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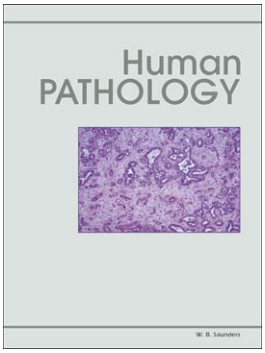

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Title: The expression of growth hormone releasing hormone (GHRH) and its receptors in breast carcinomas with apocrine differentiation – further evidence of the presence of a GHRH pathway in these tumors

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Running title: GHRH-pathway in apocrine breast tumors

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Abstract:

Apocrine breast carcinomas were evaluated for the expression of components of the growth hormone-releasing hormone (GHRH) autocrine/paracrine pathway: GHRH and its receptors (GHRH-R), as mammary apocrine carcinomas and epithelium seemed to be uniformly positive for GHRH-R in a pilot study. The apocrine phenotype was determined on the basis of hematoxylin-eosin morphology and a congruent immunohistochemical profile (estrogen receptor negativity, androgen receptor and gross cystic disease fluid protein-15 positivity). Thirty five formalin-fixed paraffin-embedded apocrine breast cancers in tissue microarrays and 24 cases using whole tissue sections were evaluated for GHRH-R and GHRH expression by immunohistochemistry using polyclonal antibodies raised against various domains of GHRH-R and one polyclonal antibody specific for GHRH. GHRH-R positivity was detected in the overwhelming majority (ranging from 90% to 100%) of apocrine breast carcinomas with all but one of the antibodies applied. The expression was usually diffuse with only isolated cases showing positivity in less then 50% of tumor cells. With the PA5-33583 antibody, GHRGH-R positivity was seen only in 73% of the cases in at least 50% of the tumor cells. GHRH expression was also present in all but one cases tested, with more than 50% of the cells expressing it in 30/34 cases. These results support a high rate of GHRH-R and GHRH expression in apocrine breast carcinomas. Whether these findings can be exploited for the targeted treatment of apocrine breast carcinomas with GHRH antagonists requires further study.

Keywords: apocrine carcinoma; breast cancer; growth hormone-releasing hormone receptor; immunohistochemistry.

1. INTRODUCTION

Apocrine carcinomas represent a peculiar estrogen receptor (ER) and progesterone receptor (PR) negative, but androgen receptor- (AR)-positive subtype of breast malignancies characterized by a specific histologic appearance on hematoxylin and eosin (H&E) stained slides, resembling the cytomorphology of apocrine metaplasia with abundant granular, eosinophilic cytoplasm and large nuclei with prominent nucleoli [1, 2, 3]. The majority of apocrine carcinomas show the growth pattern of invasive ductal / no special type (NST) carcinomas and can be viewed as a special cytomorphologic variant of it, but apocrine differentiation have also been demonstrated in special-type cancers [2,4].

Invasive carcinomas of the breast with no evidence of ER, PR expression and human epidermal growth factor receptor 2 (HER2) overexpression and/or amplification are designated as triple-negative breast cancers (TNBC). By their negativity for the above mentioned predictive factors, TNBCs are unsuitable for systemic therapies targeting the ER or HER2 pathways, and therefore identifying subsets that may be responsive to specific therapies is important [5, 6, 7].

Somewhat more than half of apocrine carcinomas represent a subgroup of TNBCs whereas nearly half of them overexpress HER2 [8]. According to Lehmann et al, TNBC can be divided into at least six distinct, relatively stable molecular subtypes [9], and one of these is the luminal androgen receptor positive (LAR) subset of cancers to which the majority of apocrine carcinomas belong. Recent studies using gene expression profiling also introduced the category of molecular apocrine tumors [10], and highlighted their relation to luminal breast carcinomas [9, 11]. The literature contains dubious data concerning the prognosis of apocrine carcinomas [12, 13]. This confusion is most likely caused by the extreme variation in the definition of apocrine differentiation, which can be based on morphologic,

immunohistochemical (IHC) and molecular features or the combination of those. Studies using rigorous morphologic and IHC criteria showed favorable prognosis of the so-designated pure apocrine carcinomas compared with NST carcinomas with similar grade and nodal status [14]. Despite their stated dismal natural prognosis and the ER and PR negativity, apocrine tumors are characterized by AR positivity and active steroid hormone metabolism [15], which suggests that this subset of TNBCs may be responsive to some specific hormonal therapeutic options, including AR antagonists [15].

