Distribution of GPR30, a Seven Membrane–Spanning Estrogen Receptor, in Primary Breast Cancer and its Association with Clinicopathologic Determinants of Tumor Progression

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Abstract

Purpose: The seven transmembrane receptor, GPR30, is linked to estrogen binding and heparan-bound epidermal growth factor release. Here, the significance of GPR30 in human breast cancer was evaluated by comparing its relationship to steroid hormone receptor expression and tumor progression variables.

Experimental Design: Immunohistochemical analysis of a National Cancer Institute–sponsored tumor collection comprised of 361 breast carcinomas obtained at first diagnosis (321 invasive and 40 intraductal tumors). Biopsies from 12 reduction mammoplasties served as controls. The distribution pattern of GPR30, estrogen receptor (ER), and progesterone receptor (PR) was correlated with clinicopathologic variables obtained at diagnosis.

Results: GPR30, ER, and PR were positive in all 12 normal controls. In contrast, GPR30 expression varied in breast tumors, in which 62% (199 of 321) of invasive tumors and 42% (17 of 40) of intraductal tumors were positive. Codistribution of ER and GPR30 was measured in 43% (139 of 321) of invasive breast tumors, whereas both receptors were lacking (ER^-GPR30^-) in 19% (61 of 321) of the tumors analyzed, indicating a significant association between ER and GPR30 ($P < 0.05$). The coexpression of PR and ER did not influence GPR30 expression, yet coexpression of GPR30 and ER was linked to PR positivity. Unlike ER, which varied inversely with HER-2/neu and tumor size, GPR30 positively associated with HER-2/neu and tumor size. In addition, GPR30 showed a positive association with metastasis ($P = 0.014$; odds ratio, 1.9).

Conclusions: GPR30 and ER exhibited distinct patterns of association with breast tumor progression variables, including HER-2/neu, tumor size, and metastatic disease. Thus, these results support the hypothesis that GPR30 and ER have an independent influence on estrogen responsiveness in breast carcinoma.

Estrogen promotes the development and homeostasis of the mammary gland, and the growth of tumors that arise from this tissue. It is widely accepted that estrogen manifests its physiologic and pathophysiologic actions through its interaction with specific receptors. Estrogen receptor (ER) α and its structural homologue, ERβ, belong to the nuclear steroid hormone family, and function indisputably as hormone-dependent transcription factors. The blockade of estrogen-binding sites on the ER has proven to be an effective means to inhibit the growth of breast tumors expressing ER, and today, this modality of treatment remains the standard endocrine therapy for ER^+ tumors. Although there is general concordance between ER expression and responsiveness to ER antagonism, as indicated in greater disease-free survival at 5-year follow-up for postmenopausal patients with ER^+ tumors receiving tamoxifen (1), roughly one in four patients do not respond to tamoxifen therapy. A variety of explanations have been offered to account for nonresponsiveness to ER antagonism, including: (*a*) intratumoral heterogeneity in ER expression, (*b*) evolution of mutant ERs with reduced affinity for ER antagonists, (*c*) drug resistance, (*d*) partial receptor antagonism, and (*e*) the presence or absence of *trans*-acting factors that influence ER functionality. These interpretations have prompted strategies better designed to assess ER activity and have served as the rationale for the discovery and use of new endocrine agents with more complete ER antagonist activity. In addition, comarkers that better predict ER functionality have been identified for the purpose of selecting patients that will respond favorably to ER antagonists. For example, coexpression of the progesterone receptor (PR), whose gene transcription is directly regulated by ER-dependent gene transactivation, has

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prognostic value for determining favorable responses to tamoxifen (2). In addition, more complete ER antagonists, such as fulvestrant, are being assessed in clinical trials for patients with primary and advanced breast cancer (3).

The existence of alternative ERs, whose action is not blocked by ER antagonists, or possibly stimulated by ER antagonists, has also been offered as a possible explanation for tamoxifen nonresponsiveness. Studies in animal and cell models have long indicated that estrogen manifests physiologic actions and biochemical effects inconsistent with its known genomic mechanism of action (reviewed in ref. 4). For instance, estrogen induces epidermal growth factor (EGF)–like activity in female reproductive tissue (5–7), and likewise activates biochemical signals typically associated with epidermal growth factor receptors (EGFR; refs. 8, 9). Estrogen also stimulates second messenger signaling characteristic of seven transmembrane–spanning receptors (7TMRs), including the activation of calcium (10, 11), cyclic AMP (12), and inositol triphosphate (13). Recent studies have linked the orphan 7TMR, GPR30, to estrogen-mediated stimulation of adenylyl cyclase (14), release of heparan-bound EGF from the surface of breast cancer cells (15), and specific estrogen binding (16, 17). GPR30 acts independently from ERα and ERβ, and triggers estrogen-dependent EGFR action. In this regard, GPR30 may play an important role in breast cancer biology because it provides a mechanism by which estrogen may promote EGF-like effects. In this manner, breast tumors that lack ERs may remain estrogen responsive by employing GPR30. This concept is particularly intriguing for patients receiving endocrine therapy because ''partial'' (tamoxifen) and ''pure'' (faslodex) ER antagonists behave similarly to estradiol, and are capable of triggering EGFR activation in breast cancer cells (14, 15).

