0
Histopathological grade [n (%)]					
I	43 (13.6)	35 (18.0)	2 (5.3)	0 (0)	0 (0)
II	129 (40.8)	97 (50.0)	14 (36.8)	6 (31.6)	5 (12.2)
III	144 (45.6)	62 (32.0)	22 (57.9)	13 (68.4)	36 (87.8)
NA	25	13	2	3	1
Ki67 staining [n (%)]					
Low	54 (25.2)	48 (34.3)	4 (16.7)	0 (0)	1 (3.2)
Intermediate	95 (44.4)	69 (49.3)	10 (41.7)	5 (41.7)	8 (25.8)
High	65 (30.4)	23 (16.4)	10 (41.7)	7 (58.3)	22 (71.0)
NA	127	67	16	10	11
p53 mutation [n (%)]					
Negative	148 (64.6)	116 (78.4)	10 (43.5)	6 (37.5)	14 (40.0)
Positive	81 (35.4)	32 (21.6)	13 (56.5)	10 (62.5)	21 (60.0)
NA	112	59	17	6	7
Lymph node metastasis [n (%)]					
Negative	160 (49.2)	101 (49.5)	18 (48.6)	10 (52.6)	21 (50.0)
Positive	165 (50.8)	103 (50.5)	19 (51.4)	9 (47.4)	21 (50.0)
NA	16	3	3	3	0
Tumor stage [n (%)]					
0	11 (3.8)	6 (3.5)	1 (3.1)	2 (9.1)	0 (0)
I	57 (19.8)	40 (23.1)	4 (12.5)	4 (18.2)	5 (12.2)
II	124 (43.1)	79 (45.7)	13 (40.6)	7 (31.8)	17 (41.5)
III	78 (27.1)	39 (22.5)	12 (37.5)	7 (31.8)	14 (41.5)
IV	18 (6.3)	9 (5.2)	2 (6.3)	2 (9.1)	2 (4.9)
NA	53	34	8	0	1

using genetic markers (Lins et al. 2009; Naslavsky et al. 2017; Parra et al. 2003; Pena et al. 2009; Pimenta et al. 2006). However, both control and BC groups were served by the Brazilian public health system (SUS) and were collected from the region of Londrina, located in the south of Brazil, a region that shows a high degree of European inheritance (Caucasoid ethnicity) (Kehdy et al. 2015; Lins et al. 2009; Parra et al. 2003; Pena et al. 2009).

DNA extraction

DNA was extracted from peripheral blood samples collected in EDTA-containing vacuum tubes using the Biopur® MiniSpin extraction kit (Biometrix, Curitiba, PR, Brazil) following the manufacturer's instructions. Solid tissues were mechanically dissociated and the DNA was extracted through salting out method.

All DNA samples were quantified in a NanoDrop 2000c spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA) at 260 nm wavelength and the ratio of absorbances at 260 nm and 280 nm was used as a measure of DNA purity. Samples with ratio below 1.7 were not used in further analyses.

A3A/B genotyping

A3A/B deletion was detected through the allele-specific polymerase chain reaction (PCR) technique described by Kidd et al. (2007). Primers for amplification of the wild-type (WT) allele were: 5′-TTGGTGCTGCCCCCTC-3′ and 5′-TAGAGACTGAGGCCCAT-3′, amplifying a fragment of 490 base-pairs (bp). For the deleted (Del) allele the primers 5′-TAGGTGCCACCCCGAT-3′ and 5′-TTGAGCATA ATCTTACTCTTGTAC-3′ amplified a fragment of 700 bp. For samples showing the amplification of Del allele and no amplification for WT allele, a second round of PCR was performed with another primer pair (5′-TGTCCCTTTTCA GAGTTTGAGTA-3′ and 5′-TGGAGCCAATTAATCACT TCAT-3′) targeted to amplify a 705 bp fragment from WT allele to ensure that the lack amplification in the first PCR round was not due to patient-specific mutations in the primers target sequences.

PCRs were performed using 1 × PCR buffer, 0.25 µM of each primer, 0.1 mM of dNTP, 1 mM of $MgCl_2$ and 0.04 U/µL of Taq DNA polymerase (Invitrogen®, Carlsbad, CA, USA) to amplify 4 ng/µL of DNA template in a final reaction volume of 15 µL. Cycling conditions were: 5 min at 95 °C followed by 40 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 45 s and a final extension step at 72°C for 7 min. A known heterozygote individual and a blank reaction (with no DNA template) were included in each PCR round as positive and negative controls. PCR fragments were visualized after electrophoresis in polyacrylamide gels (10%) using Tris–Acetate-EDTA (TAE) buffer and stained with silver nitrate.

Statistical analyses

To evaluate differences in genotype and allele distributions between groups, chi-squared (χ^2) and Fisher's exact tests were applied. To test an additive model of association, χ^2 test for trend (Cochran–Armitage test) was applied.

Binary logistic regression models adjusted by age were tested to obtain odds ratios (ORs) and 95% confidence intervals (95% CI) in case–control analyses, where the following models of association were tested: genotypic (heterozygotes or variant homozygotes vs WT homozygotes), dominant (heterozygotes and variant homozygotes as single group vs WT homozygotes) and recessive (variant homozygotes vs heterozygotes and WT homozygotes as a single group).

Kendall's rank correlation tests were applied to test the correlation between *A3A/B* and clinicopathological features. Tau-b coefficient was adopted for analyses in square contingency tables (2 × 2 or 3 × 3), while Tau-c was adopted for rectangular tables. Considering all correlation tests for each independent group of samples (subtypes and general sample), corrections for multiple comparisons were applied adopting a false discovery rate (FDR) of 0.1. Binary logistic regressions adjusted for tumor size and proliferation index were also used to further explore the association of *A3A/B* with LNM.

All statistical analyses were two-tailed, had 5% significance level and were performed in SPSS®20.0 software (IBM, Amonk, NY, USA).