According to in vitro studies, growth hormone releasing hormone (GHRH) and its receptors (GHRH-Rs) are involved in carcinogenesis acting both indirectly through the hypothalamus - pituitary gland axis by subsequent release of insulin-like growth factor 1 (IGF-1) from the liver, and more significantly directly through autocrine and paracrine regulation. Solid tumors of many organs, including breast carcinomas, express GHRH and GHRH-R [16, 17, 18]. It has been shown that the knocking down of GHRH gene expression in different cancer cell lines (e.g. breast, prostate and non-small cell lung cancer) leads to diminished cellular proliferation [18, 19]. As an additional support for the autocrine/paracrine regulation, the transfection of cell lines originally lacking GHRH-R with the GHRH-R gene results in a more pronounced cell proliferation with and even without the addition of exogenous GHRH [20]. As further evidence of the downstream intracellular influence of GHRH, in vitro supplementation with GHRH-R antagonists has been successful in reducing the invasive and metastatic potential of human cancer cell lines in several breast cancer models [21, 22, 23], by regulating cellular adhesion, migration and survival [24].

The expression of the full length pituitary pGHRH-R and/or its splice variant 1 (SV1) in cancer cells has been demonstrated on both mRNA and protein level using different techniques including RT-PCR [17, 23, 25, 26], Western blotting [21, 26], in situ hybridization [27] and IHC [20, 27].

The presence of GHRH-Rs has been demonstrated by IHC in many histological and molecular subtypes as well as in different histological grades of breast cancer including estrogen receptor dependent and independent lesions [23, 25, 28]. According to our previous results, about 30% of TNBCs showed GHRH-R expression, and the 10 apocrine carcinomas tested, as well as benign cysts with apocrine epithelium emerged as uniformly positive for this marker [28].

Based on the aforementioned results, GHRH antagonists have been proposed as potential targeted therapeutic agents for oncological treatment of breast carcinoma [29, 30], thus the presence of GHRH-R in apocrine carcinomas may theoretically have systemic treatment implications with agents targeting the GHRH-R pathway. In this study, we analyzed a larger series of apocrine breast carcinomas for the expression of GHRH and GHRH-R using antibodies raised against different parts of this latter transmembrane protein.

2. MATERIALS AND METHODS

In this retrospective study, tissue blocks of 59 patients with pure apocrine breast carcinoma were included from the archives of the Pathology Departments of the University of Szeged, the Bács-Kiskun County Teaching Hospital, the University of Turin and the University of Sarajevo.

Formalin-fixed, paraffin-embedded (FFPE) tissue blocks obtained either from breast conserving surgery or total mastectomy specimens were used for the construction of composite blocks using the tissue microarray (TMA) technique. Every tumor was represented by multiple cores. The TMA block built up in Turin consisted of triplicate cores of 1.1 mm in diameter, whereas the TMA blocks in the Hungarian departments were assembled using duplicate cores of 2.2 mm.

Twenty-two tumors were assessed in whole tissue blocks and two in needle core biopsy samples. One of the cases assessed in whole section represented an apocrine ductal carcinoma in situ with no evidence of invasive component but having a lymph node metastasis; this case was analyzed on the basis of both the tumor and its metastasis.

