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## Immunohistochemistry of the new molecular classification of breast carcinoma and its prognostic and therapeutic implications

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**Ключові слова:** рак молочної залози, імуногістохімічна діагностика, прогноз.

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### Иммуногистохимическое обоснование новой молекулярной классификации рака молочной железы, ее прогностическое и терапевтическое значение

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В последние годы в научной печати появились многочисленные данные о молекулярной биологии рака молочной железы. В связи с этим, гистологическая классификация ВОЗ (2004) оказалась устаревшей. Сегодня большинство сведений о прогнозе, лечении и клиническом исходе заболевания основано на результатах молекулярных и биологических исследований последних лет. Рассмотрены современные подходы к вопросам гистопатологической диагностики рака молочной железы на основе новых технологий. Обсуждены рекомендации по иммуногистохимическому тестированию рецепторов эстрогенов и прогестерона, а также рецепторов к эпидермальному фактору роста 2 (HERB2) в образцах опухолевой ткани. Предложен алгоритм оценки полученных данных, включая положительные, спорные и отрицательные результаты, касающиеся экспрессии белков HERB2 и амплификации гена. На основе характера экспрессии генов, изученного по технологии микрочипов с последующей иерархической кластеризацией РНК нативной ткани, выделены новые генетические профили опухоли. Показана возможность применения методов иммуногистохимии в тканях, заключенных в парафин. Рекомендована иммуногистохимическая панель для диагностики следующих типов рака молочной железы: люминальный А (Luminal A), люминальный В (Luminal B), базальноподобный и HER2/neu. Заключительная часть работы посвящена вариантам рака молочной железы с генетической восприимчивостью к BRCA1 и 2. Такие опухоли крайне агрессивны, характеризуются высокой степенью злокачественности и имеют неблагоприятный прогноз. Проанализированы новые подходы к терапии заболевания.

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The amount of information regarding breast cancer has increased spectacularly in recent years. In addition to the well-known classification of the histological types provided by the WHO (2004) and other anatomic pathological considerations (sentinel lymph node) on the biology of this disease, numerous references have appeared regarding new tests in which several molecular biological studies are considered, such as expression array analysis, that have provided more precise information on the behaviour and clinical outcome of the disease.

Two principal subjects have been proposed in relation to the diagnosis, prognosis, response to therapy and clinical outcome both in early stages and with metastatic disease: the presence of Estrogen and Progesterone Receptors in the tumour cells, and the over expression of the human epidermal growth factor receptor 2 gene ERBB2 (commonly referred to as HER2).

Due to this situation, the American Society of Clinical Oncology (ASCO) and the College of American Pathologists (CAP) in 1988 decided to pursue a study as to whether guidelines would be necessary and beneficial for patients with breast cancer. Thus a panel of experts were convened to address this issue and document evidence based on previous opinions and publications. The recommendations for both issues are summarized as follows:

*Recommendations for Immunohistochemical Testing of*

*Estrogen and Progesterone Receptors in Breast Cancer* [2]. The document includes sections dealing with ER physiology and measurement and discussion of the current issues related to ER and PgR testing for patients with breast cancer. The Panel recommends that ER and PgR status be determined on all invasive breast cancers and breast cancer recurrences. It is recommended that ER and PgR assays be considered positive if there are at least 1% positive tumor nuclei in the sample on testing in the presence of expected reactivity of internal (normal epithelial elements) and external controls. The absence of benefit from endocrine therapy for women with ER-negative invasive breast cancers has been confirmed in large overviews of randomized clinical trials (Stendahl M, 2006, Viale et al., 2008).

Recommendations for Human Epidermal Growth Factor Receptor 2 Testing in Breast Cancer [3,4]. The human epidermal growth factor receptor 2 gene ERBB2 (commonly referred to as HER2) is amplified in approximately 18% to 20% of breast cancers. Amplification is the primary mechanism of HER2 overexpression and is associated with clinical outcomes in patients with breast cancer. HER2 positivity is associated with worse prognosis (higher rate of recurrence and mortality) in patients with newly-diagnosed breast cancer who do not receive any adjuvant systemic therapy. HER2 status is also predictive for several systemic therapies. In this regard, HER2 positivity appears to be associated with

relative, but not absolute, resistance to endocrine therapies in general (Herceptin Adjuvant HERA Trial Study Team 2006).