Results

Association between *A3A/B* deletion and breast cancer susceptibility

A3A/B genotypes and allele frequencies were compared between control and BC subgroups and no significant difference was observed through univariate chi-squared and Fisher's exact tests or through chi-squared test for trend (Table 2). Also, no significant association was found for genotypic, dominant or recessive models in any subtype tested through age-adjusted logistic regressions (Table 3). The genotype frequencies were in Hardy–Weinberg equilibrium both in control and BC group.

Correlation between *A3A/B* deletion and clinicopathological parameters

To test for correlations between *A3A/B* and clinicopathological features at diagnosis, age and tumor size were treated as continuous variables, whereas other variables were categorized as shown in Table 1. Due to the low number of homozygote individuals for the deletion allele in BC group (n = 4), recessive models could not be evaluated and only additive and dominant models were tested (Table 4).

In the general BC group, no significant correlation was found, although a trend towards a significant negative

Table 2 *A3A/B* distribution among control and breast cancer groups

A3A/B	Ctrl (397)	BC (341)	HR^+ (260)	$HER2^+$ (62)	LA (207)	LB (40)	HER2 (22)	TN (42)
Genotype [n (%)]								
WT/WT	314 (79.1)	286 (83.8)	218 (83.8)	53 (85.5)	173 (83.6)	34 (85.0)	19 (86.4)	35 (83.3)
WT/Del	74 (18.6)	51 (15.0)	39 (15.0)	6 (9.7)	33 (15.9)	4 (10.0)	2 (9.1)	7 (16.7)
Del/Del	9 (2.3)	4 (1.2)	3 (1.2)	3 (4.8)	1 (0.5)	2 (5.0)	1 (4.5)	0 (0.0)
p-value[a]	–	0.2	0.26	0.13	0.17	0.25	NA	NA
p-trend[b]	–	0.07	0.10	0.56	0.1	0.69	0.63	0.39
Allele frequency [n (%)]								
WT	702 (88.4)	623 (91.3)	475 (91.3)	112 (90.3)	379 (91.5)	72 (90.0)	40 (90.9)	77 (91.7)
Del	92 (11.6)	59 (8.7)	45 (8.7)	12 (9.7)	35 (8.5)	8 (10.0)	4 (9.1)	7 (8.3)
P value[c]	–	0.07	0.11	0.65	0.09	0.85	0.81	0.47

[a]Chi-squared (χ^2) test

[b]Chi-squared test for trend (Cochran–Armitage test)

[c]Fisher's exact test

Table 3 Case–control association analyses between *A3A/B* and breast cancer subtypes

Breast cancer subgroup	Association models for *A3A/B* [OR (95%CI)][a]			
	WT/Del	Del/Del	Dominant	Recessive
General BC ($n=341$)	0.76 (0.51–1.12)	0.49 (0.15–1.61)	0.73 (0.50–1.06)	0.52 (0.16–1.69)
HR^+ ($n=260$)	0.76 (0.49–1.17)	0.48 (0.13–1.81)	0.73 (0.49–1.10)	0.51 (0.14–1.89)
$HER2^+$ ($n=62$)	0.49 (0.20–1.18)	1.96 (0.51–7.51)	0.65 (0.31–1.38)	2.17 (0.57–8.28)
LA ($n=207$)	0.81 (0.52–1.28)	0.20 (0.02–1.60)	0.75 (0.48–1.16)	0.21 (0.03–1.66)
LB ($n=40$)	0.51 (0.18–1.50)	2.05 (0.42–9.98)	0.68 (0.28–1.69)	2.26 (0.47–10.93)
HER2 ($n=22$)	0.54 (0.06–4.51)	0.24 (0.02–2.96)	0.60 (0.17–2.08)	2.06 (0.25–17.03)
TN ($n=42$)	0.85 (0.36–2.00)	–	0.76 (0.33–1.78)	–

[a]Odds ratios (OR) and 95% confidence intervals (95%CI) obtained through age-adjusted logistic regression.

correlation between age at diagnosis and *A3A/B* was noted both in additive and dominant models ($p=0.06$ for both; Table 4).

In LA subgroup, *A3A/B* was positively correlated with tumor size and proliferation index (Ki67), indicating that this CNV positively affects LA tumor growth, but negatively correlated with LNM both in additive and dominant models ($p<0.05$; $q<0.1$; Table 4).

In TN subgroup, *A3A/B* in dominant model positively correlated with histopathological grade; however, this was not significant assuming a 10% FDR (Tau-b = 0.169; $p=0.04$; $q=0.28$; Table 4). Due to the absence of deleted homozygotes, additive model could not be evaluated in this subgroup.

A3A/B independently associates with lymph node metastasis in luminal-A subtype

Since LNM is a severe latter event in BC pathogenesis that is associated with poor overall and disease-free survival, we sought to investigate if the correlation found between *A3A/B* and LNM in LA tumors was independent of other established clinicopathological features.

Initially, correlation analyses showed that, among all clinicopathological features, excluding tumor stage (Table 1), LNM was only associated with tumor size (Tau-c = 0.312; $p<0.001$) and Ki67 (Tau-c = 0.185; $p=0.028$). Then, logistic regression models setting LNM as dependent variable and *A3A/B* in dominant model along