For the selection of apocrine carcinomas we defined apocrine differentiation using both histomorphologic and IHC criteria. Only tumors diffusely (>90%) made up of cells characterized by abundant, intensely eosinophilic, granular or sometimes vacuolated cytoplasm, large nuclei and prominent nucleoli were included [1, 2]. ER- and PR- negativity, AR-positivity [8, 31], with the co-expression of gross cystic disease fluid protein-15 (GCDFP-15) [32] were considered as IHC criteria. These restrictive eligibility criteria excluded many carcinomas complying with the requirements of other commonly used apocrine carcinoma definitions, but yielded a more homogenous group of tumors sometimes designated as "pure" apocrine carcinomas. The ER, PR, HER2, AR, GCDFP15 IHC and HER2 in situ hybridization (ISH) reactions were carried out following the local protocols of the contributing departments and the result were derived from the original reports, with a few exceptions in which missing AR and GCDFP reactions had to be performed. The interpretation of the ER, PR and HER2 staining was according to the current American Society of Clinical Oncology/College of American Pathologists recommendations [33].

Four to five-micrometer-thick sections of the FFPE tissue blocks or similarly processed TMAs were used for GHRH and GHRH-R IHC. To strengthen the preliminary results gained with a single GHRH-R antibody [28], and exclude the possibility of a consistent "false" staining of the apocrine epithelium, we decided to test the cases with different antibodies developed against various domains of the GHRH-R protein and one additional antibody against the human GHRH (Figure 1, Table 1). The GHRH staining was only carried out on a subset of the tumors, i.e. the cases in the TMA blocks because of the

limited amount of antibody available, and one case was damaged and inappropriate for evaluation in both samples.

Primary antibodies and the details of the protocols used are listed in Table 1, and the domain specificity of the antibodies is shown on Figure 1.

For the GHRH-R immunostainings, normal cadaver pituitary tissue was used as positive control, whereas slides from a brain bank sample representing the hypothalamic region (a kind donation by Dr. István Bodi, London) served as positive control for the GHRH IHC. Specimens were evaluated only in the case of adequate positivity in the mentioned controls. Positive staining of breast cancer tissue was classified according to the localization of staining and percentage of positive tumor cells. Two cut-off levels for positivity were arbitrarily chosen, a more permissive cut-off level of 10% also used in our previous study [28], and a more rigorous one using 50% of the cells staining. According to Gallego et al [27], cytoplasmic and/or nuclear staining was considered positive, as well as membranous staining.

No patient related information was retrieved, materials were used anonymously and retrospectively with no influence on patient outcome or treatment. The institutional ethical committees of the involved institutions were previously consulted, and the study was approved (17/2015 Human Investigation Review Board, University of Szeged).

3. RESULTS

According to the criteria listed in the previous section, all the apocrine carcinomas studied were negative for ER and PR; 35 of them were HER2-negative, 3 were 2+ on IHC and not successfully tested by ISH (inadequate samples), and 21 tumors were 3+ on IHC or amplified with ISH for HER2. One case of apocrine DCIS was not tested for this latter marker.

There was no significant difference (Chi-square test with Yates' correction, 10% cut-off: *p= 0.6945,* 50% cut-off: *p= 0.8235*) between the GHRH-R expression of HER-2 positive and negative cases using ab76263.

The striking majority of the apocrine carcinomas tested showed strong GHRH-R positivity with all the antibodies used (Figure 2), only PA5-33583 showed somewhat lower positivity rate (Table 2).

Regarding the intracellular localization of staining, there were certain differences between the applied antibodies. Cytoplasmic staining was observed using ab76263 (Figure 2E), and PA5-33583 (Figure 2D), with no trace of the nuclear staining previously reported in non-apocrine carcinomas [27, 28]. Using ab28692, the majority of the cases showed a predominantly cytoplasmic labeling with some focal membranous positivity (Figure 2F), whereas more conspicuous and frequently diffuse membranous staining was detected along with cytoplasmic reactions using PA5-33582 (Figure 2C).