HER2 status also appears to be predictive for either resistance or sensitivity to different types of chemotherapeutic agents. Most importantly, several studies have now shown that agents that target HER2 are effective in both the metastatic and adjuvant settings. Trastuzumab (Herceptin; Genentech, South San Francisco, CA), a humanized monoclonal antibody, improves response rates, time to progression, and even survival when used alone or added to chemotherapy in metastatic breast cancer. Trastuzumab is also active as a single agent and was approved in 1998 by the US Food and Drug Administration for the treatment of metastatic disease [5,6,7].

Several prospective randomized clinical trials have demonstrated that adjuvant trastuzumab reduces the risk of recurrence and mortality by one half and one third, respectively, in patients with early-stage breast cancer. Furthermore, recently reported results suggest that a small molecule dual HER1/HER2 tyrosine kinase inhibitor of HER2 tyrosine kinase activity, lapatinib (Tykerb, GlaxoSmithKline, Philadelphia, PA) [8], improves clinical outcome in patients with advanced disease when added to capecitabine. Taken together, these results imply that HER2 is a useful marker for therapeutic decision making for patients with breast cancer, and the authors emphasize the importance of evaluating the assay accurately.

Trastuzumab therapy is not without its drawbacks: currently adjuvant trastuzumab is recommended for 12 months. The drug cost of 52 weeks of trastuzumab in the United States is approximately \$100,000. In addition, there is a requirement for 9 to 12 months of intravenous therapy after completion of adjuvant chemotherapy. Importantly, trastuzumab is associated with a small risk of serious cardiac toxicity.

The panel recommends that HER2 status should be determined for all invasive breast cancer<sup>3</sup>. An algorithm defining positive, equivocal, and negative values for both HER2 protein expression and gene amplification is recommended: a positive HER2 result is IHC staining of 3+ (uniform, intense membrane staining of > 30% of invasive tumor cells), a fluorescent in situ hybridization (FISH) result of more than six *HER2* gene copies per nucleus or a FISH ratio (*HER2* gene signals to chromosome 17 signals) of more than 2.2; a negative result is an IHC staining of 0 or 1+, a FISH result of less than 4.0 *HER2* gene copies per nucleus, or FISH ratio of less than 1.8. Equivocal results require additional action for final determination. It is recommended that to perform HER2 testing, laboratories show 95% concordance with another validated test for positive and negative assay values. The panel strongly recommends validation of laboratory assay or modifications, use of standardized operating procedures, and compliance with new testing criteria to be monitored with the use of stringent laboratory accreditation standards, proficiency testing, and competency assessment<sup>3</sup>.

#### *Molecular studies for a new approach to the classification of breast cancer*

In this context several microarray gene expressions, analysed by hierarchical clustering with fresh tissue RNA, have offered new genetic profiling of breast carcinomas. This technology has provided new subclassifications, dividing breast carcinomas into groups that facilitate a more precise prognostic and therapeutical approach. Nonetheless, the practical use of microarray technology, is at present almost impossible in daily routine, not only because of the high cost, but also due to other technical problems such as the need to have fresh tissue available for the study [9,10].

Nevertheless, these advances have provided seminal information that allows the application of more accessible and cheaper methods such as immunohistochemistry in paraffin-embedded tissues together with tissue microarray technology. Thanks to these two techniques, large series of tumors can be tested in single slides with a particular antibody, not only retrospectively, but also in prospective studies. Using these methods, carcinoma of the breast has been reclassified, not only based on the histology, but also supported by the positive or negative expression of a number of particular proteins that possess clinical, prognostic or therapeutic relevance. At present, several retrospective clinical analyses have validated this classification and new studies, which are underway, will combine the microarray gene expression analysis with this methodology, providing a better and more comprehensive view of the biology of breast cancer [11].