Table 4 Correlation between clinicopathological features and *A3A/B* deletion

Breast cancer subtype	Parameter	*A3A/B* Model [Tau (*p*-value)]	
		Additive	Dominant
General BC (341)	Age	− 0.068 (0.06)	− 0.089 (0.06)
	Tumor size	0.035 (0.3)	0.049 (0.27)
	Hist. grade	− 0.003 (0.96)	− 0.003 (0.94)
	Ki67	0.034 (0.41)	0.034 (0.41)
	p53	0.011 (0.87)	0.011 (0.87)
	LNM	− 0.064 (0.12)	− 0.091 (0.1)
	Stage	− 0.045 (0.17)	− 0.061 (0.17)
LA (207)	Age	− 0.061 (0.19)	− 0.081 (0.19)
	Tumor size	0.092 (0.03*)	0.125 (0.03*)
	Hist. grade	0.066 (0.3)	0.052 (0.32)
	Ki67	0.116 (0.04*)	0.116 (0.04*)
	p53	0.057 (0.52)	0.057 (0.52)
	LNM	− 0.119 (0.02*)	− 0.162 (0.02*)
	Stage	− 0.030 (0.45)	− 0.040 (0.45)
LB (40)	Age	− 0.128 (0.26)	− 0.170 (0.28)
	Tumor size	0.004 (0.96)	− 0.017 (0.88)
	Hist. grade	− 0.098 (0.56)	− 0.078 (0.56)
	Ki67	0.097 (0.29)	0.097 (0.29)
	p53	− 0.243 (0.29)	− 0.243 (0.29)
	LNM	0.161 (0.13)	0.227 (0.15)
	Stage	0.047 (0.54)	0.055 (0.59)
HER2 (22)	Age	− 0.118 (0.23)	− 0.140 (0.23)
	Tumor size	− 0.105 (0.41)	− 0.130 (0.42)
	Hist. grade	− 0.288 (0.1)	− 0.505 (0.1)
	Ki67	0.255 (0.28)	0.255 (0.28)
	p53	0.200 (0.30)	0.200 (0.30)
	LNM	0.100 (0.56)	0.167 (0.47)
	Stage	− 0.050 (0.66)	− 0.050 (0.73)
TN (42)	Age	–	− 0.209 (0.11)
	Tumor size	–	− 0.045 (0.74)
	Hist. grade	–	0.169 (0.04)
	Ki67	–	− 0.050 (0.58)
	p53	–	0.062 (0.71)
	LNM	–	− 0.064 (0.68)
	Stage	–	− 0.079 (0.56)

*$p < 0.05$; $q < 0.1$

with tumor size (Table 5, Model 1), Ki67 (Table 5, Model 2) or both (Table 5, Model 3) as independent variables were tested (Table 5).

In any of the tested models, *A3A/B* remained negatively associated with LNM, showing that this association is independent of tumor size and proliferation index. Ki67 lost its significance when tested along with *A3A/B* and tumor size together, indicating that the association between Ki67 and LNM was merely a reflection of the strong correlation between Ki67 and tumor size (Tau-c = 0.277; $p < 0.001$).

Discussion

APOBEC3-mediated mutagenesis has been implicated in the pathogenesis of BC (Burns et al. 2013a; Nik-Zainal et al. 2012), and of a plethora of other human cancers (Burns et al. 2013b; Roberts et al. 2013). Initial evidence has suggested that APOBEC3B was the main candidate mutational source (Burns et al. 2013a). However, it was shown that, although APOBEC3B indeed generate mutations in multiple cancers, the major burden of APOBEC-related mutations in cancer genomes are generated by APOBEC3A (Chan et al. 2015).

Consistent with this model, a copy number variation (CNV) deleting all *APOBEC3B* coding exons and linking its 3′-UTR to the full protein-coding sequence of *APOBEC3A,* generating a more stable transcript (Caval et al. 2014), was associated with increased APOBEC-mediated mutational burden (Nik-Zainal et al. 2014) and with increased BC risk in some cohorts (Long et al. 2013; Xuan et al. 2013).

In the current study, however, we did not find any significant association between *A3A/B* and susceptibility to any BC subtype. In this regard, controversial results were reported on the literature: studies in Chinese (Long et al. 2013), white Americans from USA (Xuan et al. 2013), Iranian (Rezaei et al. 2015) and in an Asian multi-ethnic cohort including Chinese, Indian and Malaysian patients (Wen et al. 2016) evidenced this association, while studies in South Indian (Revathidevi et al. 2016), Sweden (Göhler et al. 2015), Poland (Klonowska et al. 2017), Norwegian (Gansmo et al. 2018) and Moroccan (Marouf et al. 2016) cohorts failed to confirm these findings.

Indeed, two meta-analyses investigating the impact of *A3A/B* on the risk of breast and other cancers observed great heterogeneity among individual studies, suggesting that *A3A/B* may have population- as well as tumor-specific associations with cancer susceptibility, where gene–gene and gene–environment interactions may play a role (Hashemi et al. 2019; Klonowska et al. 2017).

The minor allele frequency for our control group (11.6%) was closer to that described for Caucasoid populations (5–10%) (Cescon et al. 2015; Gansmo et al. 2018; Göhler et al. 2015; Kidd et al. 2007; Klonowska et al. 2017) than that from African (0.9%), Asian (37%) or even Amerindian (58%) populations, including Brazilian native indigenous people (56% for Karitiana and 80% for Surui indigenous populations) included in the Human Genome Diversity Panel (HGDP) project (Cann 2002; Kidd et al. 2007), reinforcing the high degree of European ancestry observed in southern Brazilian population (Kehdy et al. 2015; Lins et al. 2009; Parra et al. 2003; Pena et al. 2009).

Also, previous studies by our group have shown that the SNP frequencies from our control group was similar

Table 5 Association between *A3A/B* deletion and lymph node metastasis considering other clinicopathological features

Factors in the model[a]	Models tested		
	Model 1	Model 2	Model 3
A3A/B dominant (*p*)	0.006**	0.02*	0.008**
WT/WT	Reference	Reference	Reference
WT/Del + Del/Del [OR (95%CI)]	0.31 (0.13–0.71)	0.29 (0.1–0.82)	0.22 (0.07–0.68)
Tumor size (*p*)	0.001***	–	0.006**
OR (95%CI)	1.46 (1.16–1.83)	–	1.52 (1.13–2.06)
Ki67 (*p*)	–	0.045*	0.24
Ki67 low [OR (95%CI)]	–	Reference	Reference
Ki67 intermediate [OR (95%CI)]	–	2.58 (1.16–5.71)	2.03 (0.89–4.62)
Ki67 high [OR (95%CI)]	–	2.73 (0.94–7.89)	1.71 (0.07–0.68)

*$p < 0.05$

[a]Binary logistic regression models setting lymph node metastasis as dependent variable

to that from a cohort of elderly health people from the city of São Paulo (Vitiello et al. 2018a; Vitiello et al. 2018b), available through de ABraOM database (https://abraom.ib.usp.br/) (Naslavsky et al. 2017). However, *A3A/B* was not included in that project hampering this comparison in the current study.

