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TGFβ1 pathway components in breast cancer tissue from aggressive subtypes correlate with better prognostic parameters in ER-positive and p53-negative cancers

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Abstract

Background: TGFβ signaling exerts context-specific effects in breast cancer (BC) pathogenesis and single nucleotide polymorphisms (SNPs) in TGFβ-signaling components play a role in the genetic control of their expression and in BC susceptibility and clinical presentation. However, studies investigating the association between the TGFβ-signaling molecules and BC prognosis rarely considered disease subtypes and SNPs. Therefore, the present study aimed to evaluate the expression of TGFβ-signaling components in BC tissue from patients with available data regarding *TGFB1* and *TGFBR2* SNPs and plasmatic TGFβ1 levels.

Methods: Immunostaining for TGFβ1, TGFβRII and phosphorylated (p)-SMAD2/3 was investigated in primary tumor tissue from 34 patients with luminal-B-HER2$^+$ (LB-HER2), HER2-enriched (HER2) and triple negative (TN) BC subtypes genotyped for *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) and *TGFBR2* (rs3087465) SNPs.

Results: Strong positive correlations were observed between TGFβ1, TGFβRII and p-SMAD2/3 in tumor tissue, and an inverse correlation was observed between intratumor and plasmatic TGFβ1 levels in TN BCs. In LB-HER2$^+$ tumors, p-SMAD2/3 was associated with older age at diagnosis and inversely correlated with p53 staining and lymph-node metastasis, while tumor-size negatively correlated with TGFβ1 and TGFβRII in this BC subgroup. Also, in p53-negative BCs, tumor size and Ki67 negatively correlated with both TGFβ1, TGFβRII and p-SMAD2/3. No correlation was found between SNPs and TGFβ1-signaling components expression.

Conclusion: TGFβ1 canonical signaling is activated in approximately half of BCs, and correlation between TGFβ components indicate a paracrine activation, which may exert tumor suppressor effects in p53-negative or Luminal-B-HER2$^+$ subgroups.

Keywords: Transforming growth factor beta, Breast neoplasm, Immunohistochemistry, Biomarkers, Prognosis, Polymorphisms

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Background

Breast cancer (BC) is a heterogeneous neoplastic disease comprising several phenotypically-differing histological and molecular subtypes defined by gene expression, methylation or mutational signatures (Cancer Genome Atlas N 2012; Ciriello et al. 2015) and at least four clinically-relevant subtypes identified by pathologic assessment of key markers through immunohistochemistry (Eroles et al. 2012; Perou et al. 2000; Polyak 2007). Currently, BC is responsible for approximately a quarter of cancer cases and for 15% of cancer-related deaths in women worldwide (Bray et al. 2018). Moreover, great patient-to-patient variability in disease evolution is observed even within well-defined molecular subtypes (Reis-Filho and Pusztai 2011).

Several factors are known to play a role in BC progression. 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 (Tata et al. 2019).

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 an inactive large latent complex which remains attached to the extracellular matrix until they are activated by diverse stimuli such as acidification, oxidative stress or through the activity of metalloproteinases (Kubiczkova et al. 2012).

These 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 then phosphorylate and activate cytoplasmic SMAD2 and SMAD3 transcription factors (TFs), which complex to SMAD4 and translocate to the nucleus to interact with other TFs and act as co-activators or co-repressors of TGFβ target genes (Kubiczkova et al. 2012). Alternatively, other pathways are directly activated by TGFβ signaling, such as the RAS/MAPK, PI3K/AKT/mTOR and Rho-GTPase (Vander Ark et al. 2018).

The complexity of TGFβ signaling leads to paradoxical effects in cancer: while in normal epithelial cells and in initial tumors it exerts antitumor effects by inducing apoptosis and cell-cycle arrest, in more aggressive neoplasms it can act as a pro carcinogenic factor by stimulating cell migration and epithelial-to-mesenchymal transition (EMT), by promoting angiogenesis and by inhibiting anti-tumor immunity, thereby enhancing the metastatic potential of the tumor (Bierie and Moses 2010; Bierie and Moses 2014; Tang et al. 2003; Yang et al. 2010).

