Immunohistochemical characterization of selected breast lesions

Ph.D. Thesis

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LIST OF FULL PAPERS THAT SERVED AS THE BASIS OF THE PH.D. THESIS

I. **Kővári B**, Rusz O, Schally AV, Kahán Z, Cserni G.
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II. **Kővári B**, Szász AM, Kulka J, Marusić Z, Sarcevic B, Tiszlavicz L, Cserni G.
Evaluation of p40 as a myoepithelial marker in different breast lesions.
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III. **Kővári B**, Báthori Á, Cserni G.
CD10 immunohistochemical expression in apocrine lesions of the breast.
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I. Meretoja TJ, Audisio RA, Heikkilä PS, Bori R, Sejben I, Regitnig P, Luschin-Ebengreuth G, Zgajnar J, Perhavec A, Gazic B, Lázár G, Takács T, **Kővári B**, Saidan ZA, Nadeem RM, Castellano I, Sapino A, Bianchi S, Vezzosi V, Barranger E, Lousquy R, Arisio R, Foschini MP, Imoto S, Kamma H, Tvedskov TF, Jensen MB, Cserni G, Leidenius MH.
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The use of digital images improves reproducibility of the Ki-67 labeling index as a proliferation marker in breast cancer.
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Different methods of pretreatment Ki-67 labeling index evaluation in core biopsies of breast cancer patients treated with neoadjuvant chemotherapy and their relation to response to therapy.
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IV. **Kővári B**, Donkó V, Piukovics K.
Extensive retroperitoneal extramedullary hematopoiesis mimicking disseminated abdominal cancer. (In Hungarian)
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LIST OF ABBREVIATIONS

AME	Adenomyoepithelioma
AR	Androgen receptor
BLBC	Basal-like breast carcinoma
CALLA	Common acute lymphoblastic leukemia antigen
CD 10	Cluster of differentiation 10
CGRP	Calcitonin gene-related peptide
CI	Confidence interval
CK	Cytokeratin
DAB	3,3'-Diaminobenzidine
DCIS	Ductal carcinoma in situ
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
FFPE	Formalin fixed, paraffin embedded tissue sample
GCDFP-15	Gross cystic disease fluid protein 15
GH	Growth-hormone
GHRH	Growth-hormone-releasing hormone
GHRH-R	Growth-hormone-releasing hormone receptor
HE	Hematoxylin and eosin stain
HER2	Human epidermal growth factor receptor 2

HIER	Heat-induced epitope retrieval
IARC	International Agency for Research on Cancer
IGF-1	Insulin-like growth factor 1
IHC	Immunohistochemistry
ILC	Invasive lobular carcinoma
ISH	In situ hybridization
Ki-67	Proliferation marker Ki-67 antibody
LAR	Luminal androgen receptor positive subtype of TNBC
LI	Ki-67 labeling index
MEC	Myoepithelial cell
NEP	Neprilysin
NST	Invasive carcinoma of no special type, formerly called invasive ductal carcinoma not otherwise specified (IDC NOS)
p40 or ΔNp63	Protein 40 or tumor protein 40
p53	Protein 53 or tumor protein 53
p63	Protein 63 or tumor protein 63
pGHRH-R	Full length, pituitary type GHRH-R
PR	Progesterone receptor
SiRNA	Small interfering ribonucleic acid
SMA	Smooth muscle actin
SMMHC	Smooth muscle myosin heavy chain

SV1	Splice variant 1 of GHRH-R
TBS	Tris buffer saline
TDLU	Terminal ductal lobular unit
TMA	Tissue microarray
TNBC	Triple-negative breast carcinoma
TP53	Gene encoding tumor protein 53
TP63	Gene encoding tumor protein 63
VIP	Vasoactive intestinal peptide
WHO	World Health Organization

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1. INTRODUCTION

1.1. GENERAL INTRODUCTION

According to the latest world cancer statistics published by the International Agency for Research on Cancer (IARC) in 2012, breast cancer represents 11,9% of diagnosed cancers, which means approximately 1.7 million new patients every year worldwide. This makes breast cancer the second most common malignancy, and the most frequently diagnosed cancer among women. Breast cancer is also the leading cause of cancer death among women and accounts for approximately 520 000 deaths per year. Since the previous (2008) IARC world cancer statistics, the incidence of breast cancer has increased by 20%, while mortality has increased by 14% [1, 2].

There are different approaches to the classification of breast cancer based on conventional methods like histopathological/morphological appearance, degree of differentiation (grade), extent of tumor spread (stage), but there are also relatively new categorizations, e.g. the ones based on gene expression profiling and molecular subtyping. Every approach tries to stratify breast cancer by risk and prognosis. The more recent classifications try to categorize the disease at the molecular level and give important predictive information on the potential responsiveness of the tumors to different therapeutic modalities.

As concerns the histopathological classification according to the 4th edition of the World Health Organization (WHO) Classification of Tumors of the Breast, invasive cancers not fulfilling the strict definition of one of the many specific histological types (e.g. lobular, tubular, mucinous etc.) are designated as invasive carcinoma of no special type (NST), formerly called invasive ductal carcinoma not otherwise specified. NST is the most common histological type comprising approximately 60% of invasive breast carcinomas. If a specific growth pattern of breast cancer represents more than 90% of the tumor, it can be classified as being of a specific type. These types of breast cancer are less frequently encountered, with invasive lobular carcinoma (ILC) being the most common, representing 10% of mammary cancers with other special types accounting for less than 1 to 5% each [3, 4].

Classification of tumors according to the level of differentiation is called grading and is one of the oldest and most widely accepted histologic tools to predict the prognosis of a malignant neoplasm. Grading of cancers can be performed on the basis of histologic or nuclear features or both. In current breast pathology practice, the most frequently used three-tiered (grade 1-3) grading scale is the so-called combined histological grade (the Nottingham score system, or the Elston-Ellis modification of the Scarff-Bloom-Richardson grading system). This method is based on the complex evaluation and semiquantitative scoring of ”tubule formation” (glandular differentiation) and nuclear morphology combined with the mitotic activity of the tumor [5-7].

A staging system is a standardized method to describe the anatomic extent of a disease, i.e. how far cancer has spread. The most common system used is the TMN staging system, which classifies cancers according to their local size and some features of advanced disease (T category), lymph node involvement (N category) and distant spread (M category). Staging can be performed by physical examination, radiologic imaging studies and pathologic examination following breast surgery. Pathologic evaluation is considered to be the most accurate method for the staging of tumors [8, 9].

Molecular subtypes of breast cancer have been identified using gene expression profiling. The most reproducibly identified molecular subtypes are the luminal A, luminal B, human epidermal growth factor receptor (HER2)-enriched and basal-like groups. Luminal types are characterized by estrogen receptor (ER) driven carcinogenesis, whereas the latter two groups are hormone receptor independent and cluster as ER-negative subtypes. The molecular type of breast cancer is a valuable information to assess prognosis and determine the appropriate therapy [10-12].

As in routine histopathology practice gene expression profiling based breast cancer classification is not yet widely accessible, an approximations of molecular subtypes using immunohistochemistry (IHC) and in situ hybridization (ISH) has been proposed by Goldhirsch et al [13]. This surrogate IHC method for the determination of molecular subtypes uses ER, progesterone receptor (PR), HER2 and Ki67 antibodies. The hormone receptor (ER/PR) positive luminal-like types are designated as luminal B-like on the basis of a high proliferation rate, using Ki-67, or HER2

overexpression/amplification, while luminal carcinomas with low Ki67 labeling index (LI) and HER2-negativity fall into the luminal A-like group. Hormone receptor negative cases overexpressing HER2 represent a so-called HER2-enriched type. Although the basal-like molecular type is frequently correlated with an ER, PR and HER2 negative IHC profile designated as triple-negative breast cancer (TNBC), the two categories cannot be equated. Many TNBCs do not fall into the basal-like breast cancer (BLBC) type [13, 14].

TNBC represents about 15% of breast malignancies [15]. By their negativity for the above-mentioned predictive factors, TNBCs are unsuitable for systemic therapies targeting the ER or HER2 pathways, and therefore identification of subsets that may be responsive to future targeted therapies is important.

According to the definition of the WHO, tumors made up of cells characterized by abundant, intensely eosinophilic, granular or sometimes vacuolated cytoplasm, large nuclei and prominent nucleoli are designated as invasive carcinomas with apocrine differentiation or simply invasive apocrine carcinomas. ER and PR negativity, androgen receptor- (AR) positivity [16], with the co-expression of gross cystic disease fluid protein-15 (GCDFP-15) are considered as IHC criteria of apocrine differentiation. Somewhat more than half of apocrine carcinomas represent a subgroup of TNBCs and nearly half of them overexpress HER2 [17]. Recently a gene expression profile based category of molecular apocrine tumors has also been introduced [18]. Apocrine carcinomas represent a peculiar ER-negative, but AR-positive molecular type of breast malignancies. These tumors are more closely related to luminal breast carcinomas, and they have a better prognosis [15, 19]. According to Lehmann et al, TNBCs can be divided into at least six distinct relatively stable molecular subtypes [19], and one of these is the luminal androgen receptor positive (LAR) set of cancers, to which many apocrine carcinomas belong to. Despite the ER and PR negativity, LAR tumors are characterized by AR positivity and active steroid hormone metabolism [20], which suggests that this subset of TNBCs may be responsive to some specific hormonal therapeutic options.

1.2. THE INVOLVEMENT OF GROWTH HORMONE-RELEASING HORMONE (GHRH) IN CARCINOGENESIS

Growth hormone-releasing hormone (GHRH) has been implicated in carcinogenesis as a growth factor acting both indirectly through the neuroendocrine axis involving the pituitary release of growth hormone (GH) with subsequent expression of insulin-like growth factor 1 (IGF-1) in the liver, and more significantly directly through autocrine and paracrine mechanisms (Figure 1).