The GHRH antibody labeled 33 (10% cut-off, 97%) and 30 (50% cut-off, 88%) of the 34 apocrine carcinomas assessed (Table 2, Figure 2B). GHRH and GHRH-R negativity concurred, with only a single case showing GHRH expression in the absence of GHRH-R staining.

4. DISCUSSION

Apocrine carcinomas are rare breast tumors making up between 0.3 to at most 4 per cent of breast carcinomas [3]. In an earlier study exploring the presence of GHRH-Rs in breast cancers, we found that apocrine carcinomas were among the ones demonstrating the highest rates of staining. All the 10 cases studied showed marked positivity with the polyclonal antibody ab76263 [28]. To avoid wrong conclusions derived from low case numbers and a potential consistent false labeling of apocrine cells, we sought at corroborating

our results by expanding the number of cases studied and using alternative antibodies developed against different domains of the GHRH-R protein (Figure 1, Table 1). The results obtained with antibody ab28692 (the epitope of which is a subregion of the C-terminal domain different from that of ab76263) along with antibodies PA5-33582 and PA5-33583, which were raised against the N-terminal and third cytoplasmic domain of GHRH-R, respectively, reinforces our previous findings with ab76263.

Besides the demonstration of GHRH-Rs in apocrine carcinomas of the breast, another component of the autocrine/paracrine mechanism, GHRH itself was also tested and found to be present in most tumors to strengthen the existence of such a mechanism.

As several previous papers [17, 21, 25] suggested an important role of the SV1 splice variant of GHRHR-R in cancers, an attempt was made to distinguish between the expression of pGHRH-R and SV1. Since three of the GHRH-R antibodies (ab76263, ab28692 and PA5-33583) used in our study were raised against synthetic peptides derived from the C-terminal or third cytoplasmic domain of the human pGHRH-R, and the biologically active SV1 differs from the full length pituitary receptor only in its N-terminal part [17], the antibodies recognize the full length pGRHR-R along with the SV1, but not the much shorter (145-amino acid-long) SV2. One GHRH-R antibody (PA5-33582) was developed against an epitope in the N-terminal first extracellular domain of the receptor. Although the amino acid sequence of the epitope is considered proprietary, according to the correspondence with the manufacturer, the PA5-33582 antibody should recognize the pGHRH-R along with SV1 and SV2 but not the SV3 (based on correspondence with the manufacturer's assistance team). Therefore, to our knowledge, no commercially available antibody is able to differentially stain pGHRH-R and SV1. Accordingly, with the help of IHC, we were able to demonstrate the almost universal presence of GHRH-Rs (pGHRH-R and/or SV1) and GHRH in apocrine breast carcinomas.

As defined here, all apocrine carcinomas are ER and PR negative, and somewhat more than half of them are also HER2 negative. The HER2 positive subset can be treated with anti-HER2 therapies, but the triple-negative subset is usually managed similarly to basal-like carcinomas, although evidence suggests that they represent a clinically distinct subset of TNBCs.

Understanding the complex group of TNBCs and the identification of subsets that could be characterized by biomarkers serving as prognostic or predictive factors for potential future molecular targets, could possibly "melt down" this difficultly treatable tumor category and is of great clinical significance. Some TNBCs, such as adenoid cystic carcinomas and secretory carcinomas have a better natural prognosis, whereas the identification of other subtypes has theranostic significance (e.g. poly ADP ribose polymerase inhibitor sensitivity of BRCA mutant cases).

Although the reports on the prognosis of apocrine carcinomas are inconsistent, and some publications do not distinguish between different subtypes of triple negative NST carcinomas, AR positivity and active steroid hormone metabolism suggests a relation of these tumors to luminal subtypes, and suggests a possible responsiveness to some forms of endocrine therapy. Inspired by novel therapies used for the treatment of prostate cancer, clinical trials focusing on AR-targeting drugs in breast cancer are under progress [34]. Based on our previous IHC study of various subtypes of breast cancer [28], an emerging future treatment option could be the targeting of the GHRH-R pathway.