In BC these effects are clear among different disease subgroups and stages, with tumor suppressor effects being observed mainly in luminal BCs and in initial tumors, and pro-tumor effects taking place mainly in HER2$^+$ and triple negative (TN) subtypes (Parvani et al. 2011; Tang et al. 2003; Wilson et al. 2005) and in p53-mutated tumors (Adorno et al. 2009).

Over the last years, our group have investigated single nucleotide polymorphisms (SNPs) in *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) (Vitiello et al. 2018) and *TGFBR2* (rs3087465) (Vitiello et al. 2019) genes in BC susceptibility and clinical presentation, showing that these variants hold subtype-specific effects. Also, it was shown that *TGFB1* haplotypes composed by these SNPs can impact the cytokine plasmatic levels (Vitiello et al. 2020). 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 or prognosis produced contradictory conclusions which may be reminiscent of the context-specific effects of TGFβ1 in different BC subgroups, since the subtype-specific impacts of these markers was poorly characterized by previous works (Buck et al. 2004a; Buck et al. 2004b; Ding et al. 2016; Figueroa et al. 2009; Gorsch et al. 1992; Koumoundourou et al. 2007; Lv et al. 2013; Qiu et al. 2015; Stuelten et al. 2006).

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 selected BC subtypes (Luminal-B-HER2$^+$, HER2-enriched and triple negative) with available data regarding at-diagnosis clinicopathological features, *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 immunostaining.

Material and methods

Sample selection

For the current study, 34 formalin-fixed, paraffin embedded (FFPE) tissues from equivalent number of patients diagnosed for Luminal-B-HER2$^+$ (LB), HER2-enriched

(HER2) or triple negative (TN) BC subtypes with available data regarding *TGFB1* (rs1800468, rs1800469, rs1800470 and rs1800471) and *TGFBR2* (rs3087465) SNPs from previous studies (Vitiello et al. 2019; Vitiello et al. 2018) were collected. Twenty-one of these patients also had plasmatic TGFβ1 levels measured at-diagnosis from a previous work (Vitiello et al. 2020). Clinicopathological features for patients included in this study are shown in Table 1, while information regarding their genotypes for *TGFB1* and *TGFBR2* are in Table 2.

All 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 (Hammond et al. 2010; Wolff et al. 2013) by experienced pathologists in clinical routine for BC diagnosis. Immunostainings for estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) were retrieved from patients' data and used to classify their tumors into the following BC intrinsic subtypes: Luminal-B-HER2$^+$ (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. Ductal carcinoma in situ (DCIS) were included in the sample as stage 0 BCs, as recommended by UICC and the American Joint Committee on Cancer (AJCC) (Giuliano et al. 2017; Hortobagyi et al. 2018). This classification considers DCIS as a pre-invasive BC stage that hold a malignant phenotype, which has high propensity to progress to invasive ductal carcinoma (IDC) in mid-term, despite still being confined by the ductal basement membrane on diagnosis (Chootipongchaivat et al. 2020; Erbas et al. 2006).

Other at-diagnosis clinicopathological data included: patients' age, pathologic tumor size, histopathologic grade, pathologic nodal status, proliferation index (Ki67) and p53 immunostaining, which was used as a classical indirect indicator for missense p53 mutations (Elledge et al. 1994) associated with worse disease prognosis (Banin Hirata et al. 2014; Cattoretti et al. 1988).

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 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-146, 1:100), goat anti-human TGFβRII antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-400, 1:100) and goat anti-human Ser423/425-phosphorylated-SMAD2/3 antibody (p-SMAD2/3; Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. sc-11,769, 1:100), followed by secondary antibody polymer conjugation (ImmunoDetector HRP/DAB, BioSB, Santa Barbara, CA, USA) and by color development with diaminobenzidine (Sigma Chemical Co., St. Louis, MO, USA). A 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 by an experienced breast pathologist (J.C.) who was blind regarding patients' identification, BC subtype, clinicopathological features and genotypes for *TGFB1* and *TGFBR2* SNPs. For each sample, three tumor areas with the greater TGFβ1, TGFβRII and p-SMAD2/3 immunostaining intensity were photographed (800 × 600 pixels) from 400X magnification fields using an Amscope camera (FMA050) adapted in the microscope.