To acquire baseline information regarding the potential role of GPR30 as an independent factor in human breast cancer, the relative tissue distribution of GPR30, ER, and PR was assessed in intraductal and invasive ductal carcinoma, and correlated with other known histopathologic markers of disease.

Materials and Methods

Tissue specimens. Archival paraffin-embedded, formalin-fixed biopsy specimens of normal breast tissue were obtained with Institutional Review Board approval from patients who underwent reduction mammoplasty at Rhode Island Hospital or Women and Infants Hospital. Breast tumor microarrays, consisting of 40 intraductal breast tumors and 333 invasive ductal carcinomas, were provided by the National Cancer Institute (NCI) Cooperative Breast Cancer Tissue Resource. All tumor samples present in the NCI microarrays were collected at first diagnosis (prior to adjuvant or neoadjuvant therapy). The NCI microarrays are suitable for studies measuring the association of novel markers with breast tumor progression because they are comprised of tumors collected from several institutes and reflect the reported frequencies of breast tumor progression variables (18).

Immunohistochemical analysis. GPR30 antibodies were generated in New Zealand white rabbits against a C-TER peptide (CAVIPD-STEQSDVRFSSAV) comprising the carboxyl terminal 18–amino acid residues from the deduced amino acid sequence of human GPR30, as previously described (15). Sera from immunized rabbits were affinity-purified on peptide columns before use. For GPR30 staining, formalin-fixed tissues were deparaffinized by heating slides to 60°C for 1 hour followed by three consecutive extractions in Citrisolv (Fisher Scientific, Pittsburgh, PA). Tissues were then washed in ethanol, rehydrated, and heated at 95°C for 20 minutes in 0.1 mol/L sodium citrate (pH 6.0). Endogenous peroxidase activity was quenched in 3% H_2O_2 and nonspecific binding was blocked using bovine serum albumin. Slides were exposed to GPR30 peptide antibodies for 2 hours at ambient temperature and then washed thrice in TBS containing 0.05% Tween 20. Tissue-associated rabbit antibodies were detected using a dextran-coated polymer containing horseradish peroxidase–conjugated goat anti-rabbit IgG (Envision-plus) and diaminobenezidine as a substrate (Dako Cytomation, Carpinteria, CA). Nuclei were counterstained using Mayer's modified hematoxylin (PolyScientific, Bay Shore, NY).

ER, PR, and Her-2/neu staining scores were determined by NCI-selected pathologists and were provided in the blind key that accompanied the CBCTR microarrays after the submission of GPR30 results. For the purpose of showing representative examples of ER and PR staining in the tumor microarray sets evaluated, ER and PR were immunostained by the Pathology services at Rhode Island Hospital on a Dako Autostainer using the Envision-plus detection system.

Evaluation of the immunostaining pattern for GPR30. Two observers using a semiquantitative scoring system (described in ref. 19) microscopically evaluated the intensity, extent, and subcellular distribution of GPR30. Scores were applied as follows: score 0, negative staining in all cells; score 1+, weakly positive or focally positive staining in <10% of the cells; score 2+, moderately positive staining covering 10% to 50% of the cells; and score 3+, strongly positive staining, including >50% of the cells. For statistical analysis as well as to reduce intraobserver variability, the immunohistochemical scores were further grouped into two categories: negative or weakly positive (0 and 1+) and moderately to strongly positive (2+ and 3+). Patient data were derived from a blind key provided by the NCI after reporting GPR30 scores.

Statistical analysis. Associations between steroid receptor expression categories and tumor stage were evaluated using the χ^2 test or the Fisher's exact test, as needed. Two parametric groups were compared using Student's t test for independent samples. The comparison between two nonparametric (ordinal) groups was done using the Mann-Whitney U test. Two-tailed P values of 0.05 or less were considered to be statistically significant.