We have found that the vast majority of the apocrine carcinomas show GHRH and GHRH-R positivity with all the applied antibodies, with no difference on the basis of HER2 status. In comparison to non-apocrine triple negative and HER2-enriched carcinomas, tumors with apocrine differentiation show GHRH-R expression significantly more frequently [28].

We tested 26 non-apocrine TNBCs and 13 HER2 positive non-apocrine carcinomas for GHRH-R with the ab76263 antibody, and only 8 of each group showed positivity with the 10% cut-off limit, whereas only 2 HER2 positive cases showed a positive staining in more than 50% of the cells. It is also important to emphasize the GHRH-R staining of apocrine carcinomas is usually diffuse, whereas we failed to encounter any non-apocrine TNBC with positivity in more than 50% of the tumor cells [28].

Preclinical studies using nude mice models with human cancer cell lines suggest that antagonistic analogs of GHRH do not only directly inhibit proliferation in cancers of different types but also potentiate the effects of cytotoxic chemotherapies [35]. Treatment with a GHRH antagonist showed a tumor reducing potential comparable to conventional chemotherapy with minimal side effects in xenograft transplanted nude mice models using non-apocrine human TNBC cell lines [29, 30]. The chemotherapy enhancing effect has also been demonstrated in TNBCs both with the use of docetaxel [29] and doxorubicin [30].

It is logical to think that the presence of GHRH-R reflects the ability of GHRH antagonists to act on cancer cells similarly to the presence of ER and HER2 being predictive to the effect of estrogen receptor modulators and trastuzumab, respectively Although according to our findings GHRH antagonists seem to be potential future therapeutic agents in breast cancer, at this stage, it is not yet clear, how GHRH-R IHC could be used as a potential biomarker for these therapies, e.g. what cut-off staining fraction and/or intensity should be considered for positivity.

5. CONCLUDING REMARKS

Our results are further highlighting the distinct nature of apocrine carcinomas amongst breast carcinomas, in consistency with the recently introduced molecular apocrine and LAR subtypes. The most important finding of the present study is the almost constant expression of

GHRH and GHRH-R in apocrine carcinomas, suggesting a role for these proteins in the endocrine/autocrine/paracrine regulation of these cancers. **In the light of promising preclinical investigations with GHRH antagonists, our results imply a possible responsiveness of apocrine breast cancers to targeted treatment against the GHRH pathway.** Since somewhat more than half of the apocrine carcinomas are TNBCs, the use of potent GHRH antagonists could make a significant therapeutic difference for patients with such aggressive tumors that are refractory to current targeted treatment modalities. Obviously, further investigations are warranted to elucidate the effect of GHRH antagonists in the clinical setting, and the role of GHRH-R expression as a potential biomarker for this.

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Disclosure

The authors have no conflict of interest to disclose.

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FIGURE LEGENDS AND TABLE TITLES

Figure 1. Structure and domains of the pGHRH-R protein with the target domains of different anti-GHRH-R IHC antibodies highlighted.

Figure 2. Expression of GHRH and GHRH-R in apocrine carcinomas. A: Typical acidophilic morphology of apocrine carcinomas (H&E); B: GHRH positivity; C-F and GHRH-R positivity with antibodies PA5-33582 (C), PA5-33583 (D), ab76263 (E) and ab28692 (F); all x20.

Table 1: List and applied protocols of primary antibodies.

Table 2. Distribution of GHRH and GHRH-R-positivity according to the different antibodies. (Not all cases could be evaluated for all antibodies.)

Table 1: List and applied protocols of primary antibodies.

