

GPR30 and estrogen receptor expression: new insights into hormone dependence of inflammatory breast cancer

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Abstract GPR30 is a novel G protein-coupled estrogen receptor (ER) associated with metastases in breast cancer (BC) and poor survival in endometrial and ovarian tumors. The association of GPR30 expression with inflammatory breast cancer (IBC), an aggressive and commonly hormone-independent form of BC, has not been studied. GPR30, ER, progesterone receptor (PR), epidermal growth factor receptor (EGFR), and HER-2 expression were assessed by immunohistochemistry (and FISH for HER-2) in 88 primary IBCs. GPR30 expression was correlated with

patient overall survival (OS), disease-free survival (DFS), pathologic variables, and other biomarkers. GPR30 expression was found in 69% of IBC cases. ER, PR, HER-2, and EGFR were found in 43, 35, 39, and 34% of IBC cases, respectively. GPR30 expression correlated inversely with ER expression ($P = 0.02$). Co-expression of ER and GPR30 was found in 24% of IBC samples; 19% expressed only ER and 46% expressed only GPR30. Univariate analysis showed no association between GPR30 expression and OS or DFS. However, co-expression of ER and GPR30 was associated with improved OS ($P < 0.03$) and marginally with DFS ($P < 0.06$); the absence of both ER and GPR30 was associated with worse OS and DFS ($P = 0.03$ for both). Multivariate analysis identified ER as an independent prognostic factor of OS ($P = 0.008$) and DFS ($P = 0.02$). The majority of IBC tumors are GPR30-positive, suggesting that estrogen signaling may be active in ER-negative IBC patients. These findings suggest potential new therapeutic targets for IBC such as novel endocrine agents or direct modulation of GPR30.

Hugo Arias-Pulido and Yun Gong contributed equally to this work. Melanie Royce and Massimo Cristofanilli equally contributed as senior investigators in this study.

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Introduction

Inflammatory breast cancer (IBC) is a rare but aggressive and lethal form of breast cancer with clinical and biological characteristics of a rapidly proliferating disease [1, 2]. While the incidence of non-IBC (NIBC) has decreased based on the Surveillance, Epidemiology, and End Results (SEER) registries [3], that of IBC has increased throughout the 1990s [4]. IBC patients typically present with clinical signs mimicking an inflammatory process such as diffuse breast erythema, peau d'orange, skin induration, and warmth. Tumor emboli are often identified in dermal lymphatics, although they are not always seen on skin biopsy [2, 5]. Although multidisciplinary approaches and multimodality therapy, especially the administration of neoadjuvant chemotherapy, have improved the outcomes and survival rates for locally advanced breast cancer, the prognosis of IBC remains poor [6, 7]. This is in part related to the fact that knowledge of the molecular mechanism(s) underlying IBC still lags behind that of NIBC. At the molecular level, most IBC tumors are associated with features of poorer prognosis such as lack of hormone receptor expression, HER-2 amplification and over-expression of EGFR, nuclear factor κ B (NF- κ B), E-cadherin, and caveolin-1/2, as well as mutations of p53 [8, 9]. However, these markers are not specific for IBC. Although RhoC expression is considered a genetic determinant for IBC and coordinated changes in RhoC and WISP3 expression are fundamental to the pathogenesis of IBC [10], there are no useful clinical predictive markers in IBC. Therefore, elucidating the role of novel individual molecules and their associated pathways is important as they might provide insights into the biology of and potential new therapeutic targets for IBC.