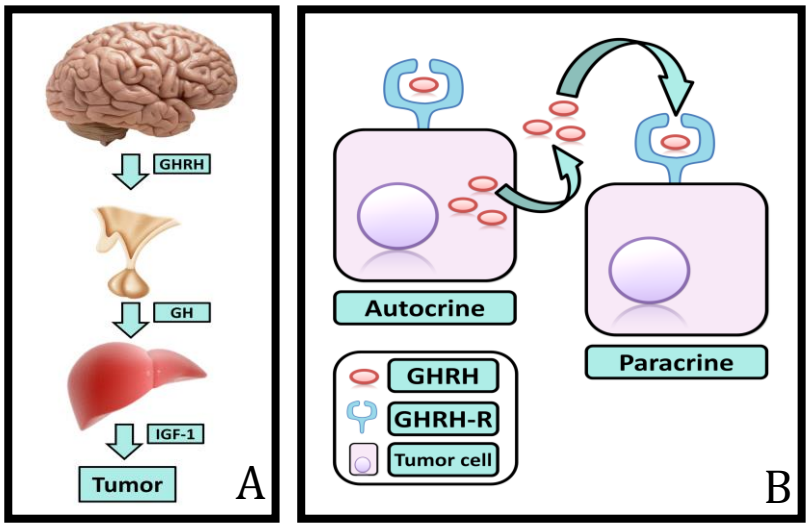

Figure 1. (A) Indirect carcinogenetic pathway by the stimulation of pituitary GHRH-receptors and the neuroendocrine axis. (B) Direct autocrine and paracrine pathways.

Many cancers of extrapituitary tissues, including breast carcinomas, express GHRH and GHRH receptors (GHRH-R) [21-23]. The presence of both the full-length pituitary GHRH receptor (pGHRH-R) and its splice variants, predominantly the splice variant 1 (SV1) have been documented in breast cancer [22, 24-26]. As evidence of an autocrine/paracrine regulatory mechanism, it has been shown that the knocking down of the GHRH gene expression in breast cancer cell lines with small interfering ribonucleic acid (SiRNA) results in reduced cellular proliferation [27]. Similar effects are produced in prostate cancer and non-small cell lung cancer cell lines [23, 27]. As additional support for an autocrine/paracrine regulation, the transfection of the MCF7 cells (originally devoid of GHRH-R) with the GHRH-R, results in increased cellular

proliferation after the addition of exogenous GHRH. This increase in proliferation is even greater when the transfection involves the SV1 receptor [28]. The transfection of MCF-7 cells with the SV1 receptor results in increased proliferation even without the addition of exogenous GHRH, suggesting a GHRH-independent activation of this truncated receptor [28]. Furthermore, GHRH-R antagonists have been found to be effective in the reduction of invasive and metastatic potential of human cancer cell lines in vitro by modifying cellular adhesion, migration and survival [29]. The antagonistic analogs of GHRH have been reported to consistently reduce or abolish the growth of several breast cancer models [26, 30, 31], and therefore such antagonists have been proposed as potential targeted therapeutic agents for breast carcinoma.

The presence of the pGHRH-R and/or the SV1 receptor in cancer cells has been demonstrated by different techniques including RT-PCR [22, 24, 31, 32], Western blotting [26, 32], in situ hybridization [33], immunohistochemistry [25, 28, 33, 34] and radioreceptor assays [35]. GHRH-Rs have been demonstrated in ER dependent as well as independent breast carcinoma cell lines [31], in both ductal NST carcinomas (in various histological grades) and ILCs [25, 33].

The presence of GHRH-R in breast carcinomas may therefore have potential systemic treatment implications with agents targeting the GHRH-R pathway. Although the presence of GHRH-R in breast cancer was documented before, no previous study systematically investigated the expression of the receptor in different subtypes of breast cancer before.

1.3. THE p53 TUMOR SUPRESSOR GENE FAMILY

The tumor suppressor gene family “p53” includes transformation-related protein 53 (TP53), transformation-related protein 63 (TP63) and transformation-related protein 73 (TP73) genes that are responsible for encoding transcription factor proteins labelled as tumor protein 53 (p53), tumor protein 63 (p63) and tumor protein 73 (p73), respectively [36, 37].

The tumor suppressor p53 enforces its anti-oncogenic functions by arresting the cell cycle, initiating apoptosis and facilitating DNA repair through its effects on the transcription of other tumor suppressor genes [38, 39].

In contrast to the ubiquitous expression of p53, p63 and p73 are expressed in limited tissue types and share partially overlapping functions with p53 by upregulating numerous p53 target genes [40, 41]. TP63 encodes for three main splice variants TAp63α, TAp63β and TAp63γ, which differ in their carboxy-terminal domains and ΔNp63 also known as p40 isoforms resulting from alternative promoter usage which lack the N-terminal (transcription activation, TA) domain [42] (Figure 2.). TAp63α and TAp63γ trigger anti-proliferative effect rendering them tumor suppressor proteins whereas the p40 isoform works as an oncoprotein and suppresses the transactivation activity of both TAp63 and p53 [43].

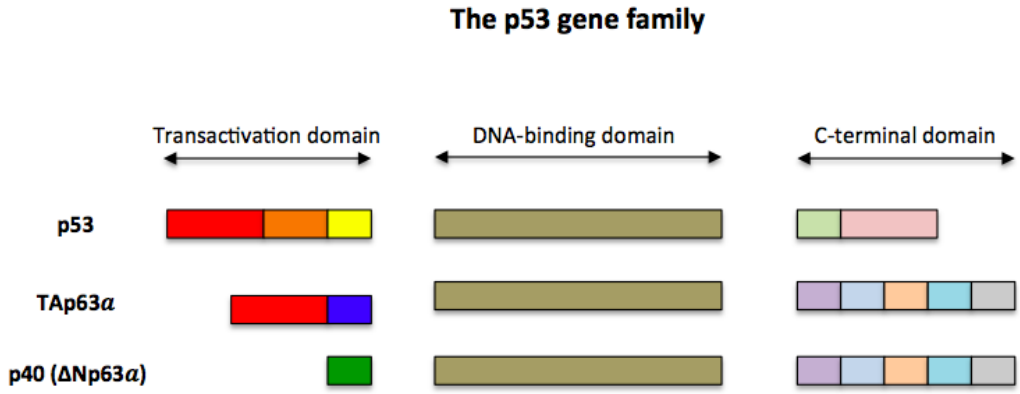

Figure 2. Schematic structure of the p53, p63 and p40 (ΔNp63 isoform) proteins.

p63 is expressed in basal epithelia in a restricted pattern, and is utilized primarily as a marker of squamous [44], myoepithelial (MEC) [45], prostate basal [46] and urothelial cells [47] in current surgical pathology practice.

In comparison to other MEC markers, p63 is slightly less sensitive but more specific than smooth muscle actin, smooth muscle myosin heavy chain and calponin [48]. p40 is the newest member of the family being used as an IHC marker and is reported to be superior to p63 for squamous differentiation in the differential diagnosis of non-small cell lung cancer [49].

Many myoepithelial / basal cell markers are also expressed in a group of breast carcinomas representing a basal-like nature or myoepithelial differentiation [50]. The most widely accepted surrogate IHC markers to identify BLBCs amongst TNBCs are cytokeratin (CK) 5, 14, 17 and epidermal growth factor receptor (EGFR)[51, 52]. Although compared to CK5, p63 is only infrequently expressed by BLBCs, there are only scant data on the expression of p40 in these tumors.

1.4. EXPRESSION OF CD10 IN BREAST TISSUE

Cluster differentiation 10 (CD10) also known as neprilysin, enkephalinase, common acute lymphoblastic leukemia antigen (CALLA) or neutral endopeptidase (NEP) is a membrane-bound zinc-dependent metalloprotease enzyme that degrades a number of small secreted peptides [53, 54]. It is a fairly ubiquitous enzyme found on the surface of many different cell types including pre-B cells, germinal center B cells, neutrophils, T-cell precursors and epithelial cells of the kidney, stomach, colon, prostate and liver canaliculi as well as in stromal cells of the endometrium and myofibroblasts [55]. In humans, CD10-related DNA sequences are found on chromosome 3 [56]. Three different splice variants of CD10 have been identified, suggesting that CD10 expression may be controlled in a tissue specific manner [57]. Physiologically, CD10 plays an important role in the metabolism of signaling peptides like natriuretic peptides, angiotensins, bradykinin, endothelin, enkephalins, oxytocin, tachykinins, substance P, calcitonin gene-related peptide (CGRP) and vasoactive intestinal polypeptide (VIP), involving it in the extracellular regulation of a number of signaling pathways of the mammalian nervous, cardiovascular, inflammatory and immune systems [53, 55]. CD10 is involved in the pathogenesis of numerous non-neoplastic diseases such as diabetic nephropathy [58] and Alzheimer's disease [59]. With IHC, it can be detected in many hematological malignancies [60-62], soft tissue neoplasia (e.g. pleiomorphic undifferentiated sarcoma, fibrosarcomas, leiomyosarcomas and malignant peripheral nerve sheath tumors) [63], as well as in carcinomas of different organs, like the skin [64, 65], the lungs [66.], the pancreas [67], the liver [68], the stomach [69], the uterine cervix [70], the kidneys [71], the urinary bladder [72] and the prostate [73]. Such a wide spectrum of expression may

suggest a limitation in the usefulness of CD10 immunostaining in routine diagnostic pathology.

As concerns the breast, CD10 has an important role in its development through modulation of cell growth and differentiation, and by having effects on epithelial-mesenchymal morphogenesis [74, 75]. CD10 is not only expressed by MECs but can also be detected on the surface of mammary stem cells, early common breast progenitor cells and in myoepithelial progenitors. CD10 protease maintains the early progenitor population in the human mammary lineage by degrading signaling proteins that would otherwise promote maturation [76]. A study using a mouse model has shown the involvement of oxytocin, a peptide cleaved by CD10, in the differentiation of MECs [77]. CD10 also has prognostic implications; its expression in breast tumor stromal cells is correlated with ER negativity, a higher grade and poor prognosis [78, 79]. CD10 has been shown to discriminate between benign, borderline and malignant phyllodes tumors of the breast and its expression has been found on IHC to correlate significantly with the occurrence of distant metastasis [80].

In diagnostic breast histopathology, CD10 IHC is used to identify MECs. Although MECs around normal structures (ducts and lobules) are nicely highlighted by this marker, in pathologic conditions such as ductal carcinoma in situ (DCIS), CD10 has a relatively low sensitivity as an MEC marker [81], and its specificity also seems compromised by the fact that, rarely, tumor cells also stain with the antibody [82], although the pattern of staining in the neoplastic mammary epithelium has not been widely studied.

Apocrine epithelium has been described to be positive for CD10 [83], and Kalof et al. [81] clearly documented the consistent luminal staining of apocrine metaplasia. While studying breast lesions immunostained for CD10 as an MEC marker, we also recognized that paratumoral apocrine cysts demonstrated a strong, predominantly apical reaction, and we have also found traces of this staining pattern in the literature [81, 83]. To our knowledge, no previous studies have systematically examined CD10 expression of apocrine lesions.

