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Biology of the estrogen receptor, GPR30, in triple negative breast cancer

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Abstract

BACKGROUND: Triple-negative (TN) breast cancer lacks a known signaling pathway amenable to targeted therapy. The authors hypothesized that the G protein–coupled receptor GPR30 may be present in TN breast cancer and serve a role for tumor growth.

METHODS: A retrospective pathology study and chart review were conducted. All patients aged ≤ 49 years from 2000 to 2008 were included (n = 24). Concurrent patients aged ≥ 50 years were randomly selected. Paraffin sections were stained for GPR30 and reviewed by a pathologist blinded to estrogen receptor and progesterone receptor status. Disease-free survival was analyzed versus age and receptor status. Means were compared using 2-sample t tests and proportions using chi-square analysis.

RESULTS: Twenty-seven patients tested GPR30 positive and 21 GPR30 negative. Seventeen of 18 TN cancers tested positive for GPR30 (P < .0001). Recurrence at a mean follow-up of 36 months was 22.2% in the GPR30-positive group and 9.5% in the GPR30-negative group.

CONCLUSIONS: GPR30 is prevalent in TN breast cancer and associated with young age and possibly recurrence.

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Triple-negative breast cancer (TNBC), defined as a lack of classical estrogen receptor (ER)–α and progesterone receptor (PR) without overexpression of human epidermal growth factor receptor 2 (HER2), constitutes 15% to 20% of all breast cancers. For breast cancers occurring in patients aged <50 years, however, the percentage increases to 25% to 30%.1 In addition to younger ages, patients with triple-negative disease frequently present at higher stages and follow more aggressive courses.2 The rate of death in patients with TNBC at 5 years is twice that of ER-α-positive tumors.3 Cytotoxic chemotherapy remains the mainstay of treatment for this patient population because specific targeted therapy has not yet been successful.

Another significant feature of the epidemiology of TNBC is its prevalence in cancers occurring in patients who have germ-line BRCA1 mutations. The BRCA1 mutation impairs deoxyribonucleic acid repair mechanisms, leading to an increased susceptibility to agents that damage deoxyribonucleic acid. Clinical trials attempting to exploit this molecular feature of BRCA1 mutation–associated tumors have studied the use of platinum-based chemotherapeutic agents and inhibitors of poly(adenosine diphosphate ribose) polymerase, a regulator of the deoxyribonucleic acid base excision mechanism. However, although 80% of germ-line
BRCA1 tumors are triple negative, only 12.5% of sporadic triple-negative tumors contain BRCA1 mutations. Basal-like breast cancers have an increased incidence of BRCA1 dysfunction through other mechanisms, leading to the concept of “BRCAanness.” Clinical trial results are contradictory at this date, with some promising phase 2 results not bearing out in subsequent trials and others still pending.

Molecular subtypes of TNBC have been proposed, highlighting the heterogeneity of this entity. Although “triple negative” is a designation based on the lack of immunohistochemical staining of the clinically relevant receptors, the so-called intrinsic subtypes luminal A, luminal B, HER2-enriched, and basal-like are based on gene expression analysis by microarray. Basal-like tumors are 68.5% triple negative, while triple-negative tumors are 78.6% basal-like. Other proposed subgroups of triple-negative tumors according to gene expression include immunomodulatory, mesenchymal, and androgen receptor luminal. Given that TNBC accounts for a minority of cancers, conducting trials of agents aimed at specific subtypes will be difficult. The challenge faced in producing a pharmaceutical agent targeted to TNBC is to find a target that might be present in the great majority of triple-negative tumors despite their heterogeneity.

A candidate biomarker and putative mechanism for growth regulation of TNBC is a novel ER, GPR30, a G protein–coupled receptor identified in 1997 by Carmeci et al as an orphan receptor without known physiologic ligands in the ERα-positive breast cancer cell line MCF-7. Recent data suggests a cellular membrane location, as opposed to the traditional nuclear position of standard hormone receptors. Evidence has linked GPR30 to various tissues such as breast and endometrium, and its presence has been associated with both cancer and aggressive disease. Further research has defined many agonists to the receptor, including 17β-estradiol, tamoxifen, and fulvestrant, which interestingly is a pure antagonist to the classic ER-α. Filardo specifically examined the response of GPR30 to 17β-estradiol and discovered a signaling pathway, which includes activation of the tyrosine kinase Src. After activation, the epidermal growth factor receptor is autophosphorylated at tyrosine, leading to the initiation of the mitogen-activated protein kinase signaling pathway. Eventual cell proliferation develops via the 17β-estradiol stimulus, despite not having a classical ER-α. This independent estrogen stimulation involving GPR30 may explain how TNBC remains responsive to the hormone.