GPR30, a recently characterized seven-transmembrane receptor belonging to the G-protein-coupled receptor family, binds estrogen with high affinity, and functions independently from the traditional nuclear estrogen receptors (ER α and ER β) to regulate cellular and physiological responsiveness to estrogen [11, 12]. Activation of GPR30 leads to multiple intracellular responses related to growth, differentiation, and proliferation [13–15]. Therefore, GPR30-mediated activity is of particular interest for patients receiving hormone treatment since it may represent a new mechanism for resistance to classical endocrine therapy by allowing tumor cells to adapt to signaling pathways that can circumvent the classical ER-pathway [16]. Tamoxifen, an ER inhibitor widely used in the clinic,

stimulates GPR30, which in turn trans-activates the EGFR signaling pathway [11, 13, 17]. This observation has led investigators to postulate that GPR30 modulates the effects of endocrine therapy by providing an alternative survival mechanism either directly or indirectly through cross-talk with growth receptors and other signaling molecules [18]. Furthermore, high expression of GPR30 in breast, endometrial, and ovarian tumors has been associated with metastases and poor survival [19–21]. In order to define the role of GPR30 as a functional estrogen-related biomarker in IBC, we analyzed GPR30 expression in tissue samples of IBC patients that have completed neoadjuvant therapy and undergone surgery. We sought to evaluate the level of GPR30 expression, correlate it with the expression of hormone receptors, growth factors receptors, and other known histopathological variables of IBC and assess the impact of expression on prognosis.

Methods

Clinical specimens

Eighty-eight patients with primary IBC who were treated at The University of Texas M. D. Anderson Cancer Center (MDACC) from September 1994 to August 2004 were included in this study. For the purposes of this study, patients were identified as having sufficient amount of residual tumor after neoadjuvant chemotherapy. Patients with pathological complete response, minimal residual tumor tissue, missing tissue, or information were not included. This study was approved by the University of New Mexico Cancer Center (UNMCC) and MDACC Institutional Review Boards. Formalin-fixed paraffin-embedded tissues were obtained from mastectomy specimens and used to build tissue microarrays (TMAs) as described elsewhere [22]. This implies that all cases included had experienced clinical response to neoadjuvant chemotherapy but still displayed persistent disease at the time of surgery. IBC was diagnosed on the basis of clinical signs such as rapid progression (i.e., clinical evolution of less than 3 months) of diffuse skin erythema, peau d'orange, tenderness, induration, and warmth, with or without an underlying palpable mass, and a histologic proof of invasive breast carcinoma with or without evidence of dermal lymphatic invasion. The modified Black's nuclear grading system was used to grade invasive tumor cells. All patients received multimodal treatment, including preoperative chemotherapy, surgery, and radiation therapy. The chemotherapy was given according to protocols described previously [23], consisting of four to six cycles of an anthracycline-based regimen that included doxorubicin (50 mg/m²), cyclophosphamide (500 mg/m²), and

5-fluorouracil (500 mg/m²) given every 21 days. The majority of the patients (89%) also received paclitaxel and three patients received trastuzumab. Endocrine therapy (Tamoxifen or aromatase inhibitor) was given to patients with ER-positive and/or PR-positive tumors.

Evaluation of ER, PR, HER-2, EGFR, and GPR30 expression

ER, PR, and HER-2 protein expression were determined by immunohistochemical staining (IHC) and HER-2 gene amplification was detected using fluorescent in situ hybridization (FISH) at MDACC; EGFR and GPR30 protein expression were assessed by IHC at UNMCC. When only limited tumor sample existed in the TMA core or the core tissue was lost during the IHC procedure, a whole section from the original block was used for the study.

ER, PR, and HER2 expression levels were evaluated using standard procedures with the modified avidin–biotin complex method in a DAKO auto-stainer (DAKO, Carpinteria, CA) using primary antibodies against ER (clone 6F11; Novocastra, Burlingame, CA; Dilution, 1:50), PR (clone 1A6; Novocastra, Burlingame, CA; Dilution, 1:30) and HER-2 (clone AB8; Neomarker/Labvision Corporation, Fremont, CA; Dilution, 1:100) as previously described [24]. Normal endometrial (ER, PR) and breast (HER-2) tissues were used as positive controls; the same tissues, incubated with an isotype-matched antibody, were used as negative controls.