2. AIMS

The aims of the present thesis are listed as follows:

To analyze a series of breast carcinomas for the expression of GHRH-R and to correlate the presence of these receptors to histological features and morphological or biological subtypes of breast cancers.

To investigate a series of apocrine breast carcinomas for the expression of GHRH-R, because of the positive immunostaining of paratumoral benign apocrine epithelium noted during the course of the study.

To test the maintenance of GHRH-R status of the primary tumors in lymph node metastases as a possible GHRH-R antagonist could also be useful in the treatment of metastatic patients.

To compare the expression of p40 versus p63 in the MEC component of normal breast structures and in breast lesions with occasional absence of or decrease in the staining for some other MEC markers and to see whether p40 was also superior to p63 as a MEC marker.

To assess and compare the expression of p63 versus p40 in TNBCs showing CK5 expression, i.e. in tumors that would be classified as BLBCs by the surrogate IHC based approach.

To analyze a series of breast lesions with apocrine differentiation for the expression of CD10, both in the epithelial and the myoepithelial components and to explore how the immunostaining varied in benign, in situ and invasive malignant lesions.

3. MATERIALS AND METHODS

3.1. GENERAL ASPECTS

The conducted GHRH-R, p40 and CD10 expression related research was all carried out retrospectively using IHC.

Tissue blocks obtained either from breast conserving surgery or total mastectomy specimens mainly from the archives of the Bács-Kiskun County Teaching Hospital and University of Szeged were used with the following exceptions:

- For the GHRH-R expression study, breast carcinomas with apocrine differentiation were also obtained from the Department of Medical Sciences, University of Turin, Via Santena 7, Turin 10126, Italy.
- For the p40 expression study, blocks of adenomyoepithelial lesions were also obtained from the Department of Pathology, University Hospital Center Sestre milosrdnice of Zagreb and the 2nd Department of Pathology of Semmelweis University, Budapest.

Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin (FFPE). Composite tissue microarray (TMA) paraffin blocks were also built up by extracting smaller cylindrical tissue samples from the donor blocks of multiple breast cancer cases. Four to five micrometer-thick whole tissue sections and similar sections of TMA blocks were used for IHC. Deparaffinization and rehydration at room temperature was followed by heat induced epitope retrieval (HIER) with the PT Link system (“Target retrieval solution”, pH 9.0 for 30 min at 94°C - DAKO, Glostrup, Denmark). After being rinsed with Tris buffer saline - EnVision FLEX Wash (TBS), the sections were placed in a Dako Autostainer Link 48 for endogenous peroxidase blockage and staining. Diaminobenzidine (DAB; Dako, Glostrup, Denmark) or VIP (Vector Laboratories, Burlingame, CA, USA) were used as chromogens. The sections were then counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene, and mounted. Negative controls were carried out by omitting the primary antibody.

Primary antibodies used in the different studies and the details of the applied protocols are listed in Table 1.

Antibody	Name (Clone)	Immunogen epitope	Company	Dilution	Incubation time / temperature
GHRH-R	ab 76263 (polyclonal)	C-terminal domain (50 amino acid)	Abcam (Cambridge, UK)	1:250	60 min / room temperature (RT)
ER	SP1	C-terminal domain	Thermo Scientific, (Waltham, MA, USA)	1:200	30 min/RT
PR	RB-9017 (polyclonal)	Not specified	Thermo Scientific, (Waltham, MA, USA)	1:100	60 min/RT
HER2	SP3	Cytoplasmic domain	Biocare Medical (Concord, CA, USA)	1:100	60 min/RT
Ki-67	MIB-1	Not specified	Dako (Glostrup, Denmark)	1:100	30 min/RT
AR	F39.4.1	Amino acids 301-320	BioGenex, (Fremont, CA, USA)	1:50	30 min/RT
GCDFP-15	23A3	Not specified	Cell Marque, (Rocklin, CA, USA)	1:200	30 min/RT
p40	BC28	Amino acids 5-17	BioCare (Concord, CA, USA)	1:200	30 min/RT
p63	4A4	TA domain	Thermo Scientific, (Waltham, MA, USA)	1:400	30 min/RT
CK5	XM26	C-terminal domain	BioCare (Concord, CA, USA)	1:1 Ready to use	60 min/RT
CD10	56C6	Not specified	Cell Marque (Rocklin, CA, USA) Dako (Glostrup, Denmark)	1:50 1:1 Ready to use	30 min/RT

Table 1. List and applied protocols of primary antibodies.

All specimens were evaluated by two pathologists (BK, GC) in parallel using a double headed microscope.

Since all patient and disease information was gathered anonymously and retrospectively with no influence on patient outcome or treatment, no ethical permission was deemed necessary according to local regulations. For the p40 related study, involving TMA fabrication from a few tumors, the institutional review board of the Bács-Kiskun County Teaching Hospital was consulted, and the study was considered non-interventional and approved.

3.2. THE EXPRESSION OF GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

In this retrospective study, tissue blocks of 100 breast cancer cases were used. Carcinomas ≤2 cm (pT1 tumors) [8, 9], were preferentially included in the study to limit the effect of tumor heterogeneity. Groups of different histological, molecular and clinicopathological types of breast cancer were selected. Histological types included invasive tubular, ductal / NST carcinomas and ILCs as defined by the WHO classification of breast tumors [3]. Grading was performed on the basis of the Nottingham scheme [6]. Molecular types were determined by means of the surrogate IHC-based approach as proposed by the St Gallen consensus meeting report valid at the time of performing the study [13]. On this basis, ER-positive tumors were classified as luminal A if they were HER2-negative and had a Ki-67 LI <14%; they were labeled as luminal B if they were either HER2-positive and/or had a Ki-67 LI >13% or both [84]. ER-negative tumors were classified either as HER2-positive or as TNBC (HER2-negative and PR-negative). Cases with casting-type microcalcification on the mammogram were also included in the study because these tumors have been reported to have an unfavorable outcome by some authors [85-87], and they are considered as a special entity by the multidisciplinary breast team at the University of Szeged. During the analysis of the cases, we observed a consistent and strong staining for GHRH-R in foci of apocrine metaplasia (Figure 3). To investigate this unanticipated phenomenon, we included 31 cases of recently diagnosed carcinomas (of any size) with apocrine differentiation (apocrine carcinomas). For the selection of

apocrine neoplasms, we defined apocrine differentiation by using both histomorphologic [3] and IHC criteria (ER and PR negativity, AR and GCDFP-15 positivity) [16].

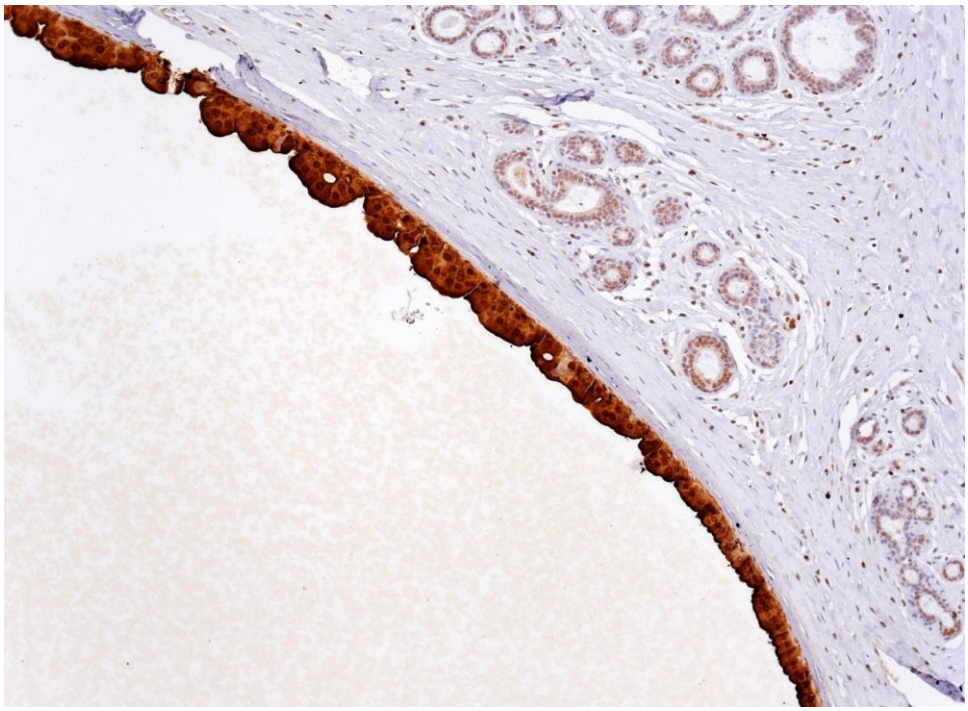

Figure 3. Strong staining for GHRH-R in foci of apocrine metaplasia (GHRH-R, 10x)

FFPE tissue blocks (from the Departments of Szeged, Kecskemét and Turin) were used for the construction of composite TMA blocks. 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 in diameter. Samples were preferentially taken from the periphery of the tumors in every institution. At the Hungarian departments each TMA block included two orientation markers; two cores of non-mammary tissues (liver and kidney) also serving as negative controls, whereas in the TMA block built in Turin, four orientation cores were placed, two pieces of non-apocrine normal mammary tissue as negative controls and two cores of apocrine DCIS as positive controls. Six 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 DCIS 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.)

Metastatic cancer tissues of lymph node positive cases were also evaluated with TMA technique using duplicate cores of 2.2 mm in diameter.

The primary antibodies and IHC protocols used are listed in Table 1. The stains for ER, PR, HER2 and in most cases Ki-67 were performed routinely and the results were available from the original reports. The interpretation of the ER, PR and HER2 staining was according to the American Society of Clinical Oncology/College of American Pathologists guidelines [88, 89]. Every slide included a pituitary gland tissue-chip to serve as positive control. Specimens were evaluated only in the case of adequate staining in the controls. Positive staining of breast cancer tissue was classified according to the localization of immune reaction and percentage of positive tumor cells. On the basis of a previous report, both nuclear and cytoplasmic stainings were accepted as positive [33]. The invasive parenchymal component of the tumors was evaluated, using a lower and higher cutoff level of 10% and 50% of tumor cell positivity.

Statistical analysis was performed with the chi-square test using the SPSS 20.0 (IBM, Armonk, NY, USA) statistical software, and the significance level chosen was $p < 0.05$.