In relation to the impact of this CNV in APOBEC3A expression, Caval et al. (2014) reported that cells from the human embrionic kidney (HEK) 293T line transfected with constructs encoding APOBEC3A fused to APOBEC3B 3′-UTR showed increased APOBEC3A mRNA and protein expression in comparison to those transfected with natural APOBEC3A construct. Otherwise, Klonowska et al. (2017) have measured the expression of *APOBEC3A*, *APOBEC3B* and of the hybrid (*A3A/B*-derived) transcripts in lymphoblast cell lines naturally harboring different *A3A/B* genotypes, and showed that the hybrid transcript expression was several times lower than the expression of either natural APOBEC3A (~ 12-fold) or APOBEC3B (> 1500-fold) transcripts.

Also, in silico analyses suggested that while 17 miRNAs could bind to APOBEC3A 3′-UTR, only 8 miRNAs putatively bind to the 3′-UTR of *APOBEC3B* and of the hybrid transcript, including 2 miRNAs (miR-138-5p and miR-34-3p) that are frequently downregulated in multiple human cancers (Revathidevi et al. 2016). Since the set of miRNAs varies among different cells, a reasonable explanation for the above-mentioned contradiction may refer to the tissue-specific effects of miRNA on the hybrid transcript expression, once these studies investigated the expression of *A3A/B* transcripts, but not of miRNAs, in different cellular models. Of note, the increased APOBEC-mediated mutational burden in cancer tissues from Del carriers is a consistent finding on the literature which argues in favor of the increased expression and activity of APOBEC3A in this context (Cescon et al. 2015; Chan et al. 2015; Chen et al. 2019; Nik-Zainal et al. 2014).

Regarding APOBEC3B, while Del/Del homozygotes for *A3A/B* completely lose this gene, heterozygote individuals were consistently shown to have diminished APOBEC3B expression compared to WT homozygotes (Cescon et al. 2015; Chen et al. 2019; Klonowska et al. 2017), leading to the conclusion that, *A3A/B* leads to increased expression of APOBEC3A protein while diminishing APOBEC3B expression in heterozygote cancer cells (Chen et al. 2019).

Besides their effects in BC initiation, APOBECs were also shown to play important roles in disease progression, prognosis and clinical outcomes: the genetic instability generated by APOBEC-mediated mutagenesis was suggested to drive tumor evolution and increase tumor heterogeneity, eventually leading to the development of treatment resistance (Law et al. 2016). In the current work, *A3A/B* positively correlated with tumor size and proliferation index (Ki67) and negatively correlated with LNM specifically in LA ($ER/PR^{+}HER2^{-}$) subtype (Table 4). We have also shown that its protective association with LNM metastasis in LA patients was independent of other clinicopathological parameters, such as tumor size and Ki67 staining (Table 5). Also, in TN subtype, a trend towards positive correlation with histopathological grade was noted, although it was not statistically significant assuming a false discovery rate of 0.1.

A study by Liu et al. (2016) have also shown an association between *A3A/*B deletion and LNM, despite no correlation trend was observed. In another work, no direct correlation between *A3A/B* and prognosis parameters was found; however, *A3A/B* negatively correlated with APOBEC3B expression in tumor tissue, and increased APOBEC3B expression was associated with worst prognostic parameters, such as the presence of LNM, higher histopathological grades and proliferation and with poor disease outcome (Cescon et al. 2015). These effects were lately found to especially take place in ER^{+} BCs (Periyasamy et al. 2015; Sieuwerts et al. 2014; Tokunaga et al. 2016), where APOBEC3B

expression was also implicated in the development of tamoxifen resistance (Law et al. 2016).

The specific effects of APOBEC3B in ER^+ is intriguing since APOBEC-mediated mutagenesis occurs in all BC subtypes and is enhanced in $HER2^+$ BCs (Chen et al. 2019; Kanu et al. 2016; Prat et al. 2015; Roberts et al. 2013). Mechanistically explaining this effect, it was shown that APOBEC3B physically interacts with ER, producing DNA double-strand breaks which promote chromatin remodeling and stimulate gene expression on ER response elements in BC cells, enhancing tumor progression (Periyasamy et al. 2015).

The enhanced mutational rate promoted by APOBEC-mediated mutagenesis, which is increased in *A3A/B* deletion carriers (Cescon et al. 2015; Chan et al. 2015; Chen et al. 2019; Nik-Zainal et al. 2014), may also contribute to tumor evolution. However, these mutations were shown to be highly immunogenic, being associated with increased immune infiltrates and neoepitope loads in tumor microenvironment (Cescon et al. 2015; Chen et al. 2019; Smid et al. 2016). This paradox was recently described in a study investigating APOBEC-generated mutations as a source of epitopes in cell-based vaccines to enhance the efficacy of immune checkpoint blockade therapy in a pre-clinical model of cancer (Driscoll et al. 2020).

Therefore, the correlations found in our study may be explained by the increased mutational rate in deleted patients (Cescon et al. 2015; Chan et al. 2015; Chen et al. 2019; Nik-Zainal et al. 2014) enhancing genomic instability and contributing to tumor cell proliferation and anaplasia, while generating immune-recognizable neoepitopes, which could inhibit metastasis of highly transformed cells (Driscoll et al. 2020). Also, corroborating with our results, the correlation between *A3A/B* and APOBEC-mediated mutational burden was shown to be greatest in LA BCs (Chen et al. 2019).

Furthermore, it has been recently shown that APOBEC3B directly interacts with CDK4 and inhibits the nuclear translocation of Ciclin-D1, delaying cell cycle progression (McCann et al. 2019), which could also provide an additional mechanistical rationale for the positive correlations found between the *APOBEC3B* deletion and cellular proliferation index and tumor size in LA BCs.