Digitally acquired images were then analyzed using 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.

Online data repositories

To complement our data on the expression of TGFβ-signaling components in BC tissue, 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.

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 as checked by 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

Table 1 Patients' clinicopathological features

Parameter	General BC ($n = 34$)	LB-HER2$^+$ ($n = 9$)	HER2 ($n = 9$)	TN ($n = 16$)
Age				
Median (IQR)	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)
Histological subtype [n (%)]				
DCIS	31 (91.2)	7 (77.8)	8 (88.9)	16 (100)
IDC	2 (5.9)	1 (11.1)	1 (11.1)	0 (0)
Mixed (IDC and ILC)	1 (2.9)	1 (11.1)	0 (0)	0 (0)
Other				
Tumor size [n (%)]				
Median (IQR)	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)
Histopathological 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 (DCIS)	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)

DCIS Ductal carcinoma in situ, *IDC* Invasive ductal carcinoma, *ILC* Invasive lobular carcinoma, *IQR* interquartile range, *SD* Standard deviation

Table 2 Genotypes for *TGFB1* and *TGFBR2* of BC patients

SNP	Total ($n = 34$)	LB-HER2$^+$ ($n = 9$)	HER2 ($n = 9$)	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)	0 (0.0)
***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)	4 (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* G74C**				
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)

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 (Benjamini and Hochberg 1995) and *q*-values were reported.

Results

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

TGFβ1 and TGFβRII expressions were predominantly cytoplasmic and/or membranous, while p-SMAD2/3 had mainly cytoplasmic staining (Fig. 1). Interestingly, TGFβ1 and TGFβRII immunostainings had bimodal distributions that were 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 (Fig. 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-HER2$^+$ 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.

Also, there was a strong correlation between the staining intensity for the three markers which was consistent among BC subtypes (Fig. 3). 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 (Fig. 4). In this analysis, SMAD7 was used as a reporter gene for SMAD2/3 activation, since this gene is directly activated as a negative feedback in this signaling pathway.

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

For 21 of the samples (6 from LB-HER2$^+$, 7 from HER2-enriched and 8 from TN subgroups), data regarding TGFβ1 plasmatic levels at diagnosis were available. 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 (Fig. 5). However, in TN subtype, but not in LB-HER2$^+$ or HER2-enriched subtypes, there was a significant negative correlation between plasmatic TGFβ1 and both intratumor TGFβ1 (Fig. 5a; Tau-b = – 0.643; $p = 0.026$) and p-SMAD2/3 staining (Fig. 5b; Tau-b = – 0.571; $p = 0.048$).

Regarding *TGFB1* and *TGFBR2* SNPs, no significant correlation was found for intratumor TGFβ1, TGFβRII or p-SMAD2/3 staining, neither in the general BC group (Table 3) nor in subtype-stratified analyses (data not shown). Similarly, no association with *TGFB1* or *TGFBR2* SNPs was found dichotomizing TGFβ1 components immunostaining as low (bellow the mean) or high (above the mean) (data not shown).

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 markers and any clinicopathological parameters in general sample or in HER2-enriched and TN subtypes (Table 4).

Otherwise, in LB-HER2$^+$ subtype, p-SMAD2/3 was positively correlated with age at diagnosis (Tau-b =