3.3. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

Groups of different histological types of breast lesions documented to demonstrate occasional alteration of MEC phenotype, including benign sclerosing lesions [90], DCIS [91] and adenomyoepithelial lesions were randomly selected on the basis of their diagnoses, and associated normal breast tissue was analyzed.

Randomly selected consecutive TNBCs expressing high molecular weight CK 5, corresponding to a subset of BLBCs on the basis of the surrogate IHC approach described by Nielsen et al [92] were used to build up a double TMA consisting of 20 carcinomas in duplicate cores of 2.2 mm in diameter. The cores were taken from the periphery of the tumors as much as possible to avoid the central necrotic areas often

present in these cancers. Two orientation markers of non-mammary tissues (liver and kidney serving as negative controls) were also included, and at least one of the cores was taken from a part of a tumor including normal lobules or ducts to serve as positive controls for MEC markers. One sample was consistently damaged and uninterpretable on all slides and in both cores, and was therefore excluded from all analyses.

The primary antibodies and protocols used for the IHC are listed in Table 1. The anti-p40 antibody is designed to selectively recognize the p40 isotype, whereas the anti-p63 antibody was developed using an immunogen incorporating the TA domain and is stated to recognize all isotypes of p63 (being a "pan p63" marker) according to the respective manufacturers' data sheets provided with the antibodies.

3.4. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

In this retrospective study, FFPE tissue blocks of 50 apocrine breast lesions were randomly selected including benign, in situ and invasive lesions. The protocol of the anti CD10 IHC used is detailed in Table 1. IHC stainings were carried out on 44 whole tissue sections (thickness: 4–5 μm) and a TMA composite block composed of 2.2 mm diameter cores in duplicate and partial overlap with the whole slide assessment. Statistical calculations were made with GraphPad QuickCalcs (San Diego, California).

4. RESULTS

4.1. GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

99 early breast cancer patients with 100 tumors (one bilateral case) were included in the present study. Cytoplasmic, nuclear, or combined GHRH-R positivity was detected in 54/100 (0.54) and 28/100 (0.28) of the cases using 10% and 50% cut-off values, respectively. Considering the most common histological types, ILCs displayed

GHRH-R positivity significantly more often (10% cut-off: $p = 0.03$; 50% cut-off: $p = 0.0003$ Pearson's chi-square) than ductal/NST carcinomas lumped together with tubular carcinomas (Table 2, Figure 4.). Seven of the ILCs were pleomorphic on the basis of cellular morphology and combined histological grade 3; all but one case were positive for GHRH-R. Positivity of staining according to the histological grade of the tumors is shown in Figure 5. Interestingly, the highest proportion of tumors demonstrating GHRH-R positivity was seen in grade 2 carcinomas, whereas this proportion was lower for grade 1 and grade 3 tumors (Table 2). Statistical analysis of GHRH-R expression in different tumor grades with the Pearson's chi-square test failed to give a significant result ($p = 0.0527$) when using the 10% cut-off, but it was possible to get significant result applying the 50% cut-off level ($p = 0.001$).

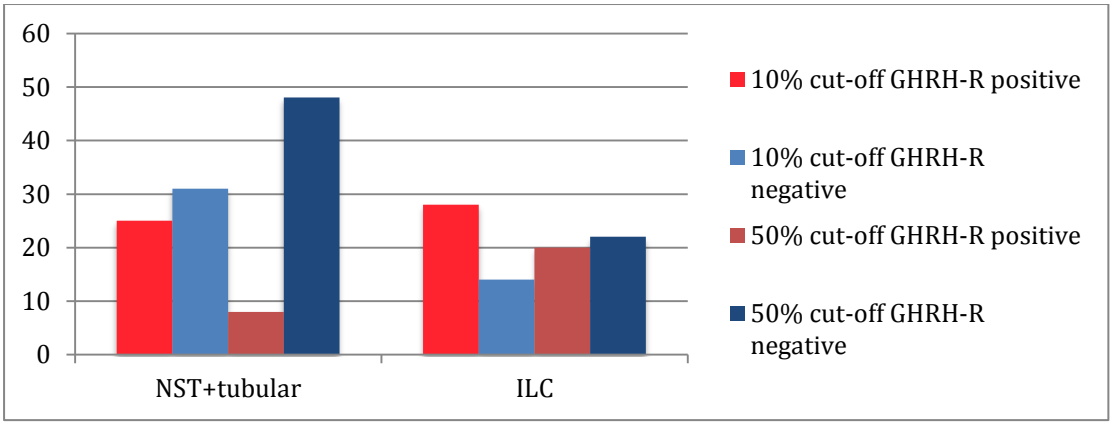

Figure 4. Distribution of GHRH-R-positive and GHRH-R-negative cases according to different cut-off values in different histological types of breast carcinomas.

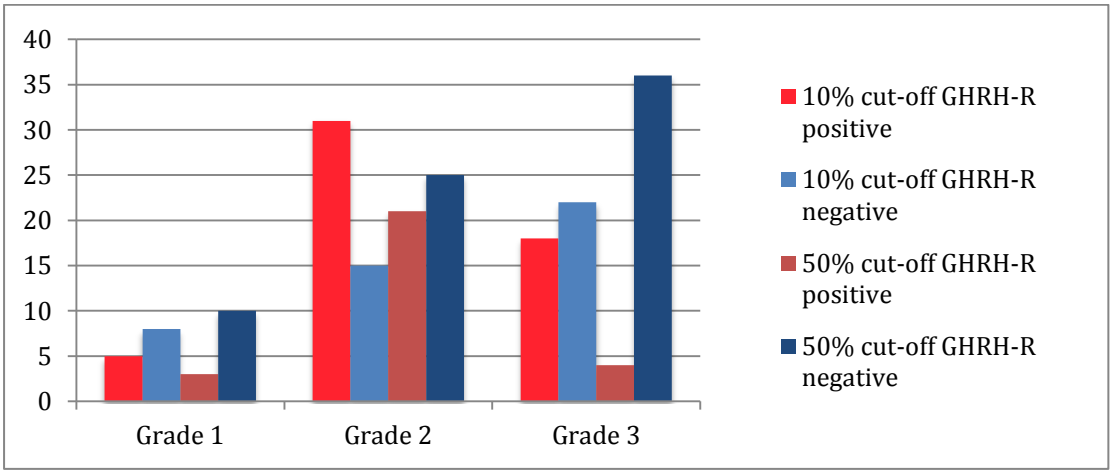

Figure 5. Distribution of GHRH-R-positive and GHRH-R-negative cases according to different cut-off values in different grades of breast carcinomas.

To assess the relation of GHRH-R expression and proliferation, the mitotic score was used, as an ordinal variable standardizing mitotic counts to the area of the high power field of the microscopes, but no association was found. On the other hand, although there was no significant difference in the Ki-67 LI of GHRH-R positive and negative tumors with the 10% cut-off level, statistical analysis using the 50% cut-off yielded a significant difference (10% cut-off: p = 0.0934; 50% cut-off: p = 0.0455; single sample t-test), albeit we need to note that Ki-67 LIs were available in only 70 cases (Figure 6.). There was no statistically significant association between nodal status and GRHR-R staining (10% cut-off: p = 0.167; 50% cut-off: p = 0.332; Pearson's chi-square) (Table 2).

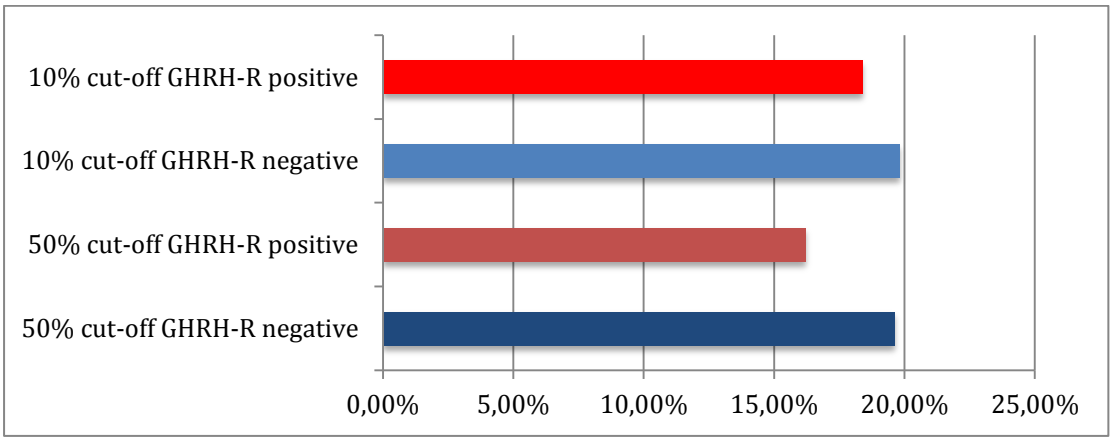

Figure 6. Average Ki-67 LIs (%) of GHRH-R-positive and GHRH-R-negative cases.

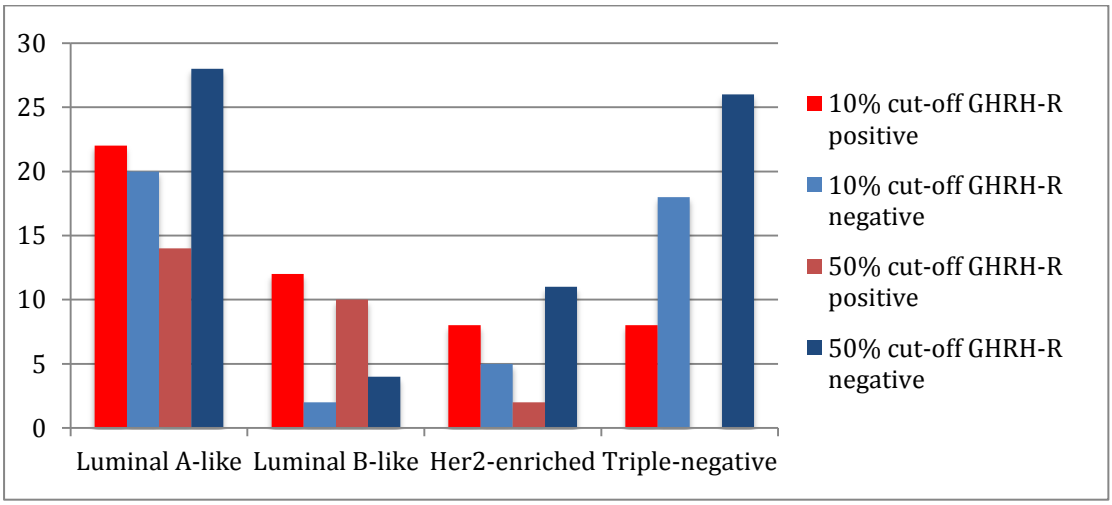

Figure 7. Distribution of GHRH-R-positive and GHRH-R-negative cases according to different cut-off values in different molecular subtypes of breast carcinomas.