Finally, our study also detected a trend towards a negative correlation between *A3A/B* and age at diagnosis in general BC group (Table 4). This is consistent with previous findings showing that the median age at diagnosis was lower in *A3A/B* deletion carriers in METABRIC cohort (Del/Del plus WT/Del = 59.8 years vs. WT/WT = 62.3 years; $p = 0.04$) and tended also to be lower in the TCGA cohort (Del/Del plus WT/Del = 54 years vs WT/WT = 58 years; $p = 0.07$) (Cescon et al. 2015). Together, these data indicate that BC in deletion carriers tend to occur at earlier ages when compared to non-deleted patients, which may relate to the increased mutational rate in *A3A/B* deleted individuals leading to earlier accumulation of cancer-driving mutations.

Conclusion

In conclusion, the current work found no association between the *A3A/B* germline deletion and BC susceptibility in a Brazilian cohort; however, *A3A/B* correlated with prognostic parameters in LA BCs, being an independent negative predictor of lymph node metastasis in this BC subtype. To the best of our knowledge, this is the first study investigating this polymorphism in admixed Brazilian patients. A limitation from the current work regards to the rarity of the studied CNV found in our sample and the relatively small sample studied, which decreases the study power. Therefore, further analyses in different and larger populations as well as mechanistic studies are encouraged to completely elucidate the effects of this germline deletion in different populations and BC subtypes, since this knowledge may reveal a valuable marker for BC susceptibility, prognosis and treatment, especially in the context of emerging immunotherapies.

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

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee for Research Involving Human Subjects from Londrina State University (CEP/UEL 189/2013 – CAAE 17123113400005231).

Informed consent Informed consent was obtained from all individual participants included in the study.

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4 CONSIDERAÇÕES FINAIS

O presente trabalho avaliou a influência de polimorfismos genéticos em genes chave relacionados à regulação de respostas imunológicas na carcinogênese mamária. Os resultados confirmam a etiologia diferencial dos subtipos de câncer de mama ao encontrar associações subtipo-específicas para a maioria das variantes, e destacam que alterações genéticas modulando o sistema imunológico modificam o risco e/ou a apresentação clínica desses subtipos da doença.

Com relação ao polimorfismo rs6897932 (Thr244Ile) do gene *IL7RA*, nossos resultados apontam que esse aumenta o risco para cânceres de mama triplo negativos, enquanto indica melhor prognóstico nesses mesmos tumores, correlacionando-se negativamente com o estágio tumoral. Já em tumores $HER2^{+}$ esse polimorfismo indica parâmetros clinicopatológicos de pior prognóstico, correlacionando-se positivamente ao grau histopatológico, a metástases em linfonodo e a maiores índices de proliferação celular.

Em relação a via do TGFβ nossos resultados revelam que os efeitos das alterações genéticas das moléculas dessa em diferentes subtipos de câncer de mama, refletem os efeitos dessa citocina nesses mesmos subtipos: variantes no gene *TGFB1* associadas à maior produção de TGFβ1 foram associadas a maior susceptibilidade a tumores de subtipos agressivos, como os $HER2^{+}$ e correlacionaram-se a parâmetros de pior prognóstico nesses tumores e em tumores triplo negativos, mas indicaram menores índices de proliferação celular em tumores luminais-A, enquanto variantes de baixa produção demonstrarm o padrão inverso. Já o polimorfismo G-875A da região promotora do *TGFBR2* (rs3087465) demonstrou uma forte associação protetora contra o câncer de mama, em especial os do subtipo luminal-A, mas correlacionou-se com maior grau histopatológico na amostra geral.

Ainda, demonstramos que uma estrutura haplotípica rara do gene *TGFB1* (ACTG) está associada a menores níveis plasmáticos de TGFβ1 comparado ao haplótipo mais frequente (GCTG). Já a ativação da via do TGFβ1 no microambiente tumoral não foi associada a nenhum dos polimorfismos genéticos analisados, mas indicou melhores parâmetros clinicopatológicos em tumores mamários do subtipo luminal-B e sem mutação em p53, consistentes com os efeitos supressores tumorais do TGFβ1 nesses subgrupos de câncer de mama.

Finalmente, em relação à deleção *APOBEC3A/B*, o presente estudo não demonstrou qualquer associação dessa variante com a susceptibilidade a cânceres de mama na presente amostra; no entanto, essa deleção correlaciona-se com maior proliferação e tamanho

tumoral e se mostrou um fator protetor independente quanto à presença de metástase em linfonodo em cânceres do subtipo luminal-A.

Assim, o presente resultado indica que genes relacionados a resposta imunológica participam da patogênese do câncer de mama de maneira subtipo-específica. Tais resultados auxiliam na compreensão da doença e apontam marcadores promissores de susceptibilidade e prognóstico na população Brasileira.

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ANEXOS

ANEXO A - Aprovações nos comitês de ética em pesquisa

UNIVERSIDADE ESTADUAL DE LONDRINA

PARANÁ
GOVERNO DO ESTADO

COMITÊ DE ÉTICA EM PESQUISA ENVOLVENDO SERES HUMANOS
Universidade Estadual de Londrina
Registro CONEP 5231

Parecer CEP/UEL:	189/2013
CAAE:	17123113.4.0000.5231
Data da Relatoria:	30/09/2013
Pesquisador(a):	Maria Angelica Ehara Watanabe
Unidade/Órgão:	Programa de PG em Patologia Experimental

Prezado(a) Senhor(a):

O "**Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina**" (Registro CONEP 5231) – de acordo com as orientações da Resolução 466/12 do Conselho Nacional de Saúde/MS e Resoluções Complementares, avaliou o projeto:

"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer."

Situação do Projeto: **Aprovado**

Informamos que deverá ser comunicada, por escrito, qualquer modificação que ocorra no desenvolvimento da pesquisa, bem como deverá apresentar ao CEP/UEL, via Plataforma Brasil, relatório final da pesquisa.

Londrina, 30 de setembro de 2013.