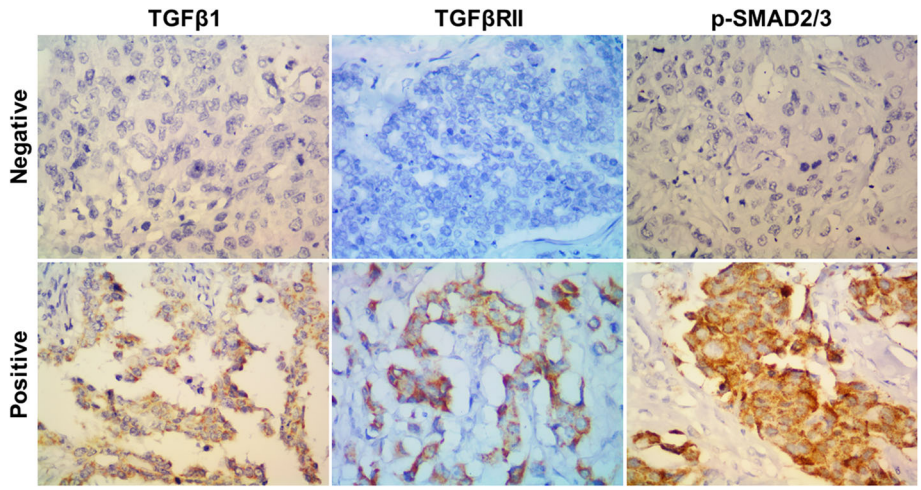

Fig. 1 Representative photomicrographs showing BC tumor sections with negative (top panel) and positive (bottom panel) staining for TGFβ1, TGFβRII and p-SMAD2/3. 400X magnification

0.551; $p = 0.004$; q = 0.084) and negatively correlated with p53 staining (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 4).

Guided by previous research indicating that p53 mutation status was an important factor switching TGFβ-signaling from a tumor suppressor to a tumor promoter (Adorno et al. 2009), correlations between TGFβ components and clinicopathological data stratifying patients by p53 status assessed through immunohistochemistry, as previously described (Elledge et al. 1994), were assessed (Table 5).

In p53-negative group, all TGFβ-signaling components negatively correlated both with tumor-size (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, $p < 0.001$, $q = 0.018$; TGFβRII: Tau-c = – 0.479, $p = 0.007$; p-SMAD2/3: Tau-c = – 0.462, $p = 0.004$, $q = 0.072$), while no correlation was observed in p53-positive group (Table 5).

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 (Adorno et al. 2009; Parvani et al. 2011; Tang et al. 2003; Wilson et al. 2005).

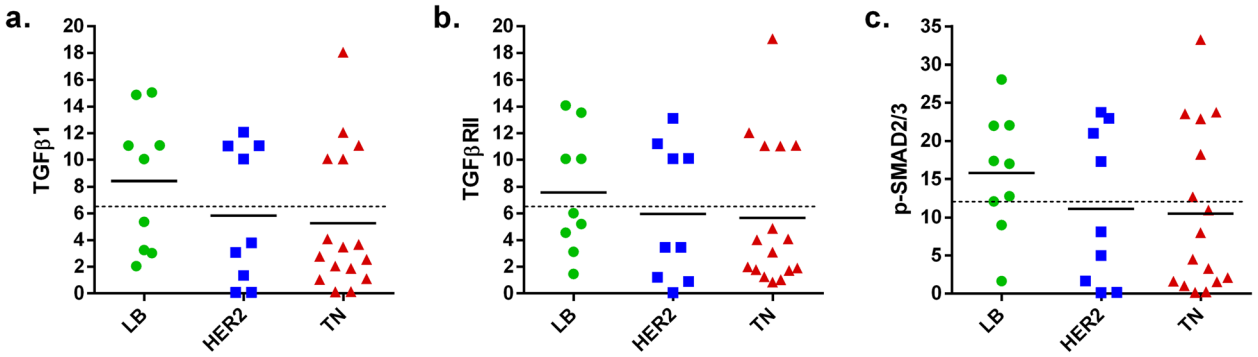

Fig. 2 Immunostaining scores for TGFβ1 (**a**), TGFβRII (**b**) and p-SMAD2/3 (**c**) among BC molecular subtypes. Data is represented as the mean percentage of positive area in 3 fields analyzed per slide. Black lines represent the mean for each subtype. Dashed lines represent the mean for TGFβ1 (**a**; 6.253), TGFβRII (**b**; 6.294) and p-SMAD2/3 (**c**; 12.056) considering all BC samples. LB: Luminal-B-HER2$^+$ subtype. HER2: HER2-enriched subtype. TN: triple-negative subtype