Results

Specificity of GPR30 peptide antibodies. The specificity of the GPR30 C-TER peptide antibodies for GPR30 protein was tested by immunoblot analysis of whole cell lysates prepared from HEK-293 cells transfected with epitope-tagged recombinant GPR30 (Fig. 1). Affinity-purified GPR30 antibodies detect a single band with an apparent molecular weight of 44 kDa in detergent lysates prepared from HEK-293 cells transfected with recombinant GPR30 containing an amino-terminal hemagluttinin tag. This 44 kDa GPR30-reactive species was also detected upon reprobing the filter with hemagluttinin-specific antibodies. However, this band was not detected in mock-transfected HEK-293 cells probed with either GPR30 or hemagluttinin antibodies. In addition, GPR30 C-TER antibodies do not detect a truncated GPR30 polypeptide (30 kDa) expressed in HEK-293 cells that lack its COOH terminus but retains the amino terminal HA epitope. These data suggest that GPR30 C-TER peptide antibodies specifically detect the COOH terminus of GPR30 protein.

Staining pattern of GPR30 in normal and breast cancer tissue. Affinity-purified GPR30 peptide antibodies were used to stain archival, paraffin-embedded breast specimens. The predominant staining pattern of GPR30 was cytoplasmic in both normal (Fig. 2) and ductal carcinoma (Fig. 3). This subcellular localization pattern is typical of seven-transmembrane receptors

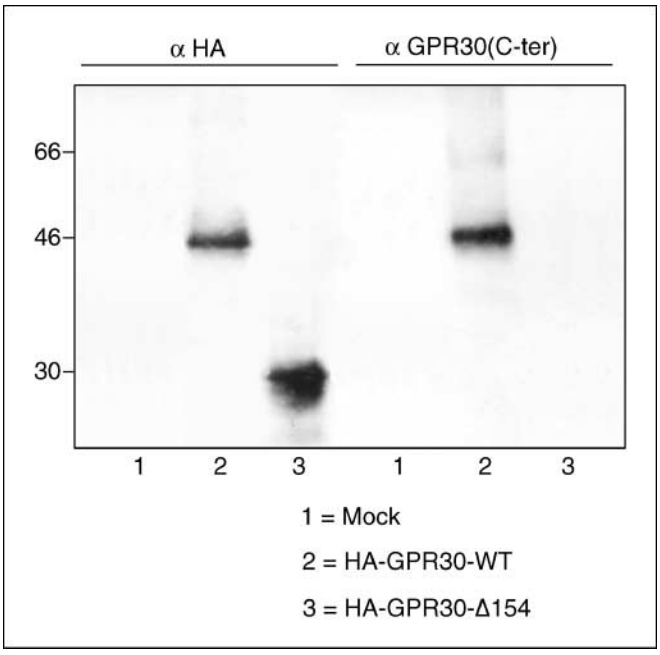

Fig. 1. Specificity of GPR30 peptide antibodies. Total protein (25 μg) from HEK-293 cells transfected with vector, HA-GPR30, or COOH-terminally truncated HA-GPR30 immunoblotted with anti-hemagluttinin (*HA*) or GPR30 C-TER peptide antibodies. Left, molecular mass standards (in kDa).

in tissue (20–22), and has been noted for GPR30 in cultured breast cancer cell lines (17). We further confirmed the specificity of the cytoplasmic GPR30 immunoreactivity pattern of peptide antibodies by demonstrating specific inhibition in immunoabsorption experiments comparing immune versus control peptide (data not shown). In normal breast tissue, ductal and lobular epithelia, myoepithelia, and stromal fibroblasts reacted strongly with GPR30 peptide antibodies, displaying a cytoplasmic staining pattern (Fig. 2A and B). No nuclear staining was observed in these cells. Vascular endothelium and smooth muscle did not exhibit detectable staining. Similar staining patterns were observed in each of the 12 mammoplasties that were evaluated for the intensity and extent of GPR30 staining. The same cell types were positive with ER and PR antibodies (data not shown). However, ER and PR immunoreactivity was exclusively confined to the nucleus with no detectable staining apparent within the plasma membrane or cytoplasm. No significant differences were measured in ER or PR expression within this normal breast tissue series. ER, PR, and GPR30 expression each varied within breast tumor specimens as described below.