At present four major types of breast carcinoma are accepted, and at least two more subtypes have been proposed<sup>12</sup>. These types are known as: Luminal A, Luminal B, Basal-like and HER2/neu, all identified using a four-marker immunopanel: ER status, PR status, HER2/neu and Ki-67 proliferation index. The addition of CK 5/6 and EGFR allows the subclassification of the Basal-like subtypes in a triple negative and in a core basal phenotype. Moreover an «Apo-crine» (AR status and GCDP-15) close to the HER2/neu and a «Claudin 1 low» stem-cell like, phenotype has been proposed. Although this immunohistochemical-molecular classification has attracted wide interest the validation at clinical level is still in progress.

The *Luminal A subtype* expresses ER and PR positivity while HER2/neu is negative, and displays a low proliferative index (KI-67) (*Table and Figure 1*). This is the most common tumor in breast mimicking normal luminal cells (positivity for luminal low-weight cytokeratins 8/18) and genes associated with an active ER pathway. Histologically it corresponds mainly to low-grade carcinomas, such as low grade ductal, tubular, cribriform and lobular carcinoma of the WHO classification<sup>1</sup>, and therefore presents low clinical stages and favorable prognosis.

*Table 1*

#### **Luminal A pattern. Clinical and morphologic findings**

- RE+, RP+ but HER2/neu negative
- Low proliferative index (KI67-).
- Low molecular weight CKs (8/18) positive
- Histology of low-grade carcinoma (low-grade ductal, tubular, cribriform mixed or lobular).
- Low clinical stage
- Good clinical outcome