EGFR expression was detected with the 31G7 clone (Zymed, Carlsbad, CA; Dilution 1:50) using the Ventana XT Benchmark auto-stainer as described by the manufacturer (Ventana Medical Systems, Inc., Tucson, AZ). Briefly, tissue sections were baked, deparaffinized in xylene, rehydrated in graded ethanol (100 and 95%), and rinsed in water. The slides were then incubated in fresh 3% hydrogen peroxide in Dulbecco's phosphate-buffered saline (DPBS: KCl/KH₂PO₄/NaCl/Na₂HPO₄·7H₂O, 2.67/1.47/133.93/8.06 mM) for 20 min followed by three 5-min rinses in DPBS. The slides were loaded on to the Benchmark auto-stainer and detection was performed using the iVIEW™ DAB detection system (Ventana Medical Systems, Inc., Tucson, AZ).

GPR30 was detected as described elsewhere [20, 21] using a detection protocol similar to EGFR except that it was carried out manually; antibody retrieval was applied for 20 min in 10 mM citrate buffer (pH 6.0), and the incubation with an antibody against the GPR30 C-terminus was performed overnight. For both EGFR and GPR30 IHC assays, normal placenta was used as a positive control; normal placenta, incubated with an isotype-matched antibody was used as a negative control.

Detection of HER-2 gene copy number by fluorescent in situ hybridization

Fluorescent in situ hybridization (FISH) was performed as previously described [25] using the PathVysion HER-2 SpectrumOrange/CEP17 SpectrumGreen kit (Vysis, Downers Grove, IL).

Scoring of IHC results

Positive status for ER and PR was defined as having nuclear staining in at least 10% of invasive tumor cells. HER-2 positive status was defined as an IHC score of 3+ or a gene copy ratio of HER-2:CEP17 of ≥ 2.0 . The tumors with an IHC score of 1+ or 2+ were confirmed by FISH.

For EGFR, signal intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). Any complete membranous staining (>1+) was considered positive. The GPR30 protein levels were scored as previously described [21] using an H-scoring system obtained by multiplying the staining intensity (graded as 0 negative; 1+, weak; 2+, moderate and 3+, strong) by the percentage of epithelial tumor cells with GPR30-positive cytoplasmic staining (0–100%). The GPR30 cut-off was initially determined in 60 normal breast epithelial biopsies (eight biopsies from non-cancer patients and 52 histologically normal breast biopsies collected from a distant site of the invasive breast tumor biopsy of the same patient). The median obtained in the normal breast samples was selected as the cut-off, and for statistical analysis as well as to reduce observer variability, the samples were grouped into negative ($H \leq \text{median}$) or positive ($H > \text{median}$) populations.

Statistical analysis

Primary outcomes for this study were overall survival (OS) and disease-free survival (DFS). OS was calculated from the date of diagnosis with death scored as an event and censoring of other patients at the date of last follow-up or of non-disease-related death. The DFS interval was calculated from the date of diagnosis to development of first recurrence. Patients without recurrence were censored at the time of last follow-up or death. Chi-square and Fisher's exact tests were used to compare demographic, clinical, and pathological data between IBC patients and IHC results between samples. The Spearman test was used to assess correlations between GPR30 expression and ER, PR, HER2, and EGFR status. For the analysis of ER, PR, HER2, EGFR, and GPR30 status, patients were divided into two groups (positive and negative) as described above. OS and DFS for the groups defined by ER, PR, HER2, EGFR, and GPR30 status and other variables (age, nodal status, lymphovascular invasion, nuclear grade, and hormone

treatment) were plotted using Kaplan–Meier curves and compared using the log-rank test. The Cox proportional hazard model with single covariates was used to obtain the hazard ratios (HRs) and associated 95% confidence intervals (CIs) for the groups compared. The primary multi-variable analyses were performed using the Cox model, with adjustments made for age (<50, ≥50 years), number of positive nodes (≤3, ≥4), lymphovascular invasion (yes, no), nuclear grade (1, 2 vs. 3), hormone treatment (yes, no), and ER, PR, HER-2, EGFR, and GPR30 status. Those variables found to be statistically significant in univariate analyses were considered for inclusion in the multivariate model. Inclusion was based on the significance of each variable as assessed by a likelihood ratio test comparing the multivariate model to the reduced model obtained by deleting variable(s) from the full model [26]. For each ordinal variable, the lowest value was used as the reference in computing hazard ratios (HR). Survival rates and HRs are presented with their 95% CIs. Wald tests were used to test for significance of HRs. Two-tailed *P* values less than 0.05 were considered statistically significant.