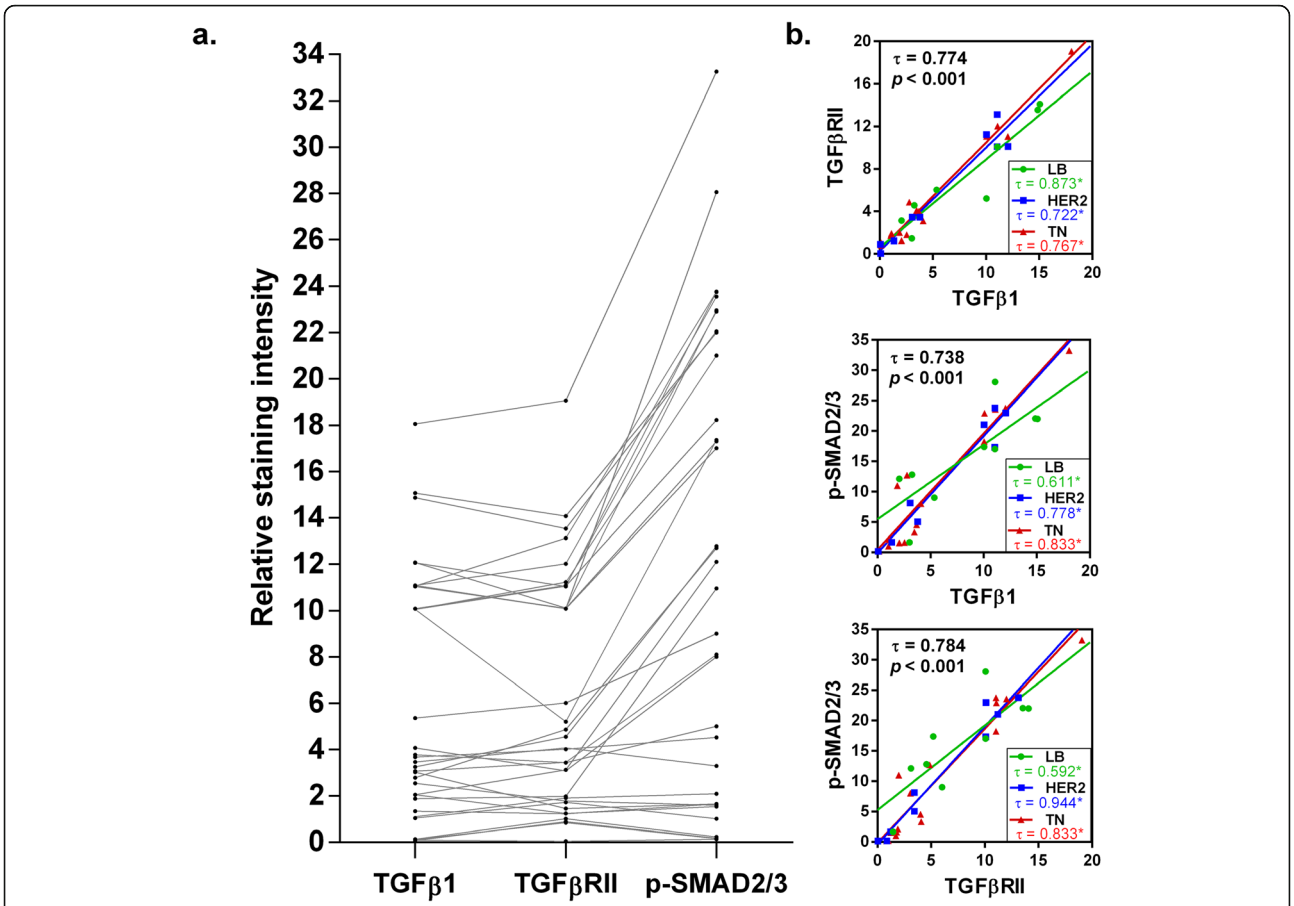

Fig. 3 Correlation between TGFβ1, TGFβRII and p-SMAD2/3 in breast cancer tissue. **a** Distribution of data regarding TGFβ1, TGFβRII and p-SMAD2/3 immunostaining in BC tissue. Lines connect data from the same patients. **b** Correlation between TGFβ1 and TGFβRII, TGFβ1 and p-SMAD2/3 and between TGFβRII and p-SMAD2/3. Tau-b correlation coefficient and *p*-values are shown within each graph. LB: Luminal-B-HER2^{+} subtype. HER2: HER2-enriched subtype. TN: triple-negative subtype