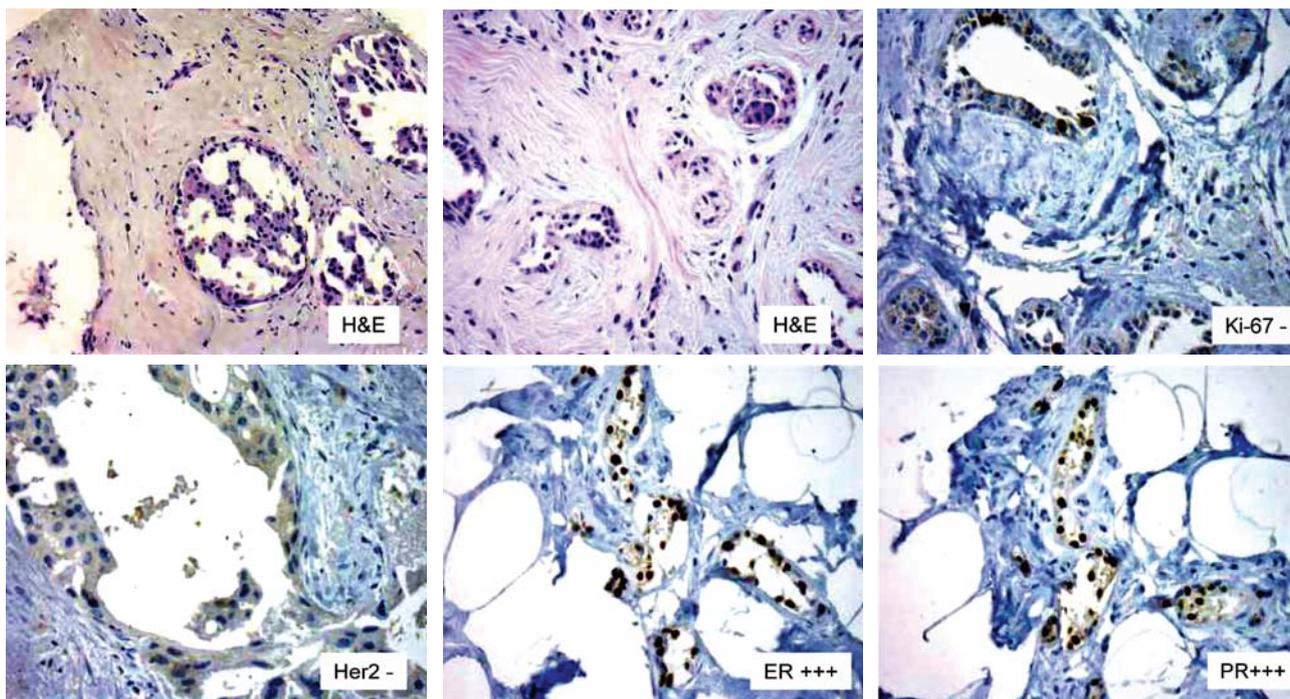


Fig. 1. Ductal-papillary breast carcinoma, luminal A.

The *Luminal B subtype* is the second more frequent breast tumor. It expresses ER but PR status is low or negative. HER2/neu is negative (Table and Figure 2), while the Ki-67 proliferative index is high. It is also constituted by derivatives of normal luminal cells (positivity for low weight cytokeratins 8/18) and has activated ER gene pathways, but simultaneously shows p53 mutations. The histological counterparts are mainly high grade ductal, NOS and micropapillary carcinomas. The clinical outcome and prognosis is worse than luminal type A, but it presents a good response to chemotherapy (TAC or FAC) and to hormonal control. The clinical stages may be more advanced (stages II and III).

Table 2

**Luminal B pattern. Clinical and morphologic findings**

- Second most frequent subtype of breast cancer.
- RE++, RP +/- but HER2/neu negative
- High Ki-67 expression (++)
- Positivity for low molecular weight Cks (8/18)
- p53 mutated and positive expressed by IMH.
- High-grade ductal carcinoma, carcinoma NOS or micropapillary type.
- More unfavorable prognosis than luminal A, but good response to chemotherapy and to hormonal control.
- Diagnosed at advance clinical stages (II or III)