Antibody	Name (Clone)	Immunogen epitope	Company	Dilution	Incubation time / temperature
GHRH-R	ab76263 (polyclonal)	C-terminal domain (50 amino acid)	Abcam (Cambridge, UK)	1:250	60 min / room temperature (RT)
	ab28692 (polyclonal)	C-terminal domain (19 amino acid)	Abcam (Cambridge, UK)	1:200	30 min/RT
	PA5-33582 (polyclonal)	N-terminal extracellular domain (18 amino acid)	Thermo Scientific, (Waltham, MA, USA)	1:600	30 min/RT
	PA5-33583 (polyclonal)	3rd cytoplasmic domain (16 amino acid)	Thermo Scientific, (Waltham, MA, USA)	1:50	30 min/RT
GHRH	MBS2006	Not specified	MyBioSource (San	1:40	30 min/RT

	543-0.1 (polyclonal)		Diego, CA, USA)		

Table 2. Distribution of GHRH and GHRH-R-positivity according to the different antibodies. (Not all cases could be evaluated for all antibodies.)

	GHRH-R antibody	**Positive/Total No. of cases**		**Percent of positive cases (95% confidence intervals (CI))**	
		10% cut-off	**50% cut-off**	**10% cut-off**	**50% cut-off**
Apocrine carcinomas	GHRH-R ab76263	57/59	53/59	97% (88-99%)	90% (83-97%)
	GHRH-R ab28692	59/59	59/59	100% (94-100%)	100% (94-100%)
	GHRH-R PA5-33582	59/59	56/59	100% (94-100%)	95% (86-98%)
	GHRH-R PA5-33583	47/52	38/52	90% (79-96%)	73% (60-83%)
	GHRH	33/34	30/34	97% (85-99%)	88% (73%-95%)