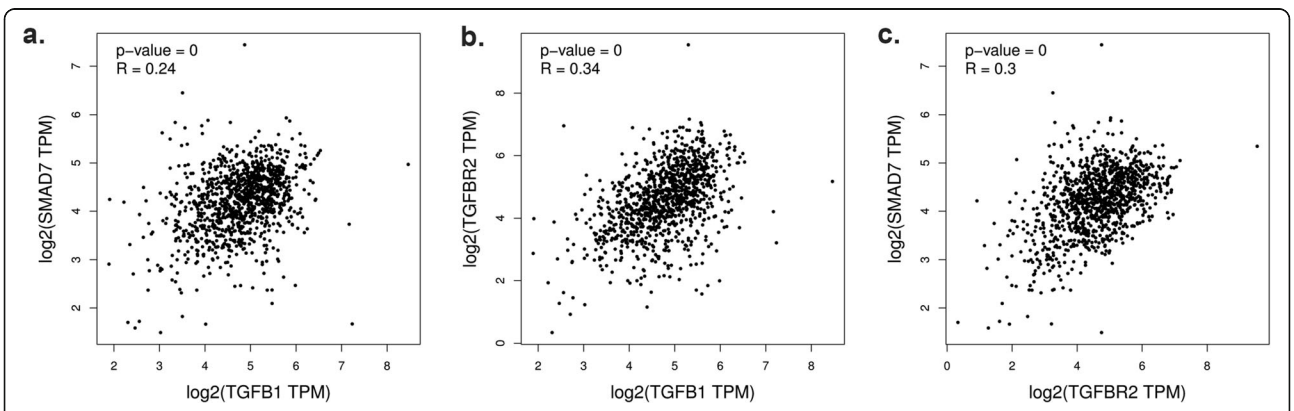

Fig. 4 Correlation between *TGFB1*, *TGFBR2* and *SMAD7* genes in breast cancer samples from TCGA project. Extremely significant correlations were evidenced between the expression of *TGFB1* and *TGFBR2* (**a**), *TGFB1* and *SMAD7* (**b**) and between *TGFBR2* and *SMAD7* (**c**) genes in tumor tissue 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)

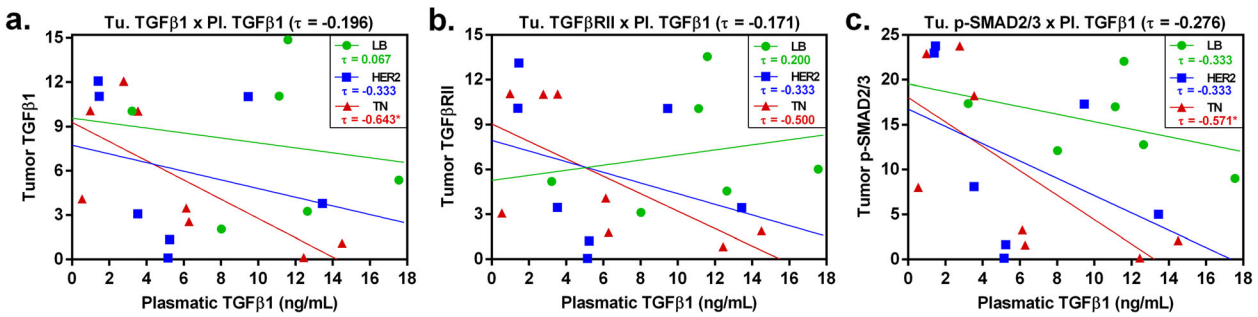

Fig. 5 Correlation between plasmatic (Pl.) TGFβ1 levels and tumor tissue (Tu.) staining for TGFβ1 (**a**), TGFβRII (**b**) and p-SMAD2/3 (**c**). Dots are shape-coded and colored according to the subtype: green circles represent samples from Luminal-B (LB) subtype, blue squares represent samples from HER2-enriched (HER2) subtype and red triangles represent samples from triple-negative (TN) subtype. Regression lines and Tau-b coefficients are also shown following the same color code. LB: Luminal-B-HER2$^+$ subtype. HER2: HER2-enriched subtype. TN: triple-negative subtype