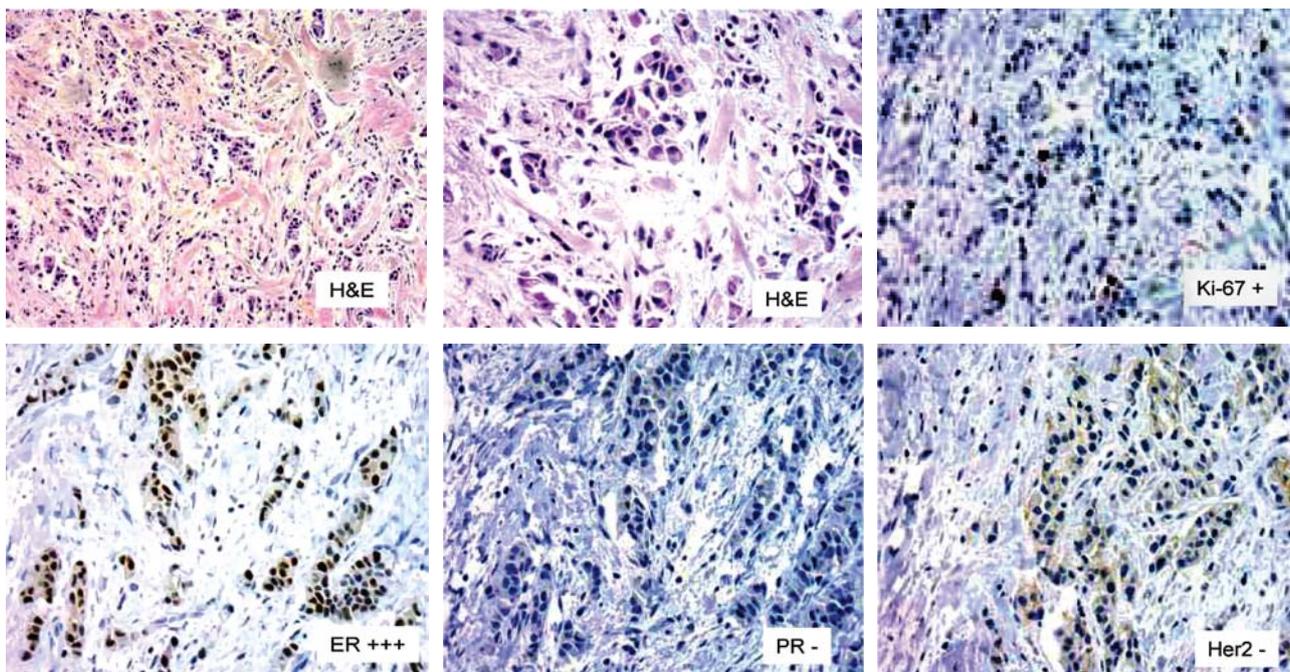


Fig. 2. Ductal-NOS breast carcinoma, luminal B.

Table 3

Basal-like subtypes including triple-negative, are more infrequent tumors (around 15% of breast carcinomas correspond to this category) but they show a possible subdivision having prognostic implications [13,14]. All basal-like carcinomas were characterized because the positivity for basal high-weight cytokeratins and specific myoepithelial cells markers (CK5/6, CK17, Caveolin1, Calponin1, P63) simultaneously lack RE, PR and HER2/neu expression (triple negative) while the Ki-67 is high, suffering also p53 mutations and DNA repair defects. There is controversy regarding these groups of tumors because not all triple negative are genetically basal-like and not all basal-like genetically-confirmed tumors display triple negative features [15].

In addition, a group of basal-like carcinomas expresses EGFR and C-KIT positivity. This last variety would display additional worse prognosis when compared to the already-known unfavorable clinical outcome and poor response to therapy of the basal-like category, which includes medullary, adenoid cystic and metaplastic carcinoma of the breast, as well a small subgroup of high grade NOS of the WHO classification. In addition, this category is more frequent in BRCA1 germline mutation carriers. For some authors, a triple negative with additional negativity for CK5/6 and EGFR should be considered as unclassified tumors awaiting further information [11].

For Foulkes et al [15], 2010 triple-negative breast cancer is a tumor characterized by lack of estrogen receptor (ER), progesterone receptor (PR), and HER2 expression (Table and Figure 3). Some investigators accept tumors as being negative for expression of ER or PR only if less than 1% of the cells are positive for ER or PR expression; others consider tumors to be negative for ER and PR when up to 10% of cells are positive for expression.

**Triple Negative type. Clinical and morphologic findings**

- Basal-Like type
- 15% of Breast Carcinoma. Any histology > Ductal NOS
- Positivity for high-weight molecular CKs and for myoepithelial markers (CK5/6, CK17, Caveolin1, Calponin1, P63)
- Triple negative for RE -, RP - and Her2 neu -
- High proliferative Index Ki-67 ++,
- P53 + (gene mutations)
- BRCA1 mutations
- Poor clinical outcome
- Triple negative tumors with CK5/6 - or EGFR - have been considered as not classifiable.

The HER2/neu subtype comprises carcinomas with definite positivity for immunostaining with this antibody (clone DAKO, 3+) and confirmed with FISH or CRIST analysis. These tumors may belong to the luminal B type, but a large number correspond to the category of ER and PR negative tumors with a high Ki-67 positivity (Table and Figure 4) and occasional low CK 5/6 expression. They are very aggressive high-grade ductal NOS carcinomas, nevertheless they respond well to the humanized monoclonal antibodies against HER2: HER2 tyrosine kinase inhibitors (Trastuzumab, Ipatinib).

Table 4

**Her2 neu positive-type. Clinical and morphologic findings**

- HER2/neu positive by IHC (clone DAKO, +++) or positive ++ but confirmed by FISH (Her2 amplification)
- Usually RE- and RP-
- High proliferative index Ki-67++
- Occasional positivity for CK 5/6
- High-grade Ductal-NOS Carcinoma with poor clinical behavior
- Good clinical control with anti-Her2 therapy (trastuzumab).