Results

Patient characteristics

Of the 88 patients included in this study, 84.1% were non-Hispanic whites, 11.4% Hispanics, and 4.5% Blacks and Asians. The median age was 49.5 years (range, 23–75 years). The majority of cases were infiltrating ductal carcinomas (IDC; 88.6%), stage IIIB (76.1%), modified Black's nuclear grade 3 (69.8%), with more than four positive nodes (63.8%) and positive for lymphovascular invasion (87.8%) at time of surgery, consistent with expectations for a cohort of patients at high risk of disease recurrence. Chemo-therapy and hormone treatment were given to 95.5 and 35.6% of patients, respectively. The characteristics of patients and tumors are summarized in Table 1. The median follow-up interval was 45.7 months (range 0.2–164.2 months). At the time of analysis, 52 patients had died (61.2%); the OS was 48.6% and the DFS was 61.0% (Fig. 1).

GPR30 expression in IBC samples

GPR30 was expressed in epithelial cells from normal breast biopsies (*N* = 60; median H-score = 125; range 0–300). In normal breast tissue, ductal and lobular epithelia displayed a cytoplasmic staining pattern with varying intensities (Fig. 2a, b). As in normal breast, IBC tissue exhibited a cytoplasmic staining pattern with staining intensity ranging from negative to strong (*N* = 73; median

Table 1 Characteristics of the IBC samples

(A) Clinical characteristics		(B) Molecular characteristics		
Variable	<i>N</i> (%)	Variable	<i>N</i> (%)	<i>P</i> *
Age ⁽⁸⁸⁾		GPR30 expression ⁽⁷³⁾		
Median	49.5	Negative	23 (31.5)	
Range	23.0–75.0	Positive	50 (68.5)	0.002
Ethnicity ⁽⁸⁸⁾		GPR30/ER patterns ⁽⁷²⁾		
White	74 (84.1)	GPR30–/ER–	8 (11.1)	
Hispanic	10 (11.4)	GPR30+/ER–	33 (45.8)	
Other	4 (4.5)	GPR30–/ER+	14 (19.4)	
Histological type ⁽⁸⁸⁾		GPR30+/ER+	17 (23.6)	0.023 ^a
Ductal	78 (88.6)	ER expression ⁽⁸⁶⁾		
Lobular	6 (6.8)	Negative	49 (57.0)	
Other	4 (4.6)	Positive	37 (43.0)	0.197
Stage ⁽⁸⁸⁾		ER/PR patterns ⁽⁸⁶⁾		
IIIB	67 (76.1)	ER–/PR–	44 (51.2)	
IIIC	13 (14.8)	ER–/PR+	5 (5.8)	
IV	8 (9.1)	ER+/PR–	12 (13.9)	
Nuclear grade ⁽⁸⁶⁾		ER+/PR+	25 (29.1)	<0.001 ^a
1	13 (15.1)	PR expression ⁽⁸⁶⁾		
2	13 (15.1)	Negative	56 (65.1)	
3	60 (69.8)	Positive	30 (34.9)	0.005
Nodal status ⁽⁸⁰⁾		Her-2 expression ⁽⁸⁵⁾		
0–3	29 (36.2)	Negative	52 (61.2)	
>4	51 (63.8)	Positive	33 (38.8)	0.039
Lymphovascular invasion ⁽⁷⁴⁾		EGFR expression ⁽⁷⁴⁾		
No	9 (12.2)	Negative	49 (66.2)	
Yes	65 (87.8)	Positive	25 (33.8)	0.005
Hormonal treatment ⁽⁸⁷⁾		Her-2/ER/PR status ⁽⁸³⁾		
No	56 (64.4)	All negative	21 (25.3)	
Yes	31 (35.6)	Others	62 (74.7)	<0.001

