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**Significance of immunohistochemistry in breast cancer**

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**Abstract**

The biological characteristics of the tumour are used to estimate prognosis and select appropriate systemic therapy for patients with (breast) cancer. The advent of molecular technology has incorporated new biomarkers along with the immunohistochemical and serum ones. Immunohistochemical markers are often used to guide treatment decisions, to classify breast cancer into subtypes that are biologically distinct and behave differently, and both as prognostic and predictive factors. Steroid hormone receptors, markers of tumour proliferation, factors involved in angiogenesis and apoptosis show further scientific interest. In this review we will provide a presentation of the immunohistochemical markers used in the management of breast cancer patients using the available data from the literature. We considered the utility of established immunohistochemical markers, and discuss challenges for integrating novel molecular markers into clinical practice.

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**Key words**: Breast cancer; Immunohistochemistry; Markers

**Core tip:** Immunohistochemistry has an important role in the pathology of breast disease, as well as in other benign or malignant tumours. There is a growing list of available products (antibodies) or antigen retrieval techniques, all contributed to the broader utility of immunohistochemistry for solving diagnostic problems or for elaborate prognosis and response to therapy in breast pathology. Myoepithelial markers are most useful in helping to distinguish benign lesions from the malign ones. The common immunohistochemical breast cancer prognostic and therapeutic markers used include: ER, HER2, Ki-67, PR, p53. In addition important are markers of angiogenesis and apoptosis.

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**INTRODUCTION**

Immunohistochemistry (IHC) is used to characterize intracellular proteins or various surfaces from cells of all tissues. Individual markers or more often panels of various marker proteins can be used to characterize various tumour subtypes, confirm tissue of origin, distinguish metastatic from primary tumour and provide additional information which may be important for prognosis, predicting response to therapy or evaluating for residual tumour post treatment. There is a growing list of available products (antibodies) or antigen retrieval techniques, all contributed to the broader utility of immunohistochemistry for solving diagnostic problems or for elaborate prognosis and response to therapy in breast pathology.

**DIAGNOSTIC MARKERS**

The most important diagnostic problems that occur in the mammary gland tumor pathology are: the differential diagnosis of various types of some benign lesions and carcinoma; differentiating between carcinoma *in situ* and invasive carcinoma, diagnosis and differentiation of micro invasion and its imitating lesions or confirming the breast as the primary site in metastatic carcinoma. In the absence of advanced molecular biological techniques IHC can be use for identifying histologic subtype or molecular phenotype. An important part of these can be solved using some IHC markers (Table 1). As we well know, normal glandular breast tissue is composed of three cell types that express different subsets of proteins: luminal, basal and myoepithelial. The luminal cells express cytokeratins (CK 7, 8, 18, 19), epithelial membrane antigen (EMA), milk fat globule membrane antigen (MFGM), α-lactalbumin, ER, PR. Myoepithelial cells express basal cell type CKs and speciﬁc markers: smooth muscle actin, calponin, S100 and p63 while basal cell types express different cytokeratins(5/6, 14, 17)[1,2,3].

**MYOEPITHELIAL MARKERS**

Myoepithelial markers are most useful in helping to distinguish invasive carcinoma from benign proliferations with a similar morphological appearance, benign proliferative lesions and most preinvasive lesions having an intact myoepithelium. Invasive carcinomas lack the myoepithelial cells layer that normally surrounds benign breast glands. There is an exception, microglandular adenosis, a benign proliferative lession that lacks the myoepithelial cell layer[3,4,5]. In the same context to assess intraductal proliferative lessions, high-molecular-weight cytokeratins (cytokeratin 14 and cytokeratin 5/6) can be helpful in distinguishing usual ductal hyperplasia from DCIS. Atypical ductal hyperplasia or *in situ* carcinoma can arise in an otherwise benign papillary lesions and it is defined as a type of a ductal hyperplasia that morphologically simulates low-grade ductal carcinoma *in situ* (DCIS). Characteristically, atypical ductal hyperplasia has a uniform population of cells and most lesions are small and focal, involving only a portion of a duct or only a few small ducts measuring under 2 mm. By IHC, positive myoepithelial staining is seen in the benign area with attenuated or absent staining in areas of atypia or *in situ* carcinoma. It is possible the area of atypia or *in situ* carcinoma may not even be represented in the limited sample of a core needle biopsy.

The smooth muscle actin (SMA) has been long used as a myoepithelial marker in breast pathology diagnosis as a sensitive marker of myoepithelial differentiation, even if it is not specific, because any cell with substantial expression of actin is positive for SMA (myofibroblasts and blood vessels are positive for SMA).