Distribution of GPR30, ER, and PR in breast tumor tissue. To compare the distribution of GPR30 and ER, PR steroid hormone receptors, adjacent serial sections collected from 361 cases of intraductal or invasive ductal carcinoma were analyzed by immunohistochemistry (Fig. 3). Unlike normal breast, tumor tissue showed variation in GPR30 staining with immunopositivity ranging from 0 to 3+ (Fig. 3C and F; Table 1). Biopsies exhibiting little or no GPR30 (1+ or 0) were considered negative, whereas tumor specimens that scored moderately or strongly (2+ or 3+) for GPR30 were categorized as positive. As observed in normal tissue, all $GPR30^+$ tumor biopsies exhibited a cytoplasmic staining pattern. No detectable cytoplasmic or plasma membrane staining was observed for ER (Fig. 3A and D) or PR (Fig. 3B and E). Among the 40 cases of ductal carcinoma *in situ*, 42% were $GPR30^+$ (Table 1), whereas 63% and 45% of the specimens were positive for ER or PR, respectively. Among the 321 cases of invasive ductal carcinoma analyzed, 40% were positive for PR (Table 1). Approximately, two-thirds (62%) of the breast tumors that were evaluated showed ER-positivity, with a similar percentage of tumors expressing the alternative ER, GPR30 (Table 1).

GPR30 was found to be strongly associated with ER ($P < 0.05$), but not with PR ($P = 0.48$; Tables 2 and 3). Forty-three percent of all invasive tumors (139 of 321) expressed ER and GPR30 (Table 2). Among the remaining categories, the following expression pattern of these two types of estrogen receptor was measured: ER^+GPR30^-, 61 of 321 (19%); ER^-GPR30^+, 60 of 321 (19%); and ER^-GPR30^-, 61 of 321 (19%). Importantly, although these data indicate a significant association between ER and GPR30, their expression in tumors is not interdependent because one-half of ER^- tumors (60 of 121) are positive for GPR30. This result implies that these tumors may remain estrogen-responsive despite the fact that they lack detectable ER (Table 2). Interestingly, however, PR expression was approximately twice as common in tumors that coexpressed GPR30 and ER compared with tumors that produced ER but not GPR30 (Table 3; 68% versus 32%). No significant differences were

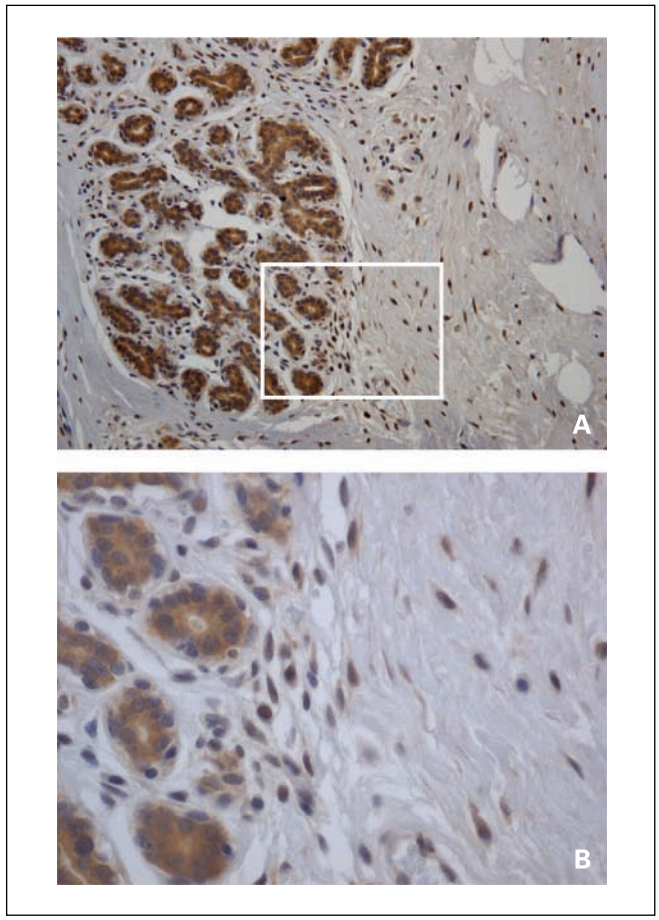

Fig. 2. *A,* reduction mammoplasty tissue immunostained with GPR30 peptide antibodies (magnification, ×200). *B,* enlarged view of boxed area in (*A*).