The number of evaluated cases among 88 patients is shown in parenthesis

^a Fisher exact test

* Chi-square test

H-score = 160; range 0–300; *P* = 0.043 for normal versus IBC specimens; Fig. 2d–f). In several samples, staining of stromal tissue, inflammatory cells, myoepithelia, and stromal fibroblasts was observed but neither was used in the grading scheme.

The majority of IBC cases were positive for GPR30 (68.5%; Table 1B). Approximately 24% of the tumors co-expressed ER and GPR30, 19% expressed only ER, 11% were negative for both ER and GPR30, and interestingly, GPR30 was expressed in 46% of tumors that failed to express ER (Table 1B). GPR30 expression was found to be inversely correlated with ER (*P* = 0.02), and hormone treatment but the latter association was not significant (*P* = 0.11); GPR30 expression did not correlate with PR, EGFR, or HER-2 (*P* > 0.1 for all) (data not shown).

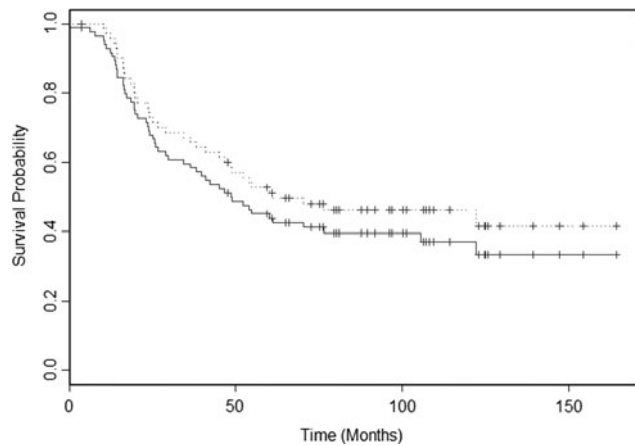


Fig. 1 Kaplan–Meier survival curves of OS (*continuous line*) and DFS (*dotted line*)

ER, PR, EGFR, and HER-2 expression in IBC samples

The majority of samples were independently negative for ER, PR, HER-2, and EGFR. Fifty-one percent of samples lacked expression of both ER and PR and 25% of the

samples were “triple negative” (ER/PR/HER-2 negative) (Table 1B).

GPR30 expression and OS and DFS

GPR30 expression was not associated with OS or DFS (Fig. 3a, b; Suppl. Table 1). However, patients who were positive for GPR30 expression showed a higher median DFS as compared to the median DFS of the whole population (122 vs. 61 months, respectively) (see Figs. 1, 3b; Suppl. Table 1). Furthermore, co-expression of GPR30 and ER (GPR30/ER patterns) was associated with OS ($P = 0.033$) but only marginally associated with DFS ($P = 0.057$; Suppl. Table 1; Suppl. Fig. 1a, b). In particular, tumors that expressed both GPR30 and ER showed a trend toward better OS and DFS than other expression patterns (Suppl. Table 1; Suppl. Fig. 1a, b). Of note, patients showing no expression of both GPR30 and ER (GPR30/ER status) were associated with significantly decreased OS and DFS ($P = 0.03$ for both; Suppl. Table 1; Suppl. Fig. 2a, b).