Profa. Dra. Alexandrina Aparecida Maciel Cardelli
Coordenadora do Comitê de Ética em Pesquisa Envolvendo Seres Humanos
Universidade Estadual de Londrina

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - UEL

UNIVERSIDADE ESTADUAL DE LONDRINA - UEL

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Implicações Prognósticas e Terapêuticas de Marcadores Genéticos e Imunológicos no Câncer

Pesquisador: Maria Angelica Ehara Watanabe

Área Temática: Genética Humana:
(Trata-se de pesquisa envolvendo Genética Humana que não necessita de análise ética por parte da CONEP;);

Versão: 1

CAAE: 73557317.0.0000.5231

Instituição Proponente: Programa de PG em Patologia Experimental

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 2.297.112

Apresentação do Projeto:

Trata-se de estudo vinculado ao Programa de Pós Graduação em Patologia Experimental/UEL. Segundo a pesquisadora o câncer ocorre decorrente da proliferação descontrolada das células devido a vários fatores, sejam eles ambientais ou genéticos, que podem culminar com invasão tecidual próxima ao tumor primário ou até mesmo o desenvolvimento de metástases. Trata-se de uma doença complexa, heterogênea, e sua evolução é dependente da interação tumor-hospedeiro. O conhecimento sobre os diferentes tipos de tumores tem sido muito explorado mas o grande desafio da oncologia tem sido o entendimento dos mecanismos moleculares que envolvem estes tumores malignos. Dentro deste contexto, os aspectos imunológicos, moleculares e epigenéticos, das citocinas e dos receptores de quimiocinas e receptores de citocinas, dos genes JAK2, ROR e p53, e também das enzimas de metabolização foram os temas escolhidos para serem abordados neste projeto, uma vez que todos estes parâmetros podem ter relevância clínica e também constituírem alvos promissores que no futuro podem ser valiosos na avaliação do prognóstico e no delineamento terapêutico. A pesquisa será realizada no Laboratório de Polimorfismos DNA e Imunologia, Departamento de Ciências Patológicas, Centro de Ciências Biológicas da UEL. Serão selecionadas um total de 1150 amostras provenientes do Hospital de Câncer de Londrina:

Endereço: LABESC - Sala 14
Bairro: Campus Universitário **CEP:** 86.057-970
UF: PR **Município:** LONDRINA
Telefone: (43)3371-5455 **E-mail:** cep268@uel.br

- 300 Amostras de tecido normal e tumoral de pacientes diagnosticadas com câncer de mama, câncer de laringe, câncer colorretal, meduloblastoma, neuroblastoma e tumor adrenocortical. a realização do ensaio de imuno-histoquímica. Essas análises serão realizadas no Laboratório de Polimorfismos DNA e Imunologia da UEL.
- 200 Amostras de tecido tumoral e saudável a fresco, de pacientes diagnosticados com cancer de mama, laringe e colorretal, provenientes de cirurgia para excisão do tumor do mesmo hospital para extração de DNA, RNA e sobrenadante.
- 300 Amostras de sangue de pacientes diagnosticadas com câncer de mama, câncer de laringe e câncer colorretal e tumores pediátricos, que serão coletadas para obtenção de DNA, RNA e plasma para estudos de polimorfismos genéticos, expressão gênica e proteica.
- 50 Amostras de medula de pacientes pediátricos diagnosticados com leucemias agudas (linfóide e mielóide) para extração de RNA e plasma.
- 300 Amostras de sangue periférico e/ou saliva de controles saudáveis, sem histórico de neoplasia, para extração de DNA, RNA e plasma.

Serão realizados análise de PCR-RFLP para estudo das variantes alélicas polimórficas, análise de imunohistoquímica, análise da expressão gênica por PCR quantitativo, análise da Expressão Proteica por ELISA. Os participantes da pesquisa serão convidados a participar do estudo durante o atendimento clínico no Serviço de Oncologia do Hospital do Câncer de Londrina. No grupo caso serão incluídas todos os pacientes que tiverem diagnóstico para câncer de mama, câncer laringe e câncer colorretal e tumores pediátricos. E no grupo controle serão incluídos indivíduos saudáveis sem histórico de neoplasias, doenças autoimunes e infecções. Serão excluídos pacientes com doenças infecciosas ou autoimunes.

Objetivo da Pesquisa:

Objetivo Primário:

- Avaliar a presença dos polimorfismos genéticos, expressão gênica e expressão proteica do fator de transcrição FOXP3, das citocinas TGFB1, IL10, IL12A, IL35 IL1B, TNFa e INFg, das quimiocinas CXCL12 e CCL5, das proteínas SMAD, IGF1, CTLA4, dos receptores de quimiocinas CXCR4, CXCR7 e CCR5 e receptores de citocinas TRII, TRIII, GIPR, IL1RN e IL7R, dos genes JAK2, ROR e p53, e também das enzimas de metabolização NQOI, GSTT1 e GSTM1, no tecido tumoral e normal, e no sangue periférico dos pacientes com câncer de mama, laringe e colorretal e nos tumores pediátricos.

Objetivos Secundários:

• Detectar a presença dos polimorfismos genéticos do FOXP3, TGF, IL10, IL12A, IL35 IL1B, TNFa e INF gama, das quimiocinas CXCL12 e CCL5 dos receptores de quimiocinas CXCR4, CXCR7 e CCR5 e receptores de citocinas TRII, TRIII, GIPR, IL1RN e IL7R, dos genes JAK2, ROR e p53, e também das enzimas de metabolização NQOI, GSTT1 e GSTM1 nos DNAs extraídos do câncer de mama, câncer de cólon, tumores de laringe e câncer colorretal e para os tumores pediátricos (tumor de Wilms, meduloblastoma, neuroblastoma, leucemias agudas, linfomas e tumor adrenocortical).

• Avaliar a expressão gênica dos genes acima citados por PCR quantitativo.

• Realizar imunohistoquímica para FOXP3, TGFb1, IL10, IL12A, IL35 IL1B, CXCL12 CCL5, SMAD, CXCR4, CCR5, p53 nos tecidos tumoral e saudável fixados em formalina tamponada e embebido em parafina, para avaliar a expressão proteica.