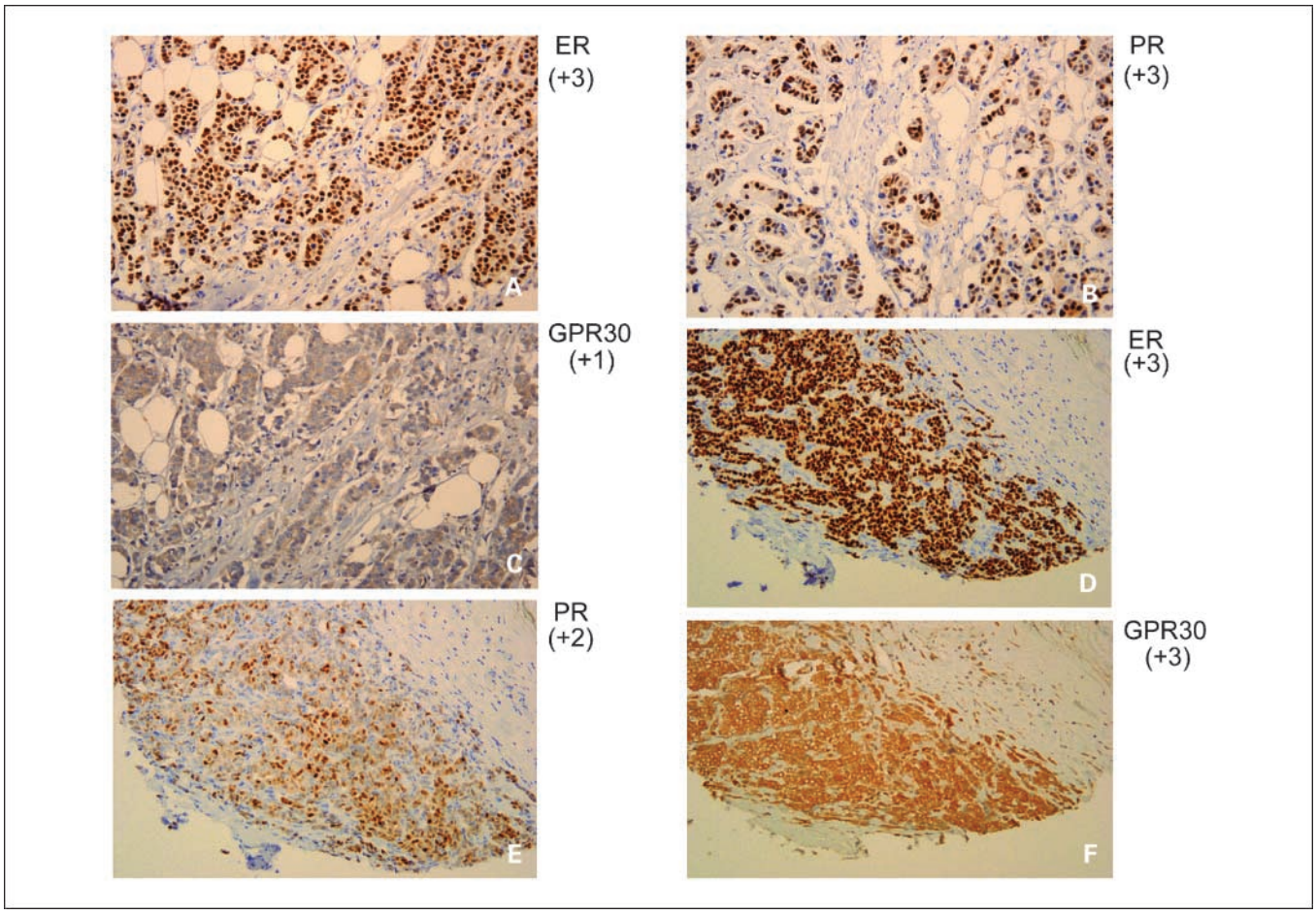

Fig. 3. Representative cases of archival, paraffin-embedded breast tumor tissue immunostained with ER, PR, and GPR30. *A-C,* GPR30$^-$ invasive carcinoma; *D-F,* GPR30$^+$ invasive carcinoma (magnification, ×200). Histochemistry scores for steroid hormone receptors are designated in each panel.

observed in the GPR30 expression levels between ER$^+$PR$^+$ and ER$^+$PR$^-$ breast tumors [73 of 107 (68%) and 66 of 93 (71%), respectively]. This observation may be noteworthy in light of the fact that PR positivity has been used as a means to predict responsiveness to estrogen therapy (23).

Association of GPR30 with HER-2/neu. Via GPR30, 17β-estradiol triggers the release of heparan-bound EGF from the surface of cultured breast cancer cells and induces tyrosyl phosphorylation of EGFRs (reviewed in ref. 24). For this reason, the relationship between GPR30 and HER-2/neu may be significant in breast cancer (Table 4). Within the 321 breast tumors included in this study, HER-2/neu data was available for 143 cases. GPR30$^+$ tumors presented higher HER-2/Neu expression scores than GPR30$^-$ tumors ($P = 0.038$, see Table 4). These findings are in contrast with the inverse association observed between ER and HER-2/neu in our study [$P = 0.003$; odds ratio (OR), 0.1] and elsewhere (25–28). These data support our prior findings *in vitro* regarding the capacity of GPR30 to promote EGFR-dependent action (15). Moreover, the fact that ER and GPR30 show distinct patterns of association with HER-2/neu supports the idea that each of these ERs may exert distinct biological effects on breast tumors.