Previous research have shown that genetic polymorphisms in *TGFB1* and *TGFBR2* genes potentially altering their expression hold subtype-specific associations with susceptibility and clinical presentation in BC, which were consistent with TGFβ1 biological effects (Vitiello et al. 2019; Vitiello et al. 2018). Also, it was shown that a rare *TGFB1* haplotype was associated with plasmatic TGFβ1 levels (Vitiello et al. 2020). 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 tissue through immunohistochemistry, and cytoplasmic staining in neoplastic cells was noted for all of them, which is corroborated by data from The Human Protein Atlas (dataset publicly available at https://www.proteinatlas.org/) and by previous research (Gorsch et al. 1992; Koumoundourou et al.

Table 3 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* G74C (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

Table 4 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)
LB-HER2$^+$	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	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)
TN	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 ($p < 0.05$)

2007; Lv et al. 2013). 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 (Figueroa et al. 2009; Koumoundourou et al. 2007; Stuelten et al. 2006), 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 (Shariat et al. 2004). 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 TGFβ1 and intratumor TGFβ signaling in BC, and indicate that plasmatic TGFβ1 may not be a good surrogate marker for TGFβ1 activity in breast 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. Furthermore, the high correlation between TGFβ1 components (including activated SMAD2/3) in tumor tissue and the staining of both TGFβ1 and TGFβRII in the cytoplasm of tumor cells, and not extracellularly and in membrane fractions, might be suggestive of an autocrine or paracrine mode of action of TGFβ leading to receptor/cytokine internalization in cancer cells. Therefore, we hypothesize that

Table 5 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-negative	**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-positive	**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 ($p < 0.05$)

the main actions of TGFβ1 in BC are mediated by its local production and consumption in tumor tissue, a phenomenon that cannot be inferred by systemic TGFβ1 quantification.

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 (Awad et al. 1998; Cao et al. 2014; Cotton et al. 2002; Dunning et al. 2003; Grainger et al. 1999; Shah et al. 2006; Silverman et al. 2004). 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 (Vitiello et al. 2020).

Previous works have also produced controversial results regarding correlations between the expression of TGFβ components and clinicopathological features (Buck et al. 2004a; Buck et al. 2004b; Ding et al. 2016; Figueroa et al. 2009; Gorsch et al. 1992; Koumoundourou et al. 2007; Lv et al. 2013; Qiu et al. 2015; Stuelten et al. 2006) or BC prognosis (Buck et al. 2004a; Buck et al. 2004b; Koumoundourou et al. 2007; Stuelten et al. 2006), 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-$HER2^+$ 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 (Figueroa et al. 2009). Another study has found that TGFβRII, but not p-SMAD2, was associated with younger age at diagnosis (Qiu et al. 2015), while others failed to observe any association between TGFβ signaling components and patients' age (Buck et al. 2004a; Buck et al. 2004b; Ding et al. 2016). However, none of these studies investigated specifically LB-$HER2^+$ BCs. Of note, in the current work 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 have also shown that TGFβ1 (Ding et al. 2016), p-SMAD2 (Figueroa et al. 2009) and TGFβRI (Buck et al. 2004a; Buck et al. 2004b) immunostainings were positively associated with LNM specifically in ER^- and TN BCs. Also, in ER^- cancers, TGFβRII staining was associated with larger tumor size (Figueroa et al. 2009). The current study, otherwise, found opposite trends in LB-$HER2^+$ tumors. Of note, once ER^- and TN cancers have increased invasive potential compared to ER^+ (luminal) BCs, these data might be consistent

with the paradoxical biological effects of TGFβ1 in promoting aggressive cancer while retaining tumor suppressor effects in less aggressive BCs (Bierie and Moses 2014; Parvani et al. 2011; Tang et al. 2003; Vitiello et al. 2018; Yang et al. 2010).