This becomes problematic in lesions where there are either myofibroblasts or blood vessels in close proximity to the epithelial lesion in cause. One pitfall is the presence myofibroblasts within desmoplastic stroma adjacent to nests/glands of invasive carcinoma being misinterpreted as myoepithelial cells, resulting in a false-negative diagnosis. This is why it is recommended to use a panel of markers(p63, calponin, smooth muscle myosin, CD10, S100) or a marker more specific like p63.

An option can be calponin, a protein belonging to the contractile apparatus in smooth muscle cells and it is considered having the same sensitivity as SMA, but as SMA as well, staining of myofibroblasts and smooth muscle in blood vessels can be obtained. As with SMA, cytoplasmic staining of myoepithelial cells tends to encircle the nucleus as opposed to the staining pattern of myofibroblasts. Compared to other markers (p63 or smooth muscle myosin heavy chain (SMMHC), it tends to show more complete staining of the myoepithelial layer.

p63 is a homolog of p53, and has been shown to be expressed exclusively in myoepithelial cells in normal breast and can be very useful in differential diagnosis involving some benign lesions such as sclerosing adenosis, radial scars and papillary lesions. The advantage of using p63 is its nuclear localization and absence of staining in smooth muscle cells, such as myofibroblasts and blood vessels. Thus, it provides almost 100% specificity, but its sensitivity has been reported to be about 90%. This is demonstrated by so called “focal gaps” in staining in the myoepithelial layer, partly due to plane of section. Also, it has now been shown that about 10% to 15% of invasive tumors, particularly high-grade and metaplastic carcinomas, express p63, although the staining is usually weaker than that seen in the myoepithelial cells. Similarly, foci of squamous differentiation stain positively.

Like other smooth muscle markers, smooth muscle myosin heavy chain (SMMHC) is associated with contractile elements and is present in all cells with such properties. It is expressed primarily in myoepithelial cells but also stains blood vessels. An advantage of SMMHC is that it demonstrates less cross reactivity to myofibroblasts than calponin and SMA. Overall, the studies so far suggest that among smooth muscle markers, SMMHC provides the best results, in terms of both sensitivity and specificity.

When the inﬂammation or reactive ﬁbrosis obscure the interface between involved ducts and adjacent stroma in some cases of DCIS, IHC can help to clarify the integrity of the duct wall. Usual ductal carcinoma cells are negative for myoepithelial cells markers: S100, smooth muscle actin, SMMHC, calponin, CK5, CK14, CK17, CD10, and p63[3, 6-14]. The speciﬁc markers among these are SMMHC, calponin, and p63, the latter as well as some basal CKs having an advantage consisting in this case in not staining myoﬁbroblasts.

In most laboratories, however, the choice between these markers depends on individual experience, preference or financial resources. A combined approach, a nuclear and a cytoplasmic myoepithelial marker is a better option to increase the diagnostic utility of these markers. Pitfalls in the use of myoepithelial markers include those related to interpretation, fixation and technical aspects, and possible biological effects. Interpretative issues include the danger of mistaking myofibroblasts for myoepithelial cells due to cross-reactivity of cytoplasmic epitopes (in particular, SMA and calponin). Fixation and technical issues include underfixed tissue not immunostaining optimally; in such situations, entrapped benign glands may be mistaken for invasive carcinoma due to lack of staining of myoepithelial cells, resulting in a false-positive diagnosis of carcinoma. The key to solve all the issues is to perfom adequate internal controls.

**LOBULAR OR DUCTAL CARCINOMA *IN SITU***

Determining whether an *in situ* lesion is lobular carcinoma or ductal carcinoma having clinical management implications is another situation in which IHC proves its worth. Generally, ductal and lobular carcinomas, either invasive either *in situ* can be distinguished on hematoxylin-eosin–stained sections. In cases with non-specific morphologic characteristics, categorization can be made through IHC and the current marker in order to differentiate the two is E-cadherin. The majority of ductal carcinomas express cytoplasmic E-cadherin, whereas most lobular carcinomas lack expression[3,15,16]. In addition, it may be used differences in CKs expression: high- molecular-weight CK (clone 34βE12) are usually expressed by lobular carcinomas but are absent or expressed at low levels in most cases of DCIS [3,17,18]. In the same context, Cytokeratin 8 stains ductal carcinoma cells with a peripheral cytoplasmic accentuation while perinuclear staining is characteristic of lobular carcinoma[19].