Association of GPR30 with other clinicopathologic variables. The relation between GPR30 and additional clinicopathologic variables was evaluated (Table 4). No significant association was observed between GPR30 and patient age ($P = 0.32$). Unlike ER or PR, which show a significantly inverse relationship with tumor grade ($P < 0.0001$ in both

Table 1. Distribution of GPR30 and steroid hormone receptors in intraductal and invasive ductal carcinoma of the breast

Tissue	PR$^+$	ER$^+$	GPR30$^+$
Ductal carcinoma *in situ*	18 of 40 (45%)	25 of 40 (63%)	17 of 40 (42%)
Invasive	129 of 321 (40%)	200 of 321 (62%)	199 of 321 (62%)

NOTE: Steroid hormone receptors were scored from 40 cases of ductal carcinoma *in situ* and 321 cases of invasive ductal carcinoma.

cases, data not shown), GPR30 was not significantly associated with grade (P = 0.60). As observed in this study, an inverse relationship was measured between ER and tumor size (P = 0.045; OR, 0.62, data not shown). In contrast, GPR30 expression varied directly with tumor size (P = 0.05; OR, 1.6). Although GPR30 expression was found equally in both large (≥2 cm) and small (<2 cm) invasive breast tumors, the lack of GPR30 expression was almost twice as common in small invasive breast cancers (Table 4), suggesting that GPR30 facilitates tumor growth. Tumor size is a well-known predictor of lymph node metastasis (29–31). However, GPR30 showed only marginal significance with regards to predicting lymph node invasion (P = 0.06), when arranging the number of lymph nodes involved into clinically relevant treatment groups (Table 4). Interestingly though, GPR30 expression at the primary tumor site was strongly associated with the development of distant metastases (P = 0.014; OR, 1.9). Among the 215 patients that showed no evidence of distant metastases, similar proportions of patients expressing (55%) or lacking (45%) GPR30 in their tumor were observed (Table 5). However, of the 106 patients presenting with distant metastases at first diagnosis, GPR30 expression was detected almost twice as commonly in the primary invasive tumor component (72% versus 28% of patients with metastases).

Tumor size, lymph node invasion, and HER-2/neu expression are the best-documented predictors of metastatic breast carcinoma (18). In our study, similar findings were observed (Table 5), thus, indicating that the tumors analyzed were representative of those included in other studies. Furthermore, the results of our study indicate that GPR30 is a significant predictor of tumor size and metastases, but not of lymph node invasion, despite the fact that GPR30 is associated with HER-2/neu overexpression. This relationship is distinct from that observed between ER and tumor size, nodal invasion, and the occurrence of distant metastases, in which the absence of ER is strongly associated with HER-2/neu, nodal tumor involvement, and the presence of distant metastases. The data presented here support the concept that ER and GPR30 represent structurally distinct estrogen receptor types that may have separate biological influences on the growth and progression of breast cancer.

Discussion

Endocrine therapy is particularly successful in patients with breast cancer, and ER expression status in breast carcinoma is one of the most important variables to be considered for the management of patients with primary and advanced breast cancer. However, a complete concordance between tamoxifen responsiveness and ER expression does not exist (1), and whether this is associated with errors in tumor sampling, intratumoral heterogeneity, and the absence of cofactors that support ER functionality is unclear. Alternatively, receptors other than the known ERs, ERα and ERβ, may be important for the growth and survival of breast cancer cells. This hypothesis is largely supported by studies which show that "pure" ER antagonists, such as ICI 182,780 (faslodex) have agonistic effects in cultured cells (32, 33) and that some estrogen effects are maintained in double estrogen receptor knockout mice (34). Support for alternative ERs comes from experiments in rodents showing the EGF-like effects of estrogen. Namely, that estrogen induces mitogenic responses in female reproductive tissues that can be ablated with neutralizing antibodies against EGF (7). Moreover, in cultured cells, estrogen promotes rapid biochemical signals typically associated with membrane receptors that either couple to heterotrimeric G proteins or harbor intrinsic tyrosine kinase activity (4). In this regard, it may be important to note that evidence of an alternative mechanism accounting for rapid estrogen action predated the discovery of the ER as a soluble binding protein in rat reproductive tissue (reviewed in ref. 24).