• Avaliar a expressão proteica por ELISA dos genes CXCL12, TGF-, FOXP3, CCL5 e INF gama.

• Avaliar a influência dos polimorfismos genéticos na expressão gênica e proteica desses genes.

• Comparar a frequência alélica dos polimorfismos dos genes supracitados e compará-los com os dados clinicopatológicos dos pacientes com os diferentes tipos de câncer.

Avaliação dos Riscos e Benefícios:

Segundo a pesquisadora o risco que os participantes da pesquisa podem ter neste projeto é quanto ao desconforto na hora da coleta de sangue periférico, porém a coleta será efetuada por profissional habilitado seguindo-se todas as normas de biossegurança, e caso ocorra algum tipo de desconforto o participante será prontamente atendido e amparado pelos coletores responsáveis. O estudo não trará benefícios diretos aos participantes, mas pretende-se obter marcadores que possam fornecer subsídios adicionais de auxílio prognóstico e delineamento terapêutico de pacientes com câncer de mama, cancer laringe e cancer colorretal e tumores pediátricos. Espera-se também obter uma integração maior entre as instituições colaboradoras, Universidade Estadual de Londrina e Hospital do Câncer de Londrina. O envolvimento de pesquisadores colaboradores permitirá a formação de profissionais qualificados para atuarem nas áreas de Imunologia e Genética do câncer, disseminando o conhecimento. Pretende-se contribuir com um maior conhecimento dos mecanismos envolvidos com a patogênese das doenças acima referidas e futuramente com a melhoria na qualidade de vida dos pacientes, através da inclusão de marcadores que, de alguma forma, possam ser aplicados futuramente na prática clínica.

Comentários e Considerações sobre a Pesquisa:

O estudo é relevante.

Considerações sobre os Termos de apresentação obrigatória:

A pesquisadora apresentou folha de rosto devidamente assinada pelo Coordenador do Programa de Pós Graduação em Patologia Experimental, cronograma adequado e orçamento detalhado. Apresentou 04 modelos de TCLE adequados em forma de convite (adultos e crianças caso e adulto e crianças controle saudáveis). Apresentou ainda termo de autorização do hospital e declaração de 02 bioquímicas responsáveis pelas coletas das amostras.

Conclusões ou Pendências e Lista de Inadequações:

Não há pendências ou inadequações.

Considerações Finais a critério do CEP:

Relatoria realizada em reunião ordinária em 25/09/2017

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_963665.pdf	11/08/2017 16:08:50		Aceito
Folha de Rosto	Folha_rosto.pdf	24/07/2017 16:01:36	Maria Angelica Ehara Watanabe	Aceito
Cronograma	9_cronograma.pdf	18/07/2017 15:57:53	Maria Angelica Ehara Watanabe	Aceito
Projeto Detalhado / Brochura Investigador	8_Projeto_Cancer_2017.pdf	18/07/2017 15:30:16	Maria Angelica Ehara Watanabe	Aceito
Outros	6_Declaracao_HCL.pdf	18/07/2017 15:29:55	Maria Angelica Ehara Watanabe	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	4_TCLE_pacientes_adultos.pdf	18/07/2017 15:28:43	Maria Angelica Ehara Watanabe	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	3_TCLE_criancas.pdf	18/07/2017 15:28:12	Maria Angelica Ehara Watanabe	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	2_TCLE_controles_saudaveis_crianca.pdf	18/07/2017 15:27:11	Maria Angelica Ehara Watanabe	Aceito

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Continuação do Parecer: 2.297.112

TCLE / Termos de Assentimento / Justificativa de Ausência	1_TCLE_controles_saudaveis_adultos.pdf	18/07/2017 15:25:12	Maria Angelica Ehara Watanabe	Aceito
Declaração de Pesquisadores	7_Termo_responsabilidade_coleta.pdf	18/07/2017 15:24:58	Maria Angelica Ehara Watanabe	Aceito
Declaração de Pesquisadores	5_Termo_de_Confidencialidade_e_Sigilo.pdf	18/07/2017 15:22:23	Maria Angelica Ehara Watanabe	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

LONDRINA, 26 de Setembro de 2017

Assinado por:
Rosana Lopes
(Coordenador)

Endereço: LABESC - Sala 14
Bairro: Campus Universitário **CEP:** 86.057-970
UF: PR **Município:** LONDRINA
Telefone: (43)3371-5455 **E-mail:** cep268@uel.br

ANEXO B -Termos de consentimento livre e esclarecido

UNIVERSIDADE
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TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

Informações sobre a pesquisa:

Você está sendo convidada a participar, como voluntária, da pesquisa intitulada **"Estudo de marcadores genéticos, epigenéticos, moleculares e imunológicos em câncer "**, que tem por objetivo analisar determinados tipo de moléculas que podem influenciar na imunidade da paciente. Você será esclarecida(o) sobre a pesquisa em qualquer aspecto que desejar. Sua participação não é obrigatória e, a qualquer momento, você poderá desistir de participar e retirar seu consentimento, sem que isso acarrete qualquer penalidade.

Procedimentos do Estudo:

Os procedimentos da pesquisa envolvem a obtenção de 5mL de sangue periférico para análise das células e moléculas do sistema imunológico. Em caso de tecido mamário tumoral, o tecido retirado durante a cirurgia será encaminhado para análise e o restante será utilizado para a realização deste projeto.

Confidencialidade da Pesquisa

As informações obtidas através desta pesquisa serão confidenciais e asseguramos o sigilo sobre sua participação. Os dados não serão divulgados de forma a possibilitar sua identificação.