**IDENTIFICATION OF SUBTYPES OF BREAST CANCER**

Analysis of both adjuvant and neoadjuvant trials has shown that not all chemotherapeutics have equal effects on each of breast cancer patients, therefore, further individualization of chemotherapy options may be bounded. Data on differences in chemotherapy sensitivity to taxanes and anthracyclines suggest that there are signiﬁcant differences across disease subtypes, which if further validated, could be used to guide the best decision making in patient treatment[20]. The St Gallen expert panel that met at the 12th International Breast Cancer Conference held at St Gallen (Switzerland) in March 2011, identified four subtypes of breast cancer according to oestrogen and progesterone receptors, over expression and/or amplification of the human epidermal growth factor receptor 2 (HER2) oncogene. The four subtypes were luminal A, luminal B, Erb-B2 over expression and basal like. The expert panel provided systemic treatment recommendations for the subtypes including endocrine therapy alone for luminal A, endocrine ± cytotoxic therapy for luminal B (HER2 negative); cytotoxics + anti-HER2 + endocrine therapy for luminal B (HER2 positive); cytotoxics + anti-HER2 for HER2 positive (non luminal); and cytotoxics for triple negative.

**METASTATIC CARCINOMA, MARKERS FOR MAMMARY ORIGIN**

In the case of small sized metastasis of infiltrating lobular carcinomas, false negative results are far more frequent than in infiltrating ductal carcinoma[21]. Medulary carcinoma metastasis or other subtypes of mammary carcinoma (lobular, sarcomatoid) can be often mistaken for malign lymphoma (with “signet ring” cells, clear cells, with carcinoma pattern, sarcomatoids). In these situations, the positive reaction for CK and lack of reactivity for lymph markers trance the diagnostic in favour of the metastasis. In as many as 24% of lymph nodes reported metastasis-free by standard histological examination, various authors found metastasis when multiple or serial sections were cut. Immunohistochemical markers also improve the specificity and accuracy of cell detection; therefore it is important to evaluate their utility in improving standard histological procedures. In the case of large metastasis in the axillary lymph nodes, IHC can demonstrate by the positive reaction for epithelial markers the carcinomatous nature of cells, difficult to appreciate as epithelial, in particular, in the case of axillary metastasis of infiltrating lobular carcinoma ( relatively uniform appearance of tumor cells and low mitotic activity ). For small metastasis of the infiltrating lobular carcinoma , false negative results are much more common than in infiltrating ductal carcinoma[21]. In addition, medullary carcinoma metastasis or other subtypes of breast carcinoma can sometimes be confused with malignant lymphoma (cell in the so called „signet ring ", clear cell); in these situations, the positive reaction for CK and lack of reactivity for lymphoma markers diagnose the metastasis.

The identification of metastatic carcinoma of the breast may be difficult in the absence of a previous history of breast cancer. Various immunophenotypic markers have been introduced to aid in this process. Markers for mammary origin include receptors for hormones, including androgen receptors and gross cystic disease ﬂuid protein 15 (GCDFP-15)[3]. GCDFP-15 is present in the liquid breast cysts and any apocrine cell: mammary glands, salivary glands, sweat, Paget's disease, etc. Therefore, carcinoma of the breast and others showed reactivity to GCDFP-15. Even in these conditions, the positive predictive value and specificity for the detection of breast cancer is 98%-99%[21], but moderately sensitive (50%–74% for breast carcinoma), which is why it is important to add other markers to the diagnostic panel such as ER, PR, AR, and HER-2/neu, mammaglobin, and CKs (7 and 20). In this context, androgen receptors and/or HER-2/*neu* are given additional value in a great number of ER-negative high-grade ductal carcinomas[3].

Lately, mammaglobin has been described as a breast cancer – specific gene, and its utility as a novel breast cancer marker has been confirmed[3,23,24]. Mammoglobin A and B identiﬁed in breast cells are overexpressed in breast cancer. Mammoglobin A is more speciﬁc for breast and gynecologic organs, while mammoglobin B may be found in a number of other tumors, especially gastrointestinal malignancies. Many studies suggested that elevated mammaglobin levels in breast cancer is associated with clinical and biological features defining a less aggressive tumor phenotype. Mammoglobin expression is not changed at the metastatic or lymph node site. It can help, in combination with other markers, to establish the correct diagnosis of metastatic breast carcinoma. Although many carcinomas would not be included in the differential diagnosis of breast carcinoma, the specificity of this marker was 92%[25] .

In the same study, when the immunohistochemical staining pattern of mammaglobin with GCDFP-15 in the breast carcinomas was compared, mammaglobin had higher sensitivity than GCDFP-15. In addition, the mammaglobin antibody cocktail stained deeper than GCDFP-15, and among the positive cases, the number of cells stained with mammaglobin is higher than with GCDFP-15. Despite some nonspecificity of the mammaglobin antibody, these data provide convincing evidence for inclusion of mammaglobin in a panel for the workup of carcinoma of an unknown primary site. When we want to diagnose a breast carcinoma, we should take into consideration the sensitivity of mammaglobin is better than that of GCDFP-15[25-27] .