As concerns the molecular types according to the IHC based classification (10% cut-off: $p = 0.009$; 50% cut-off: $p = 0.00001$ Pearson's chi-square), the luminal B-like category emerged as the molecular subtype with the highest ratio of positive cases (Figure 7). The only luminal B-like tumor with negative GHRH-R status was a HER2-negative carcinoma with high proliferation rate (Ki-67 LI: 25%). A substantial number (8/26, 31%) of triple-negative cases showed GHRH-R positivity in 10-50 % (average: 25%) of the tumor cells, but there were no cases (except the apocrine carcinomas) exceeding the 50% cut-off level (Table 2, Figure 7).

As a special clinical entity, 12 tumors with casting-type microcalcifications on the mammogram were also included in the study. Although a higher percentage of these cases showed GHRH-R positivity compared to NST carcinomas without casting type calcification (Table 2), the statistical analysis showed no significant correlation (10% cut-off: $p = 0.1092$; 50% cut-off: $p = 0.2030$ Pearson's chi-square). The carcinomas with casting-type calcifications histologically represented ductal/NST carcinomas with high-grade DCIS showing comedo necrosis. These tumors were heterogeneous in terms of hormone receptor (seven were ER-positive and five of these were also PR-positive) and HER2 status (four were positive). The GHRH-R-negative cases belonged to the luminal A (n = 2) or the HER2-enriched (n = 1) types.

All the apocrine carcinomas studied were negative for ER and PR; 21 of them were HER2-negative, 3 were 2+ on IHC and not tested by ISH, whereas 6 tumors were positive for HER2 and one case of apocrine DCIS was not tested for this marker. The striking majority of breast carcinomas with apocrine differentiation (10% cut-off: 97%, 50% cut-off: 90%) showed strong GHRH-R positivity.

Twenty-two previously examined GHRH-R expressing primary node positive tumors were evaluated. Two cases were unavailable for testing as the only metastatic sentinel node was entirely sectioned following the Hungarian recommendation for the work-up of these lymph nodes. Only a single case proved to be totally negative, and 70% (14/20) of the cases showed positivity in more than 10% of the tumor cells, whereas 30% (6/20) in more than 50% of the tumor cells.

Category		Positive/Total No. of cases		Percent of positive cases (95% confidence interval (CI))	
		10% cut-off	50% cut-off	10% cut-off	50% cut-off
Histological type	NST	25/56	8/56	45% (32-58%)	14% (7-26%)
	ILC	28/42	20/42	67% (52-79%)	48% (33-63%)
	Apocrine carcinomas	30/31	28/31	97% (84-99%)	90% (75-97%)
Tumor grade	Grade 1	5/13	3/13	38% (18-64%)	23% (8-50%)
	Grade 2	31/46	21/46	67% (53-79%)	46% (32-60%)
	Grade 3	18/40	4/40	45% (31-60%)	10% (4-23%)
Molecular type	Luminal A-like	22/42	14/42	52% (38-67%)	33% (21-48%)
	Luminal B-like	12/14	10/14	86% (60-96%)	71% (45-89%)
	HER2-enriched	8/13	2/13	62% (36-83%)	15% (4-42%)
	TNBC	8/26	0/26	31% (17-50%)	0% (0-13%)
Lymph node status	Node positive	22/30	11/30	73% (56-86%)	37% (22-54%)
	Node negative	34/60	16/60	57% (44-68%)	27% (17-39%)
Axillary lymph node metastasis of GHRH-R positive primary tumors		14/20	6/20	70% (48-85%)	30% (15-52%)
NST with comedo DCIS and casting microcalcification		9/12	4/12	75% (47-91%)	33% (14-61%)

Table 2. Distribution of GHRH-R-positive and GHRH-R negative cases according to different cut-off values and different clinicopathological groups of breast carcinomas.

4.2. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

Nineteen CK5-expressing TNBCs and thirty-six breast lesions with frequently altered MEC phenotype were included in the present study, and normal breast tissue was also evaluated in each case, where available on the selected slide (n = 31). The analyzed breast lesions were as follows: 10 adenomyoepithelial lesions [including 9 adenomyoepitheliomas (AME) and 1 adenomyoepithelial adenosis], 13 high-grade DCIS with attenuated/flattened MEC layer and 11 sclerosing lesions (including 10 complex sclerosing lesions and 1 complex fibroadenoma with areas of sclerosing adenosis). In all the cases, where appropriate (31/31), a general diffuse strong nuclear p40 positivity was detected in normal terminal ductulolobular units (TDLU) around the lesions. p40 and p63 staining patterns showed no difference in regular TDLUs (Figure 8 a, b).

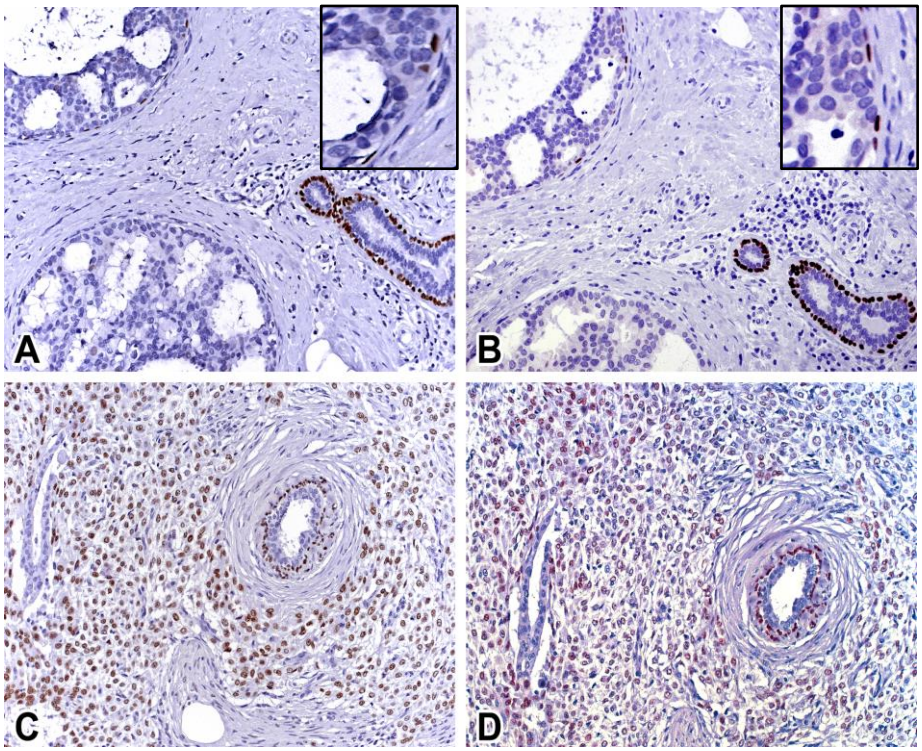

Figure 8. Parallel p40 (A, C) and p63 (B, D) staining patterns of MEC around DCIS and in AME. A, B: DCIS with scant, flattened, IHC-positive MEC in the top left of each panel (insert) and without IHC-positive MEC in the lower left of each panel; note the contrasting strong and diffuse staining of MEC around normal structures (right of each panel). C, D: AME with rather diffuse and strong staining with both markers. (A–D ×40.)

All adenomyoepithelial lesions showed nuclear p40 positivity in the MEC component ranging from weak focal (5/10) to strong diffuse (5/10). No conspicuous difference between p40 and p63 reactivity was noted (Figure 8 c, d). The attenuated/flattened MEC around DCIS showed somewhat weaker nuclear staining compared with surrounding normal TDLU, and negative cells with unequivocal MEC morphology were also detectable (Figure 8 a, b). Rarely, ducts affected by DCIS showing no positivity of the MEC were also recognized. In this set of lesions, MEC stained practically in an identical manner with p40 and p63. In 2 cases, focal positivity (few cells in an ER and PR negative but HER2-overexpressing in situ carcinoma and up to 10% in a triple-negative one) was detected in the epithelial cells of the DCIS using p40 IHC, whereas p63 staining was weaker in the first and barely perceptible (requiring high-power inspection for detection) in the second case. All 11 sclerosing lesions displayed p40 positivity of inconstant intensity, which was usually weaker than in the endogenous normal TDLUs serving as control. Focally negative MEC were also visible in multiple cases. The p63 and p40 reactions were again identical in pattern and intensity (Figure 9).

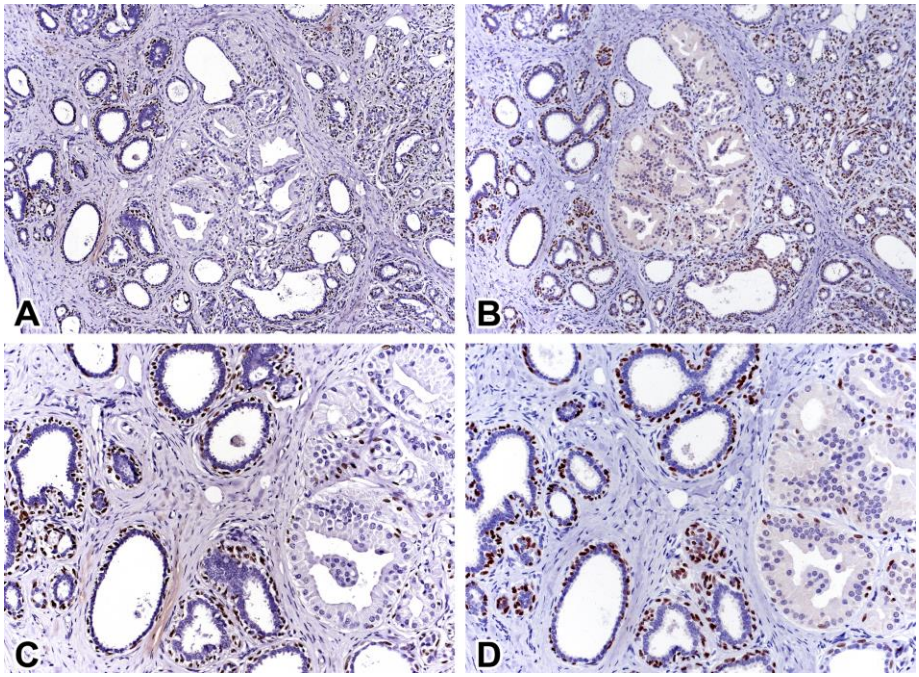

Figure 9. Staining of MEC with p40 (A, C) and p63 (B, D) in sclerosing lesions. MEC are nicely highlighted with the two stains in both the distorted sclerosing glands (A, B: top right area) and the non-distorted glands (elsewhere), but show parallel diminished staining around the centrally located apocrine glands. (A, B ×20; C, D ×40)

Of the 19 CK5-expressing TNBCs, 8 showed some p63 positivity, ranging from a few cells to 70% of the tumor cells. The intensity was generally weak and in many cases detection required scrutinous search; the intensity of staining was strong in 1 case only (Figure 10a). In contrast, p40 positivity could be seen in the majority of the cases (18/19) ranging from a few cells (<1%) to 70%. The intensity was either similar to that seen with p63 or stronger (Figure 10).