This model is in accordance with studies demonstrating that a gene-expression signature for TGFβ signaling indicated enhanced metastatic potential in ER^- BCs (Padua et al. 2008), whereas a TGFβ deficient signature correlated with metastasis in ER^+ tumors (Bierie et al. 2009; Bierie and Moses 2014). This is also corroborated by immunohistochemistry analyses showing that low TGFβ1 staining predicts longer disease-free survival (DFS) in TN BC (Ding et al. 2016) and high TGFβRII predicts shorter DFS in ER^- cancers (Buck et al. 2004a; Buck et al. 2004b), while p-SMAD2/3 staining was associated with increased DFS in ER^+ group (Koumoundourou et al. 2007).

Of note, TGFβ was shown to mediate the action of anti-estrogen therapy in ER^+ BCs, promoting apoptosis in tamoxifen-treated cells (Buck et al. 2004a; Buck et al. 2004b). Mechanistically, ER and TGFβ signaling were shown to crosstalk in breast carcinogenesis (Band and Laiho 2011) and ERα was shown to physically interact with and inhibit p-SMAD2/3 signaling by promoting their degradation, blocking TGFβ-induced EMT and migration (Cherlet and Murphy 2007; Ito et al. 2010; Malek et al. 2006). On the other hand, TGFβ signaling seems necessary to counteract ERα-induced proliferation of breast cells (Ewan et al. 2005). 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 (Cordenonsi et al. 2007), 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 (Adorno et al. 2009).

Despite this, previous research investigating 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 (Banin Hirata et al. 2014; Cattoretti et al. 1988; Elledge et al. 1994), and showed that in p53-negative group, TGFβ-signaling was associated with decreased tumor-size and proliferation, while in p53-positive BCs, no significant correlation was observed. These data might indicate that TGFβ1 exerts tumor-suppressive effects in the p53-negative group, but not in cancers associated with 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 (Zaha 2014), as it did not shown sufficient evidence to support recommendation for its use in clinical practice routine (Harris et al. 2007). However, the results reported herein and by previous data (Adorno et al. 2009) might support a role for TGFβ-signaling in conferring a clinical significance for p53 immunostaining in BC.

Conclusion

In conclusion, the present study suggests shows that TGFβ signaling components are co-expressed and activated in approximately half of tumors from Luminal-B-$HER2^+$ and HER2-enriched BCs and in a lesser proportion of triple negative BCs. Also, current data indicate that plasmatic TGFβ1 might not reflect TGFβ signaling in tumor tissue. Finally, results indicate that TGFβ signaling exert tumor-suppressive effects in luminal-B-$HER2^+$ and p53-negative BCs, consistent with the 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.

Abbreviations

ASCO: American Association for Clinical Oncology; BC: Breast cancer; DAB: 3,3′-Diaminobenzidine; DFS: Disease-free survival; ELISA: Enzyme-linked immunosorbent assay; EMT: Epithelial-to-mesenchymal transition; ER: Estrogen receptor; FDR: False discovery rate; HER2: Human epidermal growth factor receptor 2; LB: Luminal-B; LNM: Lymph node metastasis; PR: Progesterone receptor; SNP: Single nucleotide polymorphism; TCGA: The cancer genome atlas; TGFβ: Transforming growth factor beta; TGFβR: Transforming growth factor beta receptor; TN: Triple negative

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Authors' contributions

Glauco Akelinghton Freire Vitiello: Conceptualization; Data Curation; Formal Analysis; Investigation; Methodology; Visualization; Writing – Original draft. Marla Karine Amarante: Conceptualization; Methodology; Investigation; Data curation; Resources; Writing – review & editing. Jefferson Crespigio: Methodology; Investigation; Resources; Writing – review & editing. Bruna Karina Banin-Hirata: Investigation; Data curation. Nathalia de Sousa Pereira:

Investigtion; Data curation. Karen Brajão de Oliveira: Conceptualization; Resources; Writing - Review & editing; Roberta Losi Guembarovski: Conceptualization; Methodology; Resources; Funding acquisition; Project administration; Supervision; Writing - Review & editing. Maria Angelica Ehara Watanabe: Conceptualization; Methodology; Resources; Funding acquisition; Project administration; Supervision; Writing - Review & editing. The author(s) read and approved the final manuscript.

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Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
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 (CAAE 73557317.0.0000.5231). Written and signed informed consent was obtained from all individual participants included in the study.

Consent for publication
Not applicable.

Competing interests
The authors declare no conflicts of interest or competing interests.

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