A amostra de sangue e tecido obtidos, serão utilizados para obtenção de DNA e RNA para a realização deste projeto. A participação no estudo não acarretará custos para você e não haverá nehuma compemsação financeira adicional. A coordenadora do projeto é a Profª. Drª Maria Angelica Ehara Watanabe, que pode ser encontrada no endereço: Rod. Celso Garcia cid, 445, Departamento de Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, CEP: 86051-970, Tel / Fax: (43) 3371-5629, como também procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, na Avenida Robert Kock, nº 60, ou no telefone 3371 – 2490

Pesquisador Responsável

RG::_____

Consentimento livre esclarecido e informado:

Eu, _____, RG _____, declaro que estou de acordo com as informações contidas neste documento, fui devidamente esclarefcido pelo(s) pesquisador(es) dos objetivos e procedimentos da pesquisa de maneira clara e detalhada, e esclareci minhas dúvidas. Concordo em participar voluntariamente desse estudo sendo que poderei retirar meu consentimento a qualquer momento, antes ou durante o mesmo, sem penalidades ou prejuízos no meu atendimento neste projeto.

Londrina, ____ de _____, 20 ___.

Assinatura do doador (ou responsável): _____

UNIVERSIDADE ESTADUAL DE LONDRINA

Termo de Consentimento Livre e Esclarecido

"Implicações Prognósticas e Terapêuticas de Marcadores Genéticos e Imunológicos no Câncer"

Prezado (a) Participante:

Gostaríamos de convidá-lo (a) para participar da pesquisa, **"Implicações Prognósticas e Terapêuticas de Marcadores Genéticos e Imunológicos no Câncer",** a ser realizada no Laboratório de Polimorfismos DNA e Imunologia na Universidade Estadual de Londrina**.** O objetivo da pesquisa é analisar determinados tipos de moléculas que podem influenciar na imunidade da paciente e no curso da doença**.** Sua participação é muito importante e os procedimentos da pesquisa envolvem a obtenção de 4mL de sangue ou 2mL ou de saliva para análise das células e moléculas, bem como do tecido tumoral que será retirado no momento da cirurgia e encaminhado para análise histológica e o restante será utilizado para a realização deste projeto.

Esclarecemos que sua participação é totalmente voluntária, podendo recusar-se a participar, ou mesmo desistir a qualquer momento, sem que isto acarrete qualquer ônus ou prejuízo à sua pessoa. Esclarecemos, também, que suas informações serão utilizadas somente para os fins desta pesquisa e serão tratadas com o mais absoluto sigilo e confidencialidade, de modo a preservar a sua identidade. Este material coletado será suficiente para a realização desta pesquisa, portanto não restará material coletado. Esclarecemos ainda, que você não pagará e nem será remunerado (a) por sua participação. Em relação aos benefícios, espera-se no final do projeto compreender um pouco mais desta doença. O presente estudo não trará nenhum dano físico a participante, a exceção de algum desconforto relativo a coleta de sangue ou da saliva que, para ser minimizado, será realizada por profissional experiente no próprio hospital onde será realizada a cirurgia. Caso ocorra qualquer outro tipo de risco ou desconforto (tanto no âmbito físico, psíquico, moral, intelectual, social, cultural ou espiritual) o participante será prontamente atendido e amparado pelos responsáveis por este projeto, que estarão presentes em todas as coletas.

Caso você tenha dúvidas ou necessite de maiores esclarecimentos poderá contatar Dra. Maria Angelica E. Watanabe, Laboratório de Estudos e Aplicações de Polimorfismos, Departamento de

*Termo de Consentimento Livre Esclarecido apresentado, atendendo, conforme normas da Resolução 466/2012 de 12 de dezembro de 2012.

Ciências Patológicas, Centro de Ciências Biológicas, Universidade Estadual de Londrina, Rod. Celso Garcia Cid, 445, CEP: 86051-970, Tel/Fax: (43) 3371-5629, celular 99903100, email: maewatuel@gmail.com, ou procurar o Comitê de Ética em Pesquisa Envolvendo Seres Humanos da Universidade Estadual de Londrina, situado junto ao LABESC – Laboratório Escola, no Campus Universitário, telefone 3371-5455, e-mail: cep268@uel.br. Este termo deverá ser preenchido em duas vias de igual teor, sendo uma delas devidamente preenchida, assinada e entregue ao (a) participante da pesquisa.

Londrina, ___ de_____ _____de 20___.

Pesquisador Responsável:
Maria Angelica Ehara Watanabe
RG 1.280.748-1

Eu, (nome_____), declaro que fui devidamente esclarecido (a) sobre os procedimentos do estudo e concordo em participar **voluntariamente** da pesquisa descrita acima.
Assinatura (ou impressão dactiloscópica): _____
Data: _____

*Termo de Consentimento Livre Esclarecido apresentado, atendendo, conforme normas da Resolução 466/2012 de 12 de dezembro de 2012.

ANEXO C - Parecer do Hospital Universitário da Universidade Estadual de Londrina

UNIVERSIDADE ESTADUAL DE LONDRINA

PARANÁ
GOVERNO DO ESTADO

HOSPITAL UNIVERSITÁRIO
DIRETORIA SUPERINTENDENTE

PARECER PROCESSO 12901 . 2013 . 87

À Pesquisadora

Maria Angélica Ehara Watanabe

Considerando o Projeto de Pesquisa com o título "ESTUDO DE MARCADORES GENÉTICOS, EPIGENÉTICOS, MOLECULARES E IMUNOLÓGICOS EM CÂNCER" apresentado a esse Hospital Universitário, estando vinculado ao Programa de Pós-Graduação em Patologia Experimental - Centro de Ciências Biológicas/UEL;

Considerando o parecer favorável apresentado nas instâncias administrativas que envolvem a realização do estudo;

Considerando que o projeto deverá ser analisado pelo Comitê de Ética em Pesquisa do HU/UEL para posterior operacionalização, atendendo a Resolução 196/96 do Conselho Nacional de Pesquisa;

Vimos informar que **somos de parecer favorável à sua realização, resguardando-se o atendimento da legislação vigente**.

Solicitamos que, tão logo o Comitê de Ética emita parecer, que essa Diretoria Superintendente seja notificada, para os procedimentos cabíveis relacionados à documentação da pesquisa.

Solicitamos também que, uma vez realizado o estudo, uma cópia seja apresentada a esta Diretoria Superintendente, para ciência e divulgação.

Em 29/05/2013.

Profa. Dra. Margarida de Fátima Fernandes Carvalho
Diretora Superintendente do HU