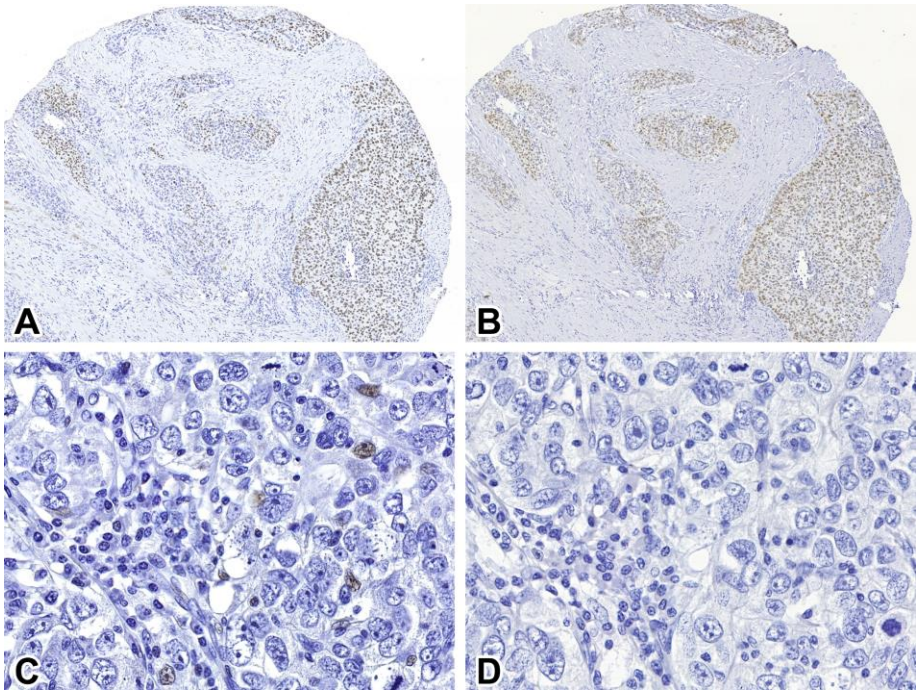

Figure 10. Staining of CK5-expressing TNBC cells with p40 (A, C) and p63 (B, D). A, B: Similar staining in the case demonstrating the most labeled cells and the strongest positivity with both antibodies. Staining of some cells with p40 (C) and lack of staining with p63 (D) in another case. A, B ×5. C, D ×200 (from digital slides).

4.3. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

Fifty apocrine lesions were included in the study: 10 cysts with or without papillary hyperplasia, 1 cyst without an MEC layer [93], 6 apocrine adenoses, 2 papillomas, 13 DCIS, 14 invasive ductal/NST carcinomas and 4 ILCs. 17/19 [0.89; 95% CI 0.68–0.97] benign apocrine lesions (Figure 11) showed complete or partial luminal CD10 staining (Figure 12a), although most cases included parts without staining and 2 lesions (an apocrine adenosis and a cyst with papillary hyperplasia)

were completely negative. The MECs in benign lesions were often but not always positive.

As concerns malignant lesions, 8/13 apocrine DCIS cases displayed no luminal staining (Figure 12/b), but 4 (0.31; 95% CI 0.13–0.58) demonstrated very focal luminal positivity. The MECs around the DCIS showed a spectrum of staining from nil to strong complete. Only 4/18 (0.22; 95% CI 0.09–0.46) invasive carcinomas demonstrated luminal/ membranous staining (Figure 12/c). Cytoplasmic CD10 positivity was seen focally in 4 invasive cancers (Figure 12/d) and in 3 DCIS, and more markedly in 1 invasive carcinoma NST (Figure 12/c); 2 of these and 1 in situ carcinoma with ‘aberrant’ cytoplasmic staining demonstrated no membranous staining. Benign lesions showed luminal/membranous staining more commonly than malignant ones (17/19 vs. 8/31; $p < 0.0001$, chi-square test with Yates correction for continuity) and this was also true for any epithelial staining including aberrant cytoplasmic labeling (17/19 vs. 11/31; $p = 0.0006$, chi-square test with Yates correction for continuity).

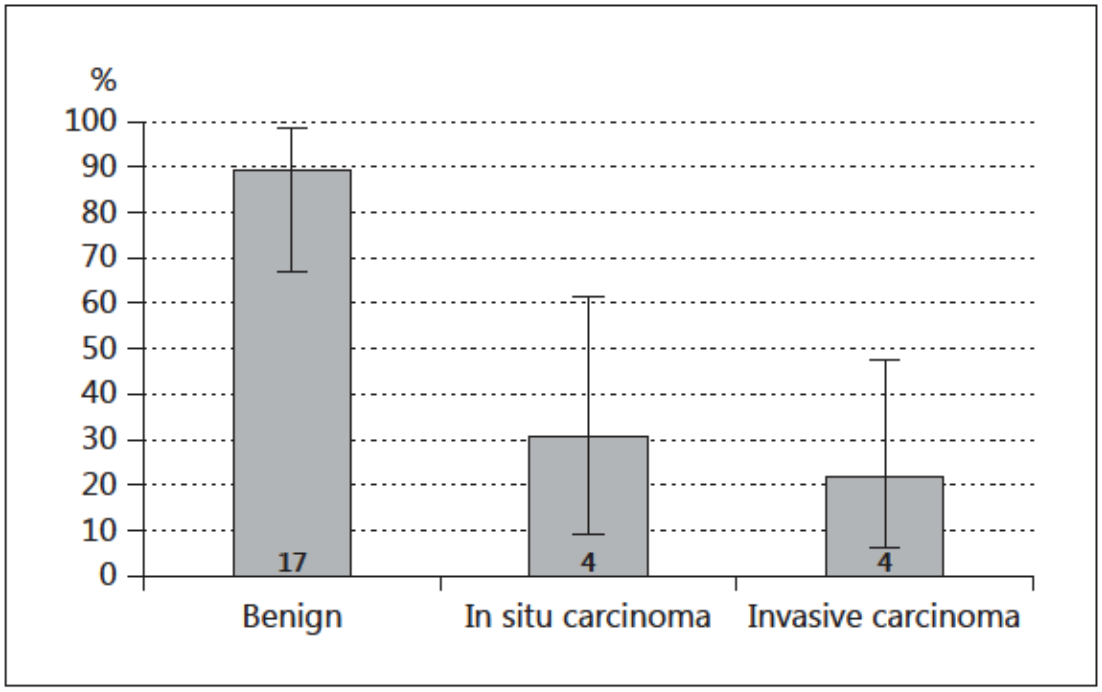

Figure 11. Proportion of benign lesions, in situ and invasive cancers showing luminal/membranous CD10 positivity. The bars represent 95% CIs.

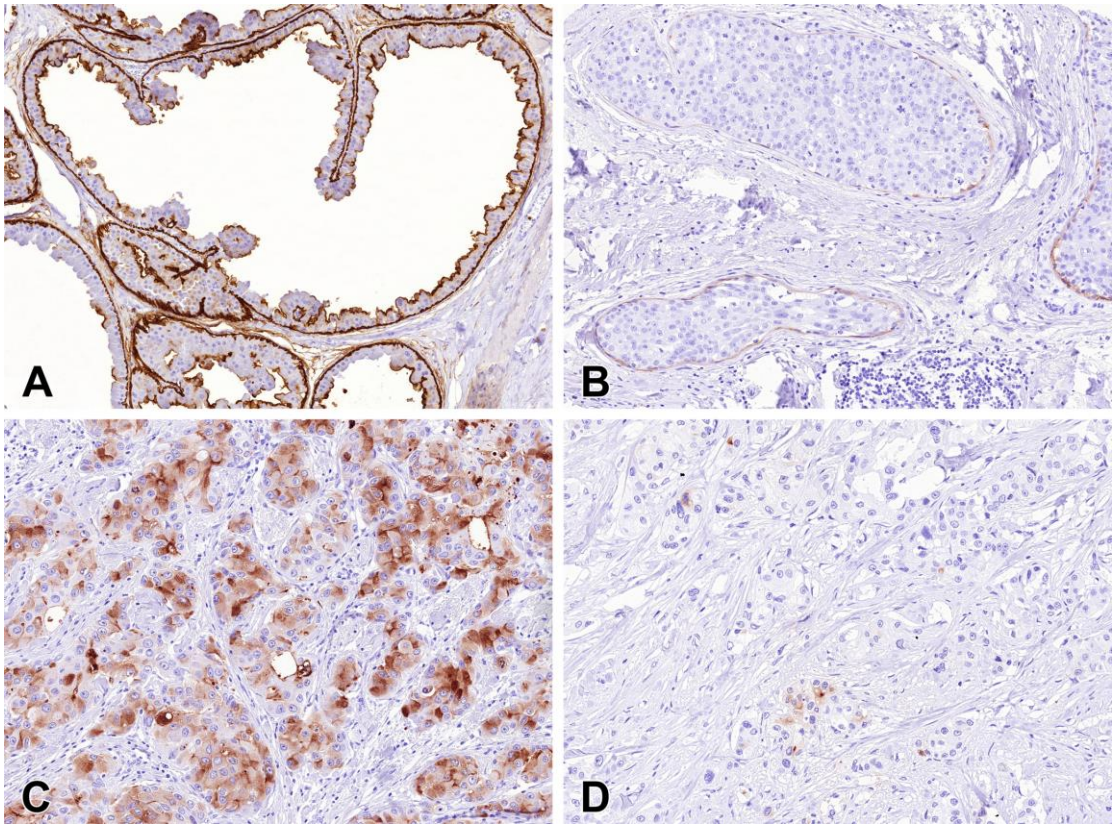

Figure 12. Examples of CD10 positivity in different lesions. **A:** Apocrine cysts with areas of papillary hyperplasia. Note focal to near-complete luminal epithelial and strong MEC positivity. **B:** DCIS with a lack of luminal/membranous staining in foci of lumen formation and weak MEC labeling. **C:** Luminal and strong cytoplasmic staining in invasive carcinoma NST. **D:** Very focal cytoplasmic labeling in invasive carcinoma. (×10)