Carcinoembryonic antigen (CEA) is a well-known tumour marker glycoprotein of 180 kDa. The polyclonal antibody reacts strongly and diffuse with ductal mammary carcinomas, the lung carcinomas and large intestine; CEAD-14 clone reacts with a small subset of mammary carcinomas, usually high grade, which is useful in the evaluation of metastatic mammary carcinoma in the lung, liver, brain and lymph nodes; 13% of breast carcinomas are positive for CEAD-14, with the focal reaction model, but possible and diffuse in high-grade carcinomas. A negative CEAD-14 pulmonary tumour is more likely to be a metastasis and not a primitive lung tumour, which is positive for other specific markers (such as thyroid transcription factor-1, TTF1)[28].

**MARKERS OF PROGNOSIS AND RESPONSE TO THERAPY**

The common immunohistochemical breast cancer prognostic and therapeutic markers used include: estrogen receptor (ER), HER2, Ki-67, progesterone receptor (PR), p53. In addition there are markers of angiogenesis and apoptosis.

***Hormone receptors***

Nowadays immunohistochemical detection of estrogen and progesterone receptors is part of the routine work-up of breast cancer, some cases of DCIS because the presence of estrogen receptors is an indication of tamoxifen therapy. There are many multiple scoring systems and many studies compared the ability to predict the treatment response and correlations with outcome. First score system counted the positive cells percentage and ignored staining intensity[29,30]. When we establish the proportion of positive stained cells, at least 1% it is considered a hormonally treatable state. According to the International Breast Cancer Study Group scheme wich is the basis of the most recent St Gallen treatment guidelines, breast cancer parts into three groups based on the the positive cells percentage: responsive (10%), response uncertain (1%–9%), and nonresponsive (0%). In other words, a thresshold of 1% positive cells indicate the option of hormonal therapy. These guidelines are widely followed in many countries from Europe and the United States, but it seems to be insufficient.

Many users report results as an Allred score, which comprises both percentage of cells positive and staining intensity[31]. A total score of 3 or more, corresponding to 1% to 10% positive cells, characterises the lowest positive and corresponds to the St Gallen endocrine response uncertain category in which case adjuvant hormone treatment can be recommended but has an uncertain beneﬁt[3,32,33]. Immunohistochemical staining for ER in DCIS, without associated invasive lession has a role in estimating potential tamoxifen positive effect. The National Surgical Adjuvant Breast and Bowel Project Protocol B-24, on patients with DCIS treated with partial mastectomy and then irradiation, to receive placebo tamoxifen for five years showed a conclusive reduction in both ipsilateral and contralateral in the adjuvant tamoxifen group[3,34,35].

**HER-2/NEU EXPRESSION**

HER-2/neu was one of the ﬁrst oncogenes studied in samples of invasive breast cancer and it is identified in 10%-20% of breast cancer patients. Its signiﬁcance consists as a marker for sensitivity to Herceptin(trastuzumab), and resistance to tamoxifen.[36]. Although Her-2/neu can be detected through many methods, only two are currently approved and recommended for its detection: IHC and FISH. Standardized immunohistochemical techniques exhibit a very good correlation with FISH methods. Such standardization requires the use of 10% neutral-buffered formalin as a fixative allowing at least 6-8 h of tissue fixation, not more than 48 h. IHC evaluates over-expression of the receptor protein at the surface of the cells, while fluorescence *in situ* hybridization evaluates the status of the *HER2* gene in the nucleus. In the majority of *HER2*-positive cancers, HER2 protein overexpression is the result of gene amplification, thus both methods should be highly correlated.

Immunohistochemistry reactions for Her-2 were scored by HercepTest where 0 and 1+ scores are negative, 2+ is weak positive and 3+ is positive. A positive HER-2 result consists of a uniform and intense membrane staining of more than 30% of tumour cells and further evaluation is unnecessary for invasive cancers that stain deﬁnitely positive and negative as well. Weak positive or equivocal or 2+ cases scored should be tested for gene ampliﬁcation by ﬂuorescence *in situ* hybridization. By this method, it is considered as a positive results more than 6 HER-2 gene copies per tumour cell nucleus or a HER-2 gene to chromosome 17 ratio of more than 2.2[3,36,37].