We have begun to characterize an alternative receptor for estrogen that is structurally distinct from ERα and ERβ. This receptor belongs to the 7TMR superfamily, and is currently known as GPR30 (24). The autonomy of GPR30 from ERα or ERβ is reflected by two measures. First, GPR30 expression is associated with estrogen-binding activity (16, 17) and estrogen-mediated intracellular signaling (14, 15) in breast cancer cell lines that do not express ERα or ERβ. Second, whereas ER antagonists compete for GPR30-dependent estrogen binding sites (16, 17), they act similarly to estrogen in their capacity to elicit estrogen-mediated signals (14, 15). Here, the biological role of GPR30 in breast cancer was explored by comparing the distribution of this alternative estrogen and the steroid hormone receptors, ER and PR, in human breast tumors.

Our results indicate that GPR30 displays a predominately cytoplasmic staining pattern in normal and tumor breast tissue (Fig. 2). This result is consistent with a prior report demonstrating a similar subcellular distribution pattern by immunocytochemistry in human SKBR3 breast cancer cells (17). Numerous other studies have shown a similar cytoplasmic location for other 7TMRs, including neurotransmitter receptors and cytokine receptors (20–22), and this phenotype is likely due to slow egress to the plasma membrane during biogenesis as well as receptor re-uptake. By immunohistochemical analysis using peptide antibodies, GPR30 protein was detected in normal breast tissue. Although some tumors

Table 2. Coexpression of GPR30 with ER or PR

Steroid hormone receptors	GPR30$^+$	GPR30$^-$	P
ER (n)			
Positive	139 of 321 (43%)	61 of 321 (19%)	<0.05
Negative	60 of 321 (19%)	61 of 121 (19%)	
PR (n)			
Positive	83 of 321 (26%)	46 of 321 (14%)	0.48
Negative	116 of 321 (36%)	76 of 321 (24%)	

Table 3. Coexpression of ER, PR, and GPR30

Steroid hormone receptors	GPR30$^+$	GPR30$^-$
ER$^+$PR$^+$	73 of 107 (68%)	34 of 107 (32%)
ER$^+$PR$^-$	66 of 93 (71%)	27 of 93 (29%)
ER$^-$PR$^+$	10 of 22 (45%)	12 of 22 (55%)
ER$^-$PR$^-$	50 of 99 (52%)	49 of 99 (48%)

Table 4. Association between GPR30 expression and factors in breast carcinoma

Variable	GPR30$^+$	GPR30$^-$	*P*
Age (mean ± SE)	60.2 ± 1.0	59 ± 1.2	0.32
Tumor grade (*n*)			
High	44	32	0.60
Intermediate	114	69	
Low	41	21	
Tumor size (*n*)			
>2 cm	103	49	0.05
≤2 cm	96	73	
Lymph node involvement (*n*)*			
0	67	49	0.06
1-3	44	73	
>4	42	24	
Distant metastases (*n*)			
Present	76	30	0.014
Absent	123	92	
HER-2/neu score (*n*)†			
3+	11	1	0.038
2+	7	3	
1+	23	5	
0	61	32	

*Lymph nodes were not excised in 65 cases.
†HER-2/neu data was available for 143 cases.

showed slightly more GPR30 expression than in normal tissue, gross overexpression of GPR30 in breast tumor tissue was not observed (Fig. 2), a finding consistent with the observation that overexpression of 7TMRs is not a common occurrence in human disease. GPR30 expression did not correlate with PR in primary tumors from patients with invasive ductal tumors. In contrast, a general pattern of agreement was observed between GPR30 and ER expression as nearly twice as many ER$^+$ breast tumors coexpressed GPR30 relative to ER$^+$ tumors that failed to produce GPR30 (Fig. 2; Table 2). However, ~50% of ER$^-$ breast tumors retained GPR30, suggesting that their expression is not interdependent (Table 2), a finding which suggests that tumors that maintain GPR30 but lack ER may remain estrogen responsive. PR expression was more than twice as common in tumors that coexpressed ER and GPR30 compared with breast tumors that produced ER but not GPR30 (72% versus 28%, respectively; Table 3). This observation is interesting in that PR expression has been used as a rational approach for refining the identification of breast tumors suitable for treatment by ER antagonism (23, 35). This rationale is predicated on the fact that the PR genes, *PR A* and *PR B*, encode estrogen response elements that interact with liganded ER to promote estrogen-dependent gene transcription. GPR30 expression may further predict ER functionality, and subsequent PR transcription, by virtue of the fact that ER gene transactivation is augmented by extracellular-regulated kinases, Erk-1 and Erk-2 (36, 37), which are activated by GPR30-dependent EGFR transactivation (15). It is important to note that although Erk-dependent ER activation is measured in heterologous cells (36, 37), it does not occur in breast cancer cells (38). However, it is also noteworthy that GPR30 is a Gs-coupled 7TMR capable of stimulating adenylyl cyclase and that cyclic AMP has a negative regulatory influence on the EGFR-to-Erk signaling axis in breast cancer cells (14).