5. DISCUSSION

5.1. THE EXPRESSION OF GHRH-R IN DIFFERENT TYPES OF BREAST CARCINOMAS

The endocrine effect of GHRH on cancer has been thought to be rendered by the stimulation of the GHRH/GH/IGF-1 axis. Recently, an additional autocrine/paracrine role in the regulation of proliferation and differentiation of cancer cells has been proposed. The latter mechanism is supported by the presence of GHRH in various malignancies as demonstrated by means of mRNA expression, by the detection of immuno-reactive and biologically active GHRH and by the identification

of its receptors in different human cancers [21-24, 32]. GHRH antagonists have been tested as potential targeted therapeutic agents in several malignancies, including breast cancers [26, 30, 31]. The incidence of GHRH-R expression in different breast cancer subtypes (histological, molecular and clinical) has not yet been investigated extensively. Since the presence of the GHRH-R could be a selection criterion for potential treatment targeting the GHRH-R, it was thought that a study identifying potential subsets of tumors preferentially expressing the receptor could be of relevance. To elucidate the possible presence of GHRH-R in the individual subtypes, we selected tissue blocks of different histological and molecular types from our archives, and examined the expression of GHRH-R with IHC. As concerns the different histologic types of breast cancer, there are many based on special features, but the two major types are ductal/NST carcinomas and ILCs. Other subtypes are less frequent, and are sometimes viewed as special types of ductal (non-lobular) carcinomas. In our study, ILCs were significantly more frequently positive for GHRH-R. A previous report has identified ILCs to have a higher rate of GHRH expression (a phenomenon which could support an autocrine/paracrine regulatory effect) than ductal/NST carcinomas. The same study failed to document a similar predilection for the distribution of SV1 using a polyclonal antibody, which is no longer available [25]. In that study, only 1 of 6 ILCs was positive for SV1. The contrary finding that a significant number of ILCs tested were positive for GHRH-R in our analysis, could probably be explained by the use of a different antibody that detects both the pGHRH-R and the SV1 receptor. Since the GHRH-R antibody used in our study was raised against a synthetic peptide derived from the C-terminal domain of the human pGHRH-R [Product datasheet - ab76263], and the biologically active SV1 differs from the full length pituitary receptor only in its N-terminal part [22], the antibody recognizes the full length GRHR-R along with the SV1, but not the much shorter (145-amino acid-long) SV2.

As concerns the grade of differentiation, significant association with the GHRH-R status was just found using the 50% cut-off value, and grade 2 tumors seemed to show GHRH-R positivity more frequently than grade 1 or 3 tumors. Reports on the distribution of GHRH-R by grade are scarce. Chatzistamou et al. suggested no predilection for any level of differentiation: 2/2 of grade 1, 5/16 of grade 2, and 6/22 of grade 3 ductal/NST carcinomas were identified as positive [25]. The reasons for finding more positive cases among grade 2 tumors are not clear, and could

be coincidental, especially in the light of molecular studies. Sotiriou and colleagues have demonstrated that gene expression profile-based genomic grades matched well histological grades 1 and 3, but breast tumors classified as histological grade 2 fell either into the category of low or high genomic grade [94]. Therefore, histologic grade 2 tumors cannot be classified morphologically into high or low grade, resulting in an intermediate prognosis due to this dual composition. Our results, as well as the previous report cited, suggest that GHRH-R positivity can occur in any grade of breast cancer, and there seems to be no strong correlation of GHRH-R expression with histologically determined tumor grade. In keeping with the results relating to the differentiation of the carcinomas, an ambiguous relation was found with proliferation depending on whether assessed by mitotic scores or the Ki-67 proliferation marker. Although there was no association between GHRH-R expression and mitotic scores, a significant correlation was found using the Ki-67 LIs. The significant association of tumor grade with the GHRH-R status using the 50% cut-off and the differences between the statistical analysis of mitotic scores and Ki-67 LIs suggest that the equivocal results may be due to the shortcomings of conventional histological grading, and maybe a stronger correlation could be found using genomic grades. There was no association of GHRH-R expression and the nodal status of breast carcinomas. The study also incorporated 12 cases with casting-type microcalcification on the mammogram. The clinical outcome of this entity is still subject to some debate with some authors and results reinforcing the finding of a poor outcome [85-87, 95-97] and others refuting it [98, 99]. Our experience supports the poor outcome of these tumors [87], and this is why such cases were separately studied for their GHRH-R expression. Although the authors originally describing this entity as one associated with poor prognosis did not specifically report the distribution of this type of carcinoma presentation according to molecular subtypes, they suggested that many of these tumors were HER2-positive [95], with HER2 positivity being three times more frequent in this subgroup than in breast carcinomas without casting-type calcifications [Tot T., personal communication 2013 July]. The present series of small tumors included carcinomas with casting-type microcalcifications with heterogeneous grade and molecular type distribution, and only one-third were HER2-positive. All cases with casting-type calcifications were associated with high-grade DCIS showing comedo necrosis and microcalcification. Using the 50% cut-off, GHRH-R positivity was observed in 33% of the cases of this clinical/mammographical entity, which is

more than double of the 14% positivity rate of ductal/NST carcinomas without casting-type microcalcification; however this difference failed to be statistically significant. The relevance of these findings is not yet known, but further study is warranted to clarify this issue. GHRH-R positivity was seen in all molecular types of breast cancer, including ER-positive and ER-negative cases, in keeping with results found with cell lines [31]. The majority of the luminal B-like tumors demonstrated strong and diffuse immune reaction with anti-GHRH-R, but as even luminal B-like tumors are heterogeneous, the significance of this finding in a relatively low number of cases is uncertain. Even though TNBCs showed GHRH-R positivity in a relatively low percentage of tumor cells (5-30%, average: 15%) and cases (31% using 10% cut-off), the unfavorable prognosis and the limited therapeutic modalities for these carcinomas emphasize the importance of this finding. Targeted anti-GHRH therapy proved to be efficient in the treatment of nude mice transplanted with human TNBC xenografts [100, 101]. An unfortunate observation was the lack of diffuse GHRH-R expression in this molecular group with no cases showing positivity in more than half of the tumor cell population. Further investigations are necessary to clarify whether TNBCs expressing the GHRH-R could be treated with GHRH-R antagonists. Whether the issue of scattered positivity highlights a limited utilisability of a possible anti-GHRH-R treatment or such low positivity rates as in the case of ER or PR could be considered enough should also be investigated in the future.

Regarding metastatic breast cancer, axillary lymph node metastases of the GHRH-R expressing primary node positive tumors were evaluated. The fact that no distant hematogenous metastases were available for testing could be explained by the early stage of the primary carcinomas examined. Although we noticed varying degree of GHRH-R staining decrease of the metastases compared to the primary carcinomas, only a single case showed total loss of GHRH-R expression, which is an important observation if we consider that any future targeted therapy looks more promising if it could also help in advanced cases.

During the analysis of the cases, we noticed a pronounced, uniform GHRH-R expression in cysts showing apocrine metaplasia. This finding inspired us to investigate the expression of GHRH-R in cancers showing apocrine differentiation and to include 31 cases of apocrine carcinoma (both in situ and invasive tumors). With 10% cut-off, 97% demonstrated strong and diffuse positivity, whereas using 50% cut-off, 90% were found positive. Apocrine carcinomas are defined as

carcinomas in which the cells demonstrate the cytological features of apocrine cells [3], they are often ER-negative and PR-negative, but AR-positive tumors [16], and express apocrine markers like GCDFP-15. As concerns the molecular types approached by IHC, somewhat more than half of apocrine carcinomas represent a subgroup of TNBCs and nearly half of them overexpress HER2 [17]. A molecular apocrine type of breast cancer with increased androgen signaling has also been described, and is characterized by ER negativity and AR positivity [3, 18, 102, 103]. This latter type is lately referred to as the LAR subtype of TNBC [19]. The overlap between breast cancers classified as apocrine on the basis of gene expression profile versus morphologic features is not complete. It has been estimated that the apocrine gene expression profile may be present in 8–14% of breast cancers, whereas apocrine carcinomas classified on the basis of morphologic appearance are relatively rare, comprising about 4% of breast carcinomas [3]; part of them may be a subset of TNBCs. Their androgen-dependent signaling pathway could also suggest a specific treatment. Whether their homogeneous positivity for GHRH-R can be translated to a targeted therapy with GHRH-R antagonists, which are under development for clinical use requires further studies.

As a caveat, it must be remembered that the present study included non-consecutively diagnosed breast carcinomas, and therefore the proportion of positive tumors may only be an estimate, requiring confirmation on a larger group of tumors. A strength, however, is that we chose to limit tumor heterogeneity by studying relatively small cancers.

5.2. p40 EXPRESSION IN BASAL-LIKE BREAST CARCINOMAS AND p40 AS A MYOEPITHELIAL MARKER IN BREAST LESIONS

The identification of an outer MEC layer is a valuable clue in the differential diagnosis of breast lesions. A broad spectrum of different cytoplasmic (e.g. smooth muscle actin (SMA), smooth muscle myosin heavy chain (SMMHC), calponin, S100 or CK5/6), nuclear (e.g. S100 and p63) and membranous (e.g. CD10) MEC markers is used by reporting pathologists. p63 protein is a commonly used MEC marker. Due to its high sensitivity (90%) and even superior specificity (up to 100% in normal TDLU) reported [45], it is preferred to cytoplasmic markers (SMA, calponin and SMMHC),

as the latter may also variably react with vascular smooth muscle cells and myofibroblasts [48]. The specificity of p63 is somewhat diminished by the observation that it is rarely expressed by tumor cells of NST carcinomas [48]. Reduced expression of markers in MEC associated with DCIS and complex sclerosing lesions is a documented phenomenon [90, 91], and such an alteration in the MEC phenotype has also been reported in AME [104]. Some markers (CD10, CK5/6 and SMMHC) show reduced expression more frequently than others (p63, SMA and calponin) [90, 91].

