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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

Glauco Akelinghton Freire Vitiello[1] · Marla Karine Amarante[1] · Bruna Karina Banin-Hirata[1] · Clodoaldo Zago Campos[2,5] · Karen Brajão de Oliveira[1] · Roberta Losi-Guembarovski[3] · Maria Angelica Ehara Watanabe[1,4]

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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

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✉ Maria Angelica Ehara Watanabe
maewatuel@gmail.com

[1] Department of Pathological Sciences, Biological Sciences Center, Londrina State University, Londrina, PR 86057-970, Brazil

[2] Department of Clinical Research, Londrina Cancer Hospital, Londrina, PR 86015-520, Brazil

[3] Department of General Biology, Biological Sciences Center, Londrina State University, Londrina, PR 86057-970, Brazil

[4] Laboratory of DNA Polymorphisms and Immunology, Department of Pathological Sciences, Biological Sciences Center, Londrina State University, Celso Garcia Cid highway (PR445), Km380, Londrina, PR 86057-970, Brazil

[5] Department of Clinical Medicine, Health Sciences Center, Londrina State University, Londrina, PR 86057-970, Brazil

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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