In 2006, Filardo et al investigated the distribution of GPR30 in relation to the presence of all 3 receptors. Coexpression of GPR30 and ER-α was present in 43% of all cases, which was a correlation but not a significant association. PR and GPR30 coexpression was demonstrated in 26% of the carcinomas, which was also not statistically significant. HER2 status was assessed in relation to GPR30, and a positive association was found. Additional analysis demonstrated GPR30 to be significantly associated with larger tumor size (>2 cm), HER2 status, and distant metastasis. Triple-negative disease and GPR30 status, however, were not specifically examined in this population.

No clinical correlation between ER-α, PR, and HER2 status and the presence of GPR30 has collectively been described. We therefore conducted a retrospective analysis on the basis of age to assess the factors that contribute to a woman’s poor prognosis when diagnosed with breast cancer at a premenopausal age (<49 years). We assessed the hormone status of the tumors, stage at diagnosis, and the prevalence or absence of GPR30. We hypothesized that GPR30 would be present in our population of patients with TNBC and that it might represent a mechanism by which estrogen in premenopausal patients serves as a driver of TNBC.

Methods

Study population

All patients aged <49 years who underwent invasive breast cancer surgery from 2000 to 2008 at Southern Illinois University School of Medicine were included in this retrospective study. An equal number of concurrent patients aged ≥50 years were randomly selected for inclusion using a table of random numbers.

Tissue specimens

Archival paraffin-embedded, formalin-fixed specimens of cancerous breast tissue were obtained for each patient. All tumor samples were collected at first diagnosis before further treatment to include chemotherapy or radiation.

Immunohistochemical analysis

A staining protocol for rabbit polyclonal GPR30 antibody (catalog no. NLS 1183; Novus Biological, Littleton, CO) was developed on the known GPR30-positive breast cancer cell line MCF-7 and MDA-MB-231 as a negative control. Cells were grown on sterile chambered slides (Labtek, Scotts Valley, CA) with normal medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 200 mmol/L glutamine, and penicillin. Cells were fixed in 4% formaldehyde at pH 7.4 for 25 minutes at 4 °C, washed twice for 5 minutes in phosphate-buffered saline at room temperature. After incubation with the primary antibody, the slides were processed according to the manufacturer’s protocol using the R.T.U Vectastain Universal Elite ABC Kit (catalog no. PK-7200; Vector Laboratories, Burlingame, CA). Primary antibody diluted 1:100 provided optimal staining in MCF-7 and minimal background staining in MDA-MB-231. A practice set of 22 consecutive in-house breast tissues and cancers was then used to evaluate the staining using the automated stainer (BenchMark; Ventana Medical Systems, Tucson, AZ) on routinely
processed, formalin-fixed, surgical pathology breast cases. Positive cases demonstrated a membrane and cytoplasmic staining pattern. Negative staining was defined as no staining or faint staining in <10% of tumor cells. Positive staining was defined as strong cytoplasmic and membrane staining in >10% of tumor cells. Normal breast epithelium was an internal control for negative staining (Fig. 1).

Retrieved, archival paraffin-embedded tumor blocks were cut into 5-μm sections, deparaffinized, and permeabilized and then blocked for endogenous peroxidase activity, rinsed, and blocked for nonspecific binding of the secondary antibody with normal goat serum. Slides were incubated with primary rabbit polyclonal GPR30 antibody (dilution 1:100) and incubated with biotinylated goat antirabbit secondary antibody (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Immunoperoxidase staining was performed using a biotin/streptavidin peroxidase complex and diaminobenzidine substrate. Sections were counterstained with hematoxylin for 3 minutes. Standard hematoxylin and eosin slides of each specimen were also prepared to verify the presence of invasive carcinoma.

**Evaluation of immunostaining pattern for GPR30**

GPR30 staining on tumor cells was scored by a pathologist blinded to the ER-α, PR, and HER2 status that was previously determined. A corresponding chart review for stage, hormone status, and disease-free survival was then performed.

**Statistical analysis**

SAS version 9.1 (SAS Institute Inc, Cary, NC) was used to assess the statistical significance of our numeric results. Analysis was completed using the chi-square test of independence. Means were compared using 2-sample t tests and proportions using chi-square analysis.

**Results**

Forty-eight patients were included in our study to assess the presence of GPR30. Twenty-seven patients tested positive for GPR30, and 21 patients did not have the receptor. In comparing the GPR30-positive and GPR30-negative groups, we looked at several variables, including age, hormone status, stage, and recurrence (Table 1).

The average age in the GPR30-positive group was 45.8 years, and the average age in the GPR30-negative group was 61.7 years. There was a statistically significant difference between the ages of onset in the 2 groups (Table 2). This finding is consistent with younger women having more aggressive tumor types.