Fig. 2 Representative IHC for GPR30 expression in IBC samples. Normal breast with negative (a) and strong (b) GPR30 expression. IBC cases with negative (d), moderate (e), and strong (f) GPR30 expression. Placenta (c) was used as a positive control and the *inset* depicts the negative control. Slides were scanned and digitized using the Aperio digital system (Vista, CA) (20 \times)

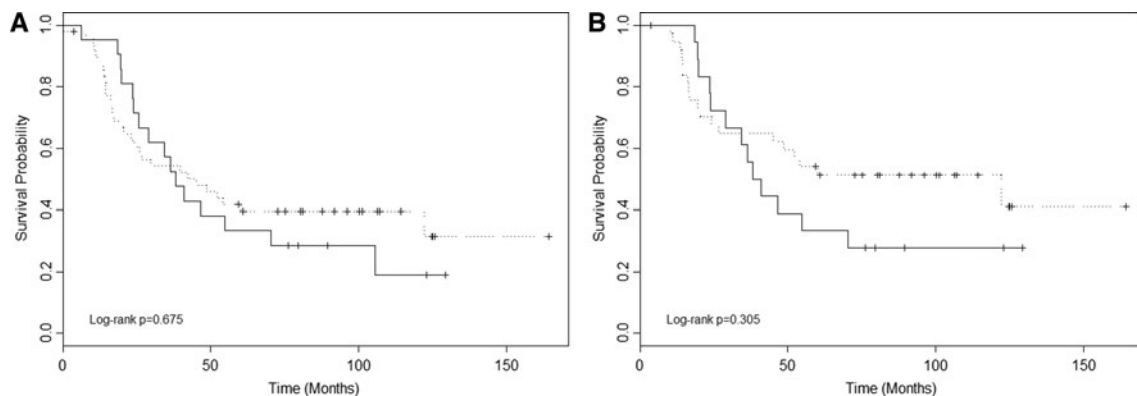
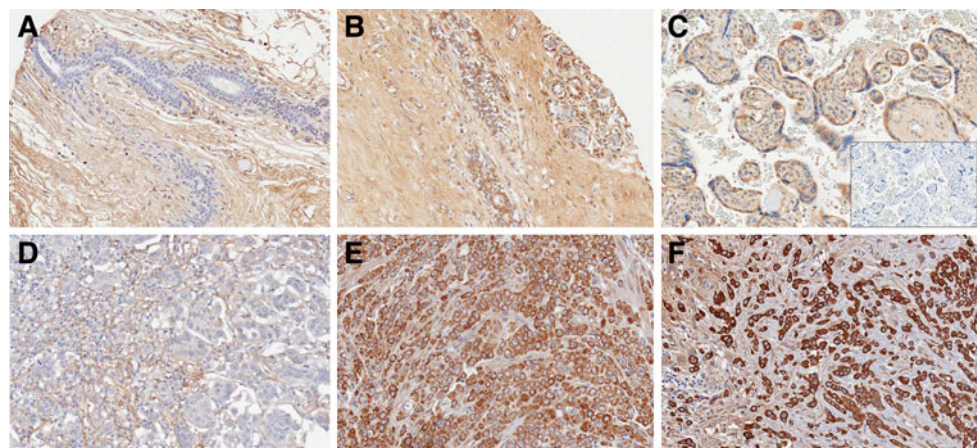


Fig. 3 Kaplan–Meier survival curves of OS (a) and DFS (b) time according to GPR30 expression. *Dotted line* represents GPR30-positive cases, *continuous line* GPR30-negative IBC cases

ER, PR, EGFR, HER-2 expression, clinical parameters and OS and DFS

ER expression was positively correlated with PR and hormone treatment ($P < 0.001$ for both), and an inverse correlation was observed with EGFR ($P < 0.001$) and HER-2 ($P = 0.02$) expression. EGFR expression showed an inverse correlation with hormone treatment ($P < 0.05$) but no correlation with HER-2 expression ($P = 0.60$; data not shown).

Of the clinico-pathological variables (age, number of positive nodes, lymphovascular invasion, nuclear grade, and hormone treatment) and biomarkers analyzed, hormone treatment, ER expression, and ER/PR expression patterns were associated with improved OS and DFS (Suppl. Table 1). Specifically, ER, either alone or co-expressed with PR, was significantly associated with an improved OS and DFS as shown by the Kaplan–Meier survival curves (Suppl. Table 1; Suppl. Fig. 3a, b).