Via ER and GPR30, estrogen promotes signaling mechanisms whose effects are measured with distinctive kinetic patterns, suggesting that they function independently (14, 15, 17). Evidence of their autonomy is provided further by the fact that their expression in breast tumors is not interdependent (Table 2). Our current data also support the concept that GPR30 and ER promote distinct biological responses (Tables 4 and 5). Perhaps the most notable distinction is that GPR30 and ER independently predict the development of metastatic disease. Primary breast tumors expressing GPR30 are almost twice as likely to result in metastasis as compared with GPR30$^-$ primary tumors ($P = 0.014$; OR, 1.9). No significant association was found between ER expression and the presence of metastatic disease ($P = 0.11$). On the other hand, GPR30 shows a diametrically opposed relationship with HER-2/neu; its expression varies directly with HER-2/neu (Table 4), which may be consistent with the capacity of GPR30 to transactivate EGF-related receptors through the release of heparan-bound EGF from breast cancer cells (15).

Tumor size and invasion of axillary lymph nodes by breast tumor cells are the most important pathologic variables that predict the occurrence of distant metastases. Primary tumors, ≥2 cm (T_2), are associated with a greater risk for developing metastatic disease and this is also reflected in an increase in the prevalence of lymph nodes containing invading tumor cells (27). Although GPR30 was found to significantly associate with tumor size in a direct manner, the likelihood of the relationship between GPR30 expression and lymph node invasion was lower. A trend of significance was observed ($P = 0.06$; Table 4), when GPR30 scores applying the standard binary scoring system reported by separating samples that are

Table 5. Association between clinicopathologic variables and metastatic disease

Variable	Nonmetastatic, *n* = 215	Metastatic, *n* = 106	*P*
Age (mean ± SE)	58 ± 0.9	62 ± 1.3	0.008
GPR30 (*n*)			
Positive	123	76	0.014
Negative	92	30	
ER (*n*)			
Positive	127	73	0.08
Negative	88	33	
PR (*n*)			
Positive	88	41	0.7
Negative	127	65	
HER-2/neu score (*n*)*			
3+	4	8	<0.0001
2+	2	8	
1+	18	10	
0	71	22	
Tumor grade (*n*)			
High	54	22	0.17
Intermediate	115	68	
Low	46	16	
Tumor size (cm ± SE)	2.1 ± 0.1	2.6 ± 0.1	0.003
Lymph node involvement			
n ≥ 4	43	23	<0.0001
n = 1-3	64	15	
n = 0	108	3	

*HER-2/neu data was available for 143 cases.

$GPR30^-$ (0, 1+) from $GPR30^+$ (2+, 3+). This trend improved slightly ($P = 0.04$) when GPR30 expression was stratified by its raw score (0, 1+, 2+, 3+). Similarly, when grouping these variables in categories using the best cutoff values, a marginally significant positive association was noticed between GPR30 and lymph node invasion, with a tendency for $GPR30^+$ primary tumors to affect invasion into two or more lymph nodes ($P = 0.06$; OR, 1.83). Although future studies comprised of larger data sets may help to resolve whether GPR30 is associated with nodal invasion, GPR30 was clearly linked to distant metastases. Its expression in primary breast cancer strongly predicted the development of frank metastases ($P = 0.014$; OR, 1.9; Table 4). The relationship between HER-2/neu and nodal invasion is controversial. Whereas some reports show a direct association between HER-2/neu and nodal invasion (39, 40), other studies have shown no link between these variables (26, 41–43). In the NCI data set studied here, a significant relationship was measured between HER-2/neu and metastasis ($P < 0.01$), in agreement with previously published data (18). Our findings might indicate the complexity regarding the relationship between GPR30 and progression to the metastatic phenotype. Alternatively, GPR30-dependent tumors may more commonly disseminate by a mechanism that does not involve lymph node trafficking and adhesion. In this regard, it is noteworthy that GPR30 promotes intracellular signals through its ability to transactivate EGFRs, and EGFR stimulation has been linked to increased tumor cell survival, growth, and invasion into the surrounding and/or distant tissues.

Our data support the hypothesis that GPR30 has biological significance in human breast cancer. These data show that GPR30 expression varies directly with HER-2/neu, and therefore, are consistent with the hypothesis that GPR30 is an alternate estrogen receptor that facilitates both ER-dependent and EGFR-dependent action. More specifically, they show that GPR30 is linked to the risk of developing metastatic disease, a variable that most clearly reflects breast tumor progression and influences the therapeutic decisions in these patients.

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