We then examined the receptor status of each patient. In looking specifically at the triple-negative cases, 17 of the 18 patients tested positive for GPR30 ($P < .0001$), illustrating our hypothesis that poor-prognosis tumors are more likely to harbor GPR30.

Next, we compared cancer stage and GPR30 status. There were more stage 2 and fewer stage 1 patients in the GPR30-positive group, though not statistically significantly. This is consistent with the recognized tendency for younger women to have higher stages at diagnosis compared with women aged >50 years.

In examining recurrence rates, 6 patients had recurrences in the GPR30-positive group (22.2%), whereas only 2 patients had recurrences in the GPR30-negative group (9.5%) (Fig. 2). Although there was a trend toward more recurrences in the GPR30-positive group, the difference
between these 2 groups was not statistically significant. This may have been a result of the small number of events. Longer follow-up might have resulted in a significant difference, for GPR30 has a poorer prognosis with a trend toward increased recurrences. The mean follow-up period for both groups was 36 months.

**Discussion**

Our series of triple-negative breast cancers strongly demonstrates the presence of GPR30 specifically in premenopausal women. We propose that growth in these tumors lacking the classical ER, PR, and HER2 may in fact be stimulated by this novel ER. Its presence in this population is supported by in vitro studies showing a role for GPR30 signaling in breast cancer cell lines. Human epidermal growth factor activated the epidermal growth factor receptor–mitogen-activated protein kinase transduction pathway and stimulated a regulatory loop, which subsequently engaged estrogen through GPR30 to boost the proliferation of the ER-negative breast cancer cell lines SkBr3 and BT20.\(^\text{15}\) Knockdown of GPR30 expression in the triple-negative cell lines MDA-MB-435 and HCC-1806 reduced estrogen-stimulated proliferation by 74% and 90%, respectively.\(^\text{16}\) Taken collectively, it appears that GPR30 is prevalent in TNBC and may be important to the growth of these tumors.

Previous studies have indicated that 80% of patients with the *BRCA1* germ-line mutation have triple-negative disease. Prophylactic oophorectomy without mastectomy will decrease the risk for breast cancer by approximately 50% in this subgroup.\(^\text{17}\) Removing the effect of estrogen appears to be beneficial and protective, begging the question of whether estrogen is acting on some receptor to induce stimulation. Additionally, our study shows that GPR30 is more prevalent in premenopausal women who have higher serum levels of estrogen compared with a postmenopausal subset of patients. Because TNBC is more prevalent in women aged <50 years, it is possible that the higher estrogen concentration has an effect on tumor growth but does not work through the classical ER. GPR30 may be a key factor because of its presence in more aggressive tumors in high-estrogen, premenopausal states.

GPR30 signaling is mediated not only by estrogen but also by estrogen-like compounds such as phytoestrogens,
gations are now under way evaluating growth inhibition of disease among premenopausal women is needed, because understanding of the role of GPR30 in triple-negative breast cancer. Future work should focus entirely on this class of receptors. Therefore, GPR30 represents a potential target for therapy with an agent other than the endocrine agents in current use. The feasibility of this depends on an increased understanding of the physiologic function of GPR30 and the potential adverse effects of its inhibition.

Limitations of our study include a small patient population and a relatively short follow-up period. Breast cancer in premenopausal women consists of a smaller percentage of patients; thus, our population over the designated study period was expectantly limited in number. Because most recurrences in triple-negative disease occur in the first 3 years after diagnosis and treatment, our follow-up period was likely adequate to assess relapsing disease. Despite these factors, however, the study is strengthened by our ability to fully ascertain receptor status, monitor recurrence, and determine the survival of our patients.

The antibody we used is for research purposes and has not been validated for clinical use. It is possible that even if GPR30 has an important function in TNBC, a standardized antibody or an assay of gene expression, such as quantitative real-time polymerase chain reaction or fluorescence in situ hybridization, could be a more clinically useful biomarker for response to targeted therapy.

Our study did not include testing for the presence of ER-β, which some studies have reported to be present in 42% of triple-negative tumors in BRCA carriers. However, ER-β is associated with an improved prognosis, as is BRCA-related TNBC, whereas GPR30-expressing tumors may have a worse prognosis. It will therefore be interesting in future studies to examine whether ER-β and GPR30 expression are counterregulatory.

Overall, more investigation is necessary to evaluate the exact function of GPR30 in TNBC. Future work should focus on defining the biochemical mechanism of action of this novel receptor in such aggressive tumors. A greater understanding of the role of GPR30 in triple-negative disease among premenopausal women is needed, because of its predominance as demonstrated in our study. Investigations are now under way evaluating growth inhibition of triple-negative disease when GPR30 is blocked in a knockdown model. All of these efforts will be directed at understanding the role of GPR30 in TNBC with the hopes of proceeding to a clinical trial.

References