“Triple negative status” was associated with poor OS and DFS ($P < 0.01$ for both; Suppl. Table 1). The lack of GPR30 expression in these IBC cases was marginally associated with worse OS ($P = 0.06$) but not with DFS ($P = 0.11$; data not shown). Expression of EGFR was associated with decreased OS and DFS ($P < 0.05$ for both; Suppl. Table 1).

Univariate and multivariate analysis

In the univariate analysis, hormone treatment, ER expression, GPR30/ER patterns, GPR30/ER status, and ER/PR patterns were associated with improved OS and DFS (Table 2). Triple negative status and EGFR over-expression were associated with worse OS and DFS (Table 2). However, the multivariate analysis identified ER expression as the only independent prognostic factor associated with improved OS (HR = 0.14; 95% CI 0.03–0.59; $P = 0.008$) and DFS (HR = 0.17; 95% CI 0.03–0.78; $P = 0.02$) (Table 2).

Discussion

IBC is an aggressive disease associated with resistance to standard multimodality therapy translating into a dismal prognosis [7]. The biological features of IBC have not been clearly defined but the disease is typically characterized by biomarkers of aggressive phenotypes such as high nuclear grade, lack of hormone receptor expression and HER-2 amplification [8–10]. Those characteristics have limited the use of treatment modalities otherwise effective in non-IBC, such as hormone treatment. Endocrine therapy has been employed in treatment and prevention of breast cancer and its efficacy is usually predicted on the basis of ER expression level. More recent research indicates a lack of concordance between tamoxifen responsiveness and ER expression [27], and suggests the existence of alternate routes critical for the growth and survival of breast cancer cells [16, 28]. GPR30 has been identified as an alternate route triggering activation of the EGFR and downstream molecules such as MAP kinases, as well as up-regulation of Bcl-2 and cyclin D2 [13–15]. We have previously shown that GPR30 is expressed in 48 of ovarian and 50% of endometrial tumors [20, 21]. In endometrial tumors, GPR30 expression is predictive of many parameters associated with adverse outcome (high grade and biologically aggressive histologic subtypes, cervical involvement, and advanced stage) and is associated with lower survival rates [20]. The same association with poor outcome is observed for GPR30 in ovarian cancer [21]. In non-IBC specimens, conflicting associations have been reported. In one study, GPR30 expression was shown to be a significant predictor of tumor size and metastases [19]. However, in a subsequent report, GPR30 expression was not associated with either HER-2 expression or survival [29]. While, the former study employed immunohistochemistry to assess GPR30 expression exclusively in epithelial tumor cells, the later investigation reported GPR30 expression from total RNA without isolating epithelial tumor cells. The use of

Table 2 Univariate and multivariate analyses of OS and DFS

Variable	Overall survival			Disease-free survival		
	HR	95% CI	<i>P</i> value	HR	95% CI	<i>P</i> value
Univariate analysis						
Hormonal treatment	0.36	0.18–0.69	0.002	0.37	0.19–0.73	0.004
ER expression	0.44	0.24–0.80	0.007	0.48	0.25–0.89	0.020
GPR30/ER patterns	0.65	0.47–0.88	0.01	0.67	0.48–0.94	0.02
GPR30/ER status	0.43	0.20–0.94	0.03	0.47	0.19–1.13	0.09
ER/PR patterns	0.78	0.63–0.97	0.03	0.81	0.65–1.02	0.07
Her-2/ER/PR status	2.12	1.70–3.84	0.01	2.20	1.17–4.14	0.02
EGFR expression	1.93	1.07–3.46	0.03	1.74	0.91–3.30	0.09
Multivariate analysis						
ER expression	0.14	0.03–0.59	0.008	0.17	0.03–0.78	0.023

different techniques may explain the discrepancies between the results of these two studies.