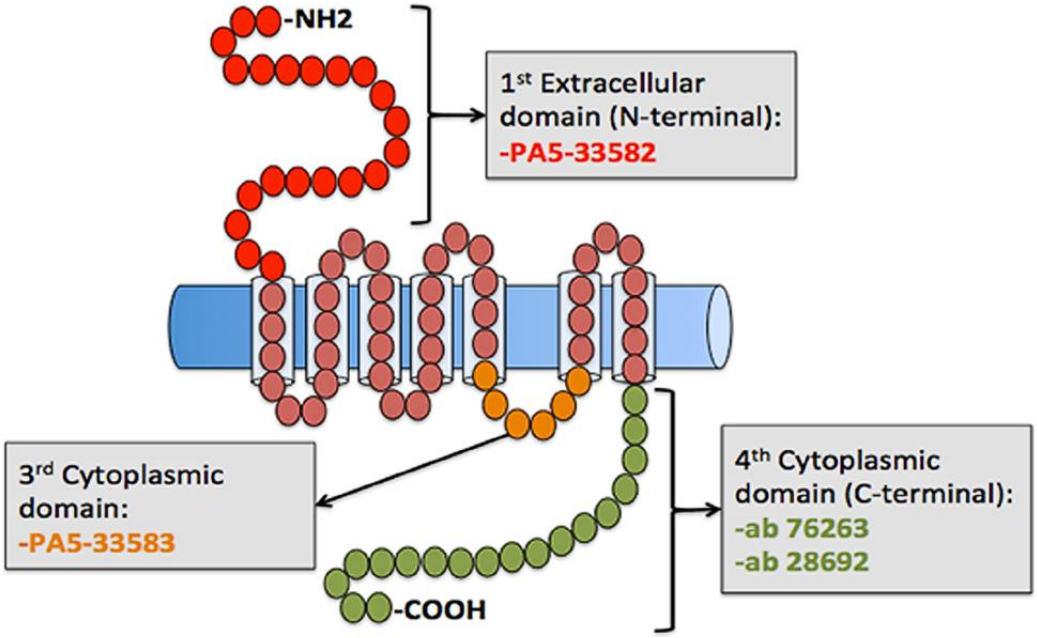

Figure 1

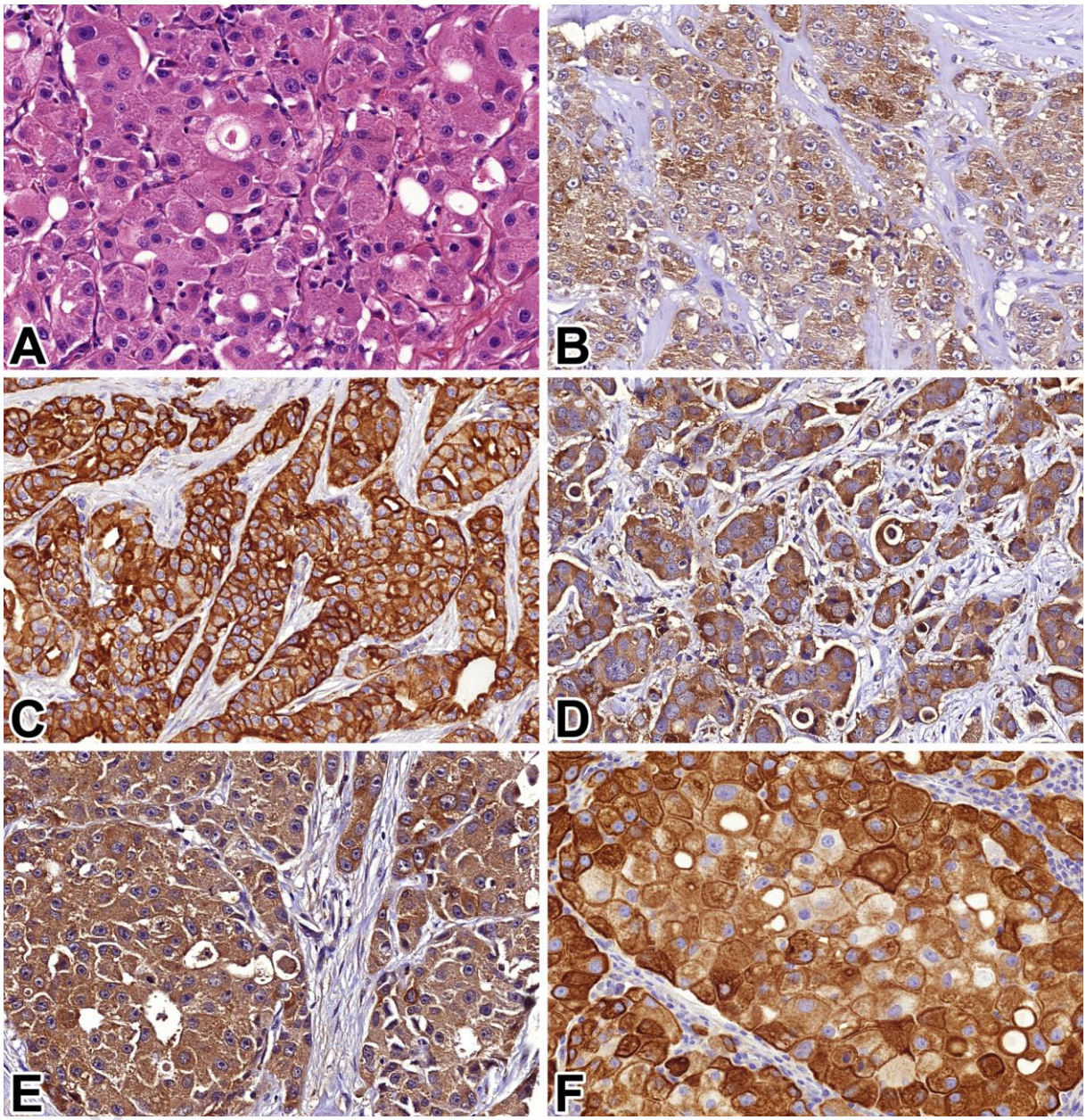

Figure 2

Highlights

- Almost all apocrine breast carcinomas showed expression of both GHRH and GHRH-R.
- These proteins probably play a role in the autocrine/paracrine regulation of apocrine cancers.
- The presence of the GHRH and GHRH-R pathway in these cancers suggest a potential role for targeted anti-GHRH therapies