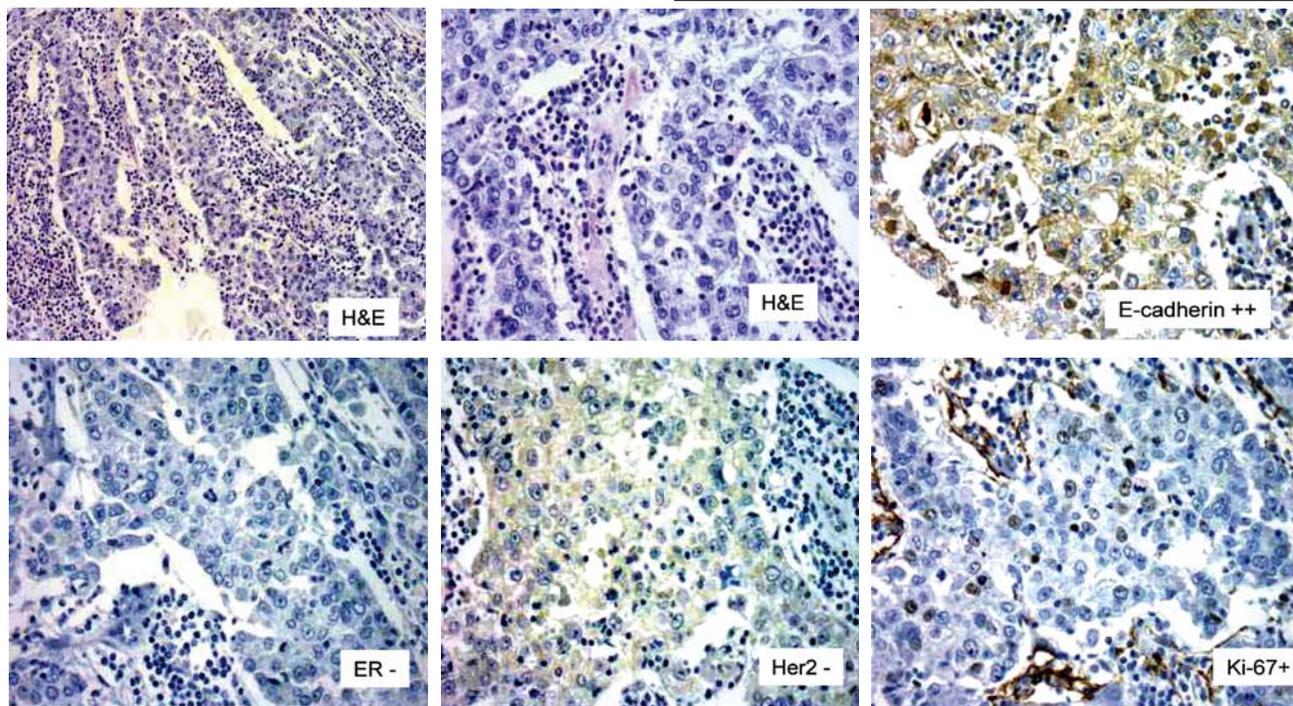


Fig. 3. Medullary breast carcinoma, triple negative.