This study represents the first to investigate the role of GPR30 in relation to other known biomarkers and its impact on prognosis in IBC patients. While we found no association between GPR30 expression and outcome, it needs to be highlighted that the patients that were included in the study already had a dismal prognosis because they had extensive residual disease after neoadjuvant chemotherapy. The study confirmed the expression of hormone and growth factor receptors within the range of published data, indicating that the tumors analyzed in this study were representative of those included in other studies. Furthermore, we demonstrate several important biological correlations that may translate into significant clinical implications. First, we found that the majority (68.5%) of IBC tumors were GPR30-positive whereas ER was found in only 43% of tumors. There was no difference in incidence between the tumors that co-expressed ER and GPR30 (23.6%) and those ER-positive tumors that failed to express GPR30 (19.4%). However, twice as many (46%) of the ER-negative IBC tumors retained GPR30 (Table 1B), suggesting that ER and GPR30 expression in IBC is not interdependent and one can further postulate that it may be possible to exploit novel endocrine therapies that work through non-classical estrogen-dependent pathways as potential therapies for IBC tumors despite the lack of detectable ER. Second, in contrast to NIBC [19], there was no correlation between GPR30 expression and HER-2 or between GPR30 and EGFR, as we previously observed in endometrial cancer [20]. These findings suggest that GPR30 may not use the EGFR and/or HER-2 routes as alternate pathways to promote tumor cell growth in IBC and/or it is possible that other yet unidentified routes may be activated by GPR30. Another explanation of these findings may lie in the intrinsic nature of IBC tumors, which are thought to be metastatic at the onset of the disease [8, 10] and, therefore, EGFR and/or HER-2 routes may not be critical or bypassed in IBC tumors. This contrasts other studies performed in pre-clinical models and tissue specimens [19] and therefore required confirmatory studies. Third, we found that patients with GPR30 over-expression had prolonged disease control as documented by the fact the DFS was twice that of the studied population although it did not reach statistical significance. It should be noted that this result is opposite to what we have previously reported in endometrial and ovarian cancers, where GPR30 over-expression was a prognostic factor of poor survival [20, 21]. GPR30 over-expression seems to be associated with improved OS and DFS in ER-positive IBC patients, raising important biological questions related to the interplay between these two important receptors and their regulation of hormone-dependence in IBC.

Tamoxifen behaves as an agonist in GPR30-positive cells, resulting in activation of pro-survival signaling pathways such as ERK and PI3K/Akt [11, 13, 17]. Therefore, on one hand, this activation may be detrimental for GPR30-positive patients receiving tamoxifen. On the other hand, a brain tumor cell line model demonstrated that the biological effect of tamoxifen is dose dependent with high doses of tamoxifen resulting in an increase of glioma cell apoptosis, suppression of tumor growth in vivo and improvement of survival in glioma xenograft models as a result of activation of pro-apoptotic genes, including NF- κ B and its targets genes [30]. Furthermore, GPR30 over-expression inhibits urothelial cell proliferation as a result of decreased cyclin D1 expression [31]. Given the protective effect of GPR30 expression observed in our study, we performed a subset analysis in our ER/PR-positive patients that received tamoxifen to analyze the effect of GPR30 expression in those patients. Although we found a trend for improved OS and DFS in GPR30-positive as compared to GPR30-negative patients, it did not reach statistical significance (data not shown) probably due to the small size of samples. Further analyses in a larger population are needed to confirm whether GPR30 may activate pro-apoptotic or anti-proliferative mechanisms that may result in a better response in ER/PR-positive patients treated with tamoxifen. Whether the same NF- κ B or cyclin D pathways are activated in IBC patients remains unknown; ongoing studies in our group will clarify these associations.

A word of caution should be taken when analyzing these results: our study has the drawbacks inherent to retrospective studies and the small size of samples. Notwithstanding these limitations, the description by our group of a specific GPR30 agonist (G1) and antagonist (G15) [32, 33] fuels our enthusiasm to further assess the effects and mechanisms of action of both agents in IBC cell lines and tumor xenografts.

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