This study specifically focused on lesions that have been reported to demonstrate an altered MEC phenotype, i.e. a change in the expression of MEC markers compared with MEC of the normal breast parenchyma: sclerosing lesions [90], high-grade DCIS [91] and adenomyoepithelial lesions [104]. Our results suggest that p63 and p40 perform similarly in all these settings. In normal breast tissue, MEC are nicely highlighted by both antibodies, and when the expression of one is reduced in a pathological condition, the other shows a similar reduction in expression; focal losses of expression occurred in parallel. Although p40 has been reported to have superior specificity than p63 as a squamous cell carcinoma marker in the differential diagnosis of non-small cell lung cancer [49], it seems to perform similarly in breast lesions acknowledged to show altered expression of MEC markers. It is, therefore, suggested that both antibodies can be used interchangeably for the demonstration of MEC. A recent study performing a TMA analysis of a larger number of breast lesions [32 adenoses, 34 intraductal papillomas, 31 DCIS, 257 ductal/NST carcinomas and 36 metaplastic carcinomas] with monoclonal pan-p63 and polyclonal p40 antibodies from different sources (but with an identical clone and identical target epitopes, respectively) reached a similar conclusion regarding the corresponding staining of MEC with p63 and p40 antibodies [105]. However, despite the identical epitopes used to generate the polyclonal p40 antibodies, one of the two tested (Diagnostic Biosystems, Pleasanton, Calif., USA) showed a much higher proportion of cancer cell labeling, highlighting the different sensitivities and specificities of the two antisera. This latter antibody was also less specific for MEC in DCIS. On the basis of the surrogate IHC based molecular classification, the highest proportion of p63 or p40 staining was found in TNBCs [105]. Identical staining of MECs with monoclonal p63 and p40 antibodies was also reported in a small series of 10 breast excision specimens [106].

The fact that the p63 antibody we used (a pan-p63 marker) should theoretically label more cases than the p40 antibody identifying only the ΔNp63 isoforms makes our finding of a higher rate of cancer cell labeling with p40 somewhat enigmatic. The explanation may perhaps lie in the differences in sensitivities alluded to in the previous paragraph in connection with two polyclonal p40 antibodies. However, the small sample size, a limitation of the present study, may also play a role.

In conclusion, p40 seems to be similar to p63 as an MEC marker both in normal breast tissue and in lesions with observed alterations in the MEC immunophenotype. The presence of tumor cell positivity in NST carcinomas demonstrating an IHC staining profile mostly in keeping with a BLBC did not interfere with MEC detection but should be acknowledged, and the preference of p40 for highlighting this subset of carcinomas rather than other subtypes should be further investigated.

The molecular subtype of breast cancer carries valuable information and can help to predict prognosis and determine the appropriate therapy. As long as determination of molecular subtypes based on gene expression profiling is not yet available in routine histopathology practice, surrogate IHC methods are expansively used for the molecular classification of the cases. Using the IHC based method, BLBC is defined as an ER, PR and HER2 negative tumor expressing proteins usually found in basal/ myoepithelial cells of the normal breast. Although CK5 and EGFR are the most frequently used and accepted, other markers e.g. high-molecular-weight cytokeratins as CK6, CK14 and CK17, P-cadherin, CD117, nestin, p16 and p53 [107] can also be used alone or as a part of an IHC panel [108]. As concerns the p53 tumor suppressor gene family, both p53 and p63 expression can be used as markers of basal phenotype. The anti-p53 antibody has a specificity of 80-85% and a sensitivity of 50-60% [109, 110], whereas the detection of the p63 protein expression is reported to have a very high specificity (94%), but low sensitivity (14%) [108]. The anti-p63 antibody is also useful in the diagnosis of metaplastic breast cancer particularly in the case of the squamous/spindle cell variant [111, 112]. A relatively newly discovered member of the p53 gene family, the p40 isoform of the p63 protein, was recently introduced as a commercially available antibody and was not previously tested in BLBC. One obvious difference between the staining patterns of p63 and p40 is the different proportion of focal positivity in carcinoma cells. CK5-expressing TNBCs

seem to express p40 more frequently than p63. Whether this phenomenon is restricted or preferential in BLBCs expressing CK5 has not been examined, and is the subject of an ongoing investigation, but one of the DCIS cases showing a few p40- and p63-positive cells was a HER2-overexpressing lesion.

5.3. CD10 EXPRESSION IN APOCRINE LESIONS OF THE BREAST

The fact that CD10 is a ubiquitous enzyme found on the surface of many different normal cell types and pathologic lesions has a negative impact on its specificity and thus on its possible utility in routine histopathological differential diagnosis. Therefore, CD10 IHC reactions should be only used to answer specific differential diagnostic questions in well-known circumstances.

Overall, breast epithelium rarely expresses CD10. Only focal labeling of luminal ductal epithelium was reported by Kalof et al. [81]; the limited number of invasive and in situ carcinomas (n = 46) that they studied were all negative. Bains and Sidhu [113] reported on a case of invasive breast carcinoma, showing cytoplasmic CD10 staining associated with an in situ component and intraductal papilloma, demonstrating the same type of labeling. Although no mention of receptor status was included in their description, on the basis of the figures, none of these lesions demonstrated the characteristic apocrine morphology. The authors concluded that CD10 positivity in metastatic tumors cannot rule out the breast as primary, and related the phenotype to the CD10-positive progenitor cells capable of differentiating towards luminal epithelial cells and MECs described by Stingl et al. [114]. NST carcinomas and ILCs are rarely positive for CD10 [83], but some subsets may be different in this respect: of 40 ER-positive tumors, none demonstrated CD10 positivity (defined with a cut-off of 10% staining) and only a single case showed <10% labeling, whereas 12 of 77 ER-negative carcinomas (16%) showed cytoplasmic or membranous staining in 30–100% of the cells [115]. A subset of ER-negative breast cancers is also negative for PR and HER2, and is therefore labeled as TNBC. Some TNBCs express basal (i.e. MEC) markers (CK 5 and/or EGFR) and this feature has been suggested for the delineation of the BLBC subgroup of breast cancers on IHC [92]. Not surprisingly, some of these carcinomas may also express CD10, an MEC marker in a substantial number of cases (16/20 of spindle-cell metaplastic carcinomas and carcinosarcomas) [116], similarly to the rare cases that demonstrate straightforward myoepithelial

differentiation [117]. Apocrine carcinomas are also generally ER- and PR-negative [118], and might have been included in previous studies of ER-negative carcinomas, but without distinct identification of this subset. Smollich et al. [119] identified cytoplasmic (and occasional membranous) CD10 (neprilysin) staining of tumor parenchymal (epithelial) cells in 33/126 (26%) of breast cancers and found this labeling to be associated with better prognosis, in contrast to the CD10 staining of the stromal myofibroblast reported to indicate worse prognosis.

CD10 positivity has been described in benign apocrine epithelium [81, 83], but no data on CD10 expression in various other types of apocrine breast lesions have been available until now. Our results indicate that benign apocrine epithelium (metaplasia) is typically positive for CD10 with a luminal staining pattern, although there are exceptions to the rule. Malignization or apocrine differentiation in malignant lesions seems to be associated with a partial or complete loss of this staining pattern, which is therefore rarer in in situ carcinomas and even rarer in invasive ones, and cytoplasmic (aberrant) staining may also occur in this subset.

Although the staining of MECs was not the primary aim of our study, our findings are in keeping with earlier works on the subject, and suggest that the sensitivity of CD10 as an MEC marker is lower than that of other markers like p40 and SMA. Its proportional sensitivity is even further diminished in certain lesions like benign sclerosing lesions [90] and DCIS [91], known for their reduced expression of MEC markers. Based on the literature [81, 91] and supported by our experience, CD10 is not an ideal MEC marker. However, occasional CD10 staining of epithelial cells should be kept in mind, as its occurrence may interfere with the identification of some cells as epithelial or myoepithelial, especially in apocrine lesions, some of which may turn out to be benign, even without the presence of a MEC layer [93].

6. CONCLUSIONS

Our work demonstrates that the distribution of GHRH-R among breast carcinomas is not restricted to histological type, differentiation grades or molecular subtypes. ILCs were found to express this marker more frequently than ductal/NST carcinomas. The finding of a relatively high proportion of positivity among ductal/NST carcinomas with casting-type microcalcification is of uncertain

significance. Even though TNBCs showed GHRH-R positivity in a relatively low percentage of tumor cells (5- 30%, average: 15%) and cases (31% using 10% cut-off), with no cases showing positivity in more than half of the tumor cell population, the unfavorable prognosis and the limited therapeutic modalities available for these patients highlight the importance of this finding, which is further emphasized by the fact that targeted anti-GHRH-R therapy proved to be efficient in the treatment of nude mice transplanted with human TNBC xenografts. The most remarkable finding of this study, we feel, is that apocrine epithelium (both benign and malignant) stains diffusely and strongly for GHRH-R. However, the genuinity of our related results require confirmation, and we have started further investigation of apocrine tumors by courtesy of a grant sponsored by the University of Szeged.

Whether our findings can be used for targeting breast carcinomas with GHRH-R antagonists is to be clarified in future studies.

The p40 protein seems to be similar to p63 as a MEC marker both in normal breast tissue and in lesions with observed alterations in the MEC immunophenotype. The presence of tumor cell positivity in NST carcinomas demonstrating an IHC staining profile mostly in keeping with a BLBC did not interfere with MEC detection but should be acknowledged, and the preference of p40 for highlighting this subset of carcinomas rather than other subtypes should be further investigated.

CD10 positivity is luminal/membranous in most benign apocrine lesions, the staining being non-universal and sometimes focal. Analogous staining in apocrine malignancies seems rarer in DCIS and even rarer in invasive apocrine carcinomas, but atypical cytoplasmic positivity may also occur. CD10 is not an ideal MEC marker in apocrine lesions. When using CD10 immunohistochemistry as a MEC marker or in the case of a carcinoma of unknown primary it should be important to know that benign and malignant apocrine lesions of the breast can also express CD10. The fact that CD10 is a ubiquitous enzyme found on the surface of many different normal cell types and pathologic lesions has a negative impact on its specificity and thus on its possible utility in routine histopathological differential diagnosis. Therefore, CD10 IHC reactions should be only used to answer specific differential diagnostic questions in well-known circumstances.

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