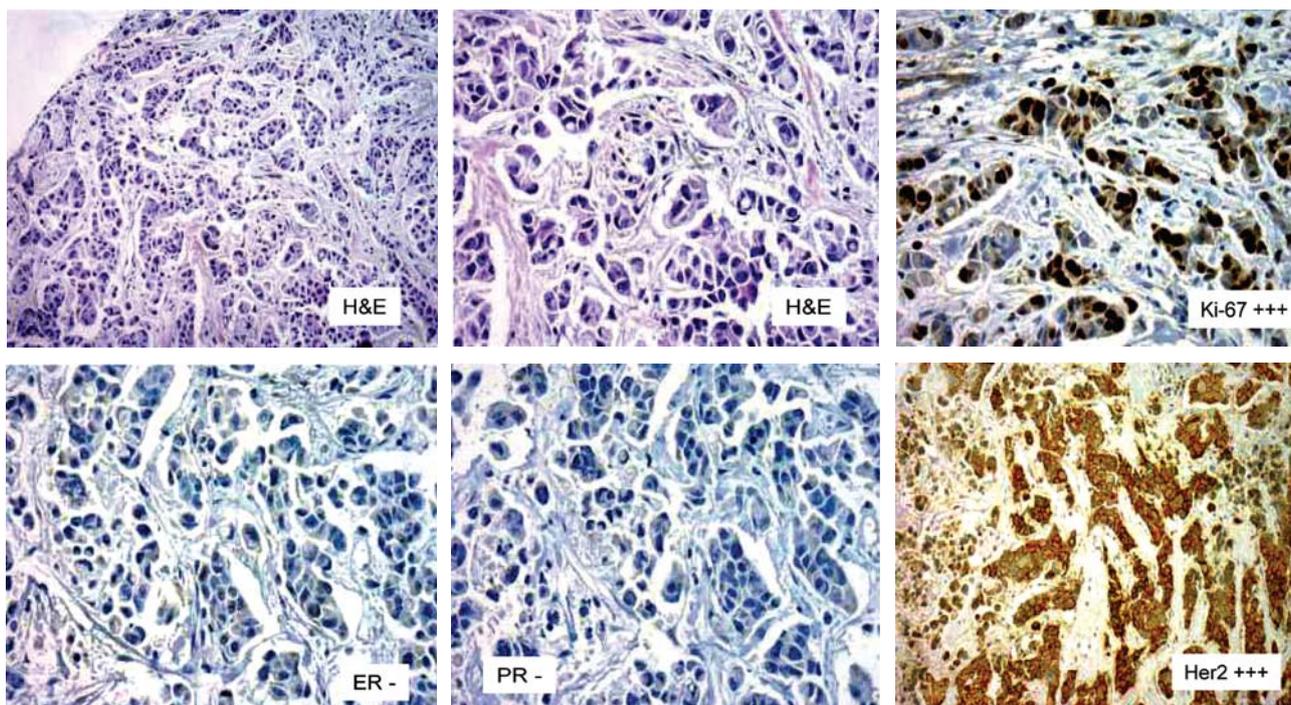


Fig. 4. Signet ring cell mucinous carcinoma Her2 positive.

*Apocrine type* is very infrequent, and the great majority correspond to ductal NOS carcinomas with focal apocrine features and only exceptionally are pure histological apocrine carcinomas diagnosed, while apocrine metaplasia is very common in benign ductal dysplasia and less frequent in in-situ carcinoma. Clinically they correspond to high grade tumors, are negative for ER and PR, and present AR positivity together with intense but focal GCDP-15 and occasionally HER2/neu 3+ . Their genetic profile has recently been partially identified [16–18]. However, it is not clear if this group, as is also the case of the recently described «*Claudin1 low stem cell like carcinoma*» [19], configures particular clinical entities or should be included within any of the above-indicated categories.

*Normal cell breast-like type* carcinoma has been considered by some authors as another specific entity [20], but the personality of this tumor detected by means of unsupervised hierarchical clustering analysis by the Stanford group [21] is not clear because it mimics normal epithelial cells, and the histology and clinical significance has still to be determined [11]/

*BRCA Associated breast Cancer.* Inherited mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 are associated with a high risk of breast and ovarian cancer. These two genes affect the capacity of cells to undergo homologous recombination with DNA repair, accelerating the number of somatic mutations accumulating in the genome. Breast cancers with BRCA1 mutant are triple negative, high-grade tumors bearing a poor prognosis. In several phase II clinical trials, this breast cancer appears to be sensitive to a new type of drug, the Poly(ADP-ribose)polymerase(PARP) inhibitors. In these cancers the blockade of PARP1 with a PARP inhibitor results in DNA repair arrest and cell death.

This is due to the fact that the cancer cells rely on an alternative pathway to repair DNA of non-homologous end joining. Several clinical trials are in progress to evaluate this possibility and the preliminary results are promising [22,23].

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