The selection of the best treatment, especially if the patient is a candidate for HER2-targeted therapy, depends on the accurate laboratory results assessing the HER2 status. All aspects of the test are performed in a highly standardized fashion with good quality control and the quality controls must be continuously monitored. The standardization includes aspects of pre-analytical sample tissue handling, the type and duration of fixation, tissue processing, assay performance, interpretation, and reporting[37].

Due to the intrinsic results subjectivity of the two techniques, IHC and FISH, the search of new methods with less subjectivity needs to be developed. Chemiluminescent technique have been used as a quantitative assay recently[39,40]. It has many benefits such as high sensitivity and accuracy, stability of reagents, easy-to-use protocols and non-photodegradable products, and indication of a good correlation between IHC and immunohistochemiluminescence results.

**KI-67**  **PROLIFERATION INDEX**

The Ki-67, a non-histone protein, involved in the early steps of polymerase I- dependent ribosomal RNA synthesis as a predictive and prognostic marker in cancers has been deeply studied. When Ki-67 level is above 10%–14% it is deﬁned a high-risk breast cancer group[41,42]. According to the St. Gallen Consensus (2009) the Ki-67 index is useful for selecting the patients with hormone receptor-positive breast cancers, for addition of chemotherapy to endocrine therapy. Thus, breast tumours are classiﬁed as low, intermediate, and highly proliferating according to the value of Ki-67 labelling index of under 15%, 16%–30%, and over 30%, respectively. Data from the clinical cancer registry Regensburgshows Ki-67 expression was associated with the common histopathological parameters, especially grading and survival, but is an additional independent prognostic parameter for disease free survival and overall survival in breast cancer patients[43].

The neoadjuvant setting is useful for analyzing the value of Ki67 as a predictive and prognostic tool. The majority of studies investigating the complete pathological response have identified a high Ki67 proliferation rate as a predictive factor for a higher rate of a complete pathological response[44,45]. However, it was found that

patients in whom progression occurred had a higher proliferation rate than in those who responded to chemotherapy during neoadjuvant chemotherapy. This suggests the nonlinear effect of Ki67 on the treatment response and probably on the prognosis as well [44,45].

Using Ki-67 expression, the effects of different doses of tamoxifen on breast cancer proliferation. have been investigated [42,46]. The change in Ki-67 expression induced by lower doses of tamoxifen was comparable to that achieved with the standard dose, concluding that tamoxifen retains antiproliferative activity at low doses [46,47]. Dowsett *et al*[48] in asmall study showed that the higher Ki-67 labelling index after two weeks of neoadjuvant treatment with tamoxifen was associated with shorter recurrence-free survival whereas higher Ki67 expression at baseline was not. According to Ellis *et al*[49] a great reduction of Ki-67 index after a short-term (a few weeks) hormonal treatment might be a simple and affordable way to select patients with ER-positive breast cancer who may not beneﬁt from adjuvant chemotherapy.

Another proliferation marker in a tumour tissue is the Ki-S2 antibody. It recognises a proliferation-speciﬁc nuclear protein expressed exclusively in the cell cycle phases S, G2, and M. Therefore, actively proliferating cells that constitute a subset of the population recognised by Ki-67 were speciﬁcally labelled. The cycling ratio is deﬁned as the ratio of the Ki-S2 labelling index to the Ki-67 labelling index and represents the relative fraction of cells in proliferation. Alterations of cell cycle regulation at the G1–S transition strongly inﬂuence breast cancer progression[42,50]. Prognosis is probably indicated by the percentage of cells in S through M phases of the cell cycle and the measurement of the Ki-S2 index also may improve to make an accurate prognosis and to identify patients with a low risk of recurrence who may not need adjuvant therapy[42, 51].

Regarding the molecular breast cancers, high Ki-67 proliferation index can be used for classify triple negative breast cancer into subtypes with different prognosis or responses to the treatment. For this purpose, it was determined the number of Ki-67 positive cells among the total number of counted tumour cells and the high expression of Ki-67 was defined as ≥ 10%. As we know, triple negative breast cancers have a poorer survival, despite their higher response rate to neoadjuvant chemotherapy and those with high Ki-67 were associated with a more aggressive clinical feature[52].

**MARKERS OF APOPTOSIS AND CELL PROLIFERATION**

Using of adjuvant chemotherapy in the early breast carcinoma is controversial, many advocating its use in high-risk patients as defined by specific pathologic parameters. Both BCL2 and p53, being involved in apoptosis and cell proliferation, play an important role in determining tumour growth and may help to define high-risk patients more accurately. In breast cancer patients BCL2 expression is significantly associated with hormonal receptor status and p53 is an important prognostic marker in early breast cancer[53].

BCL2 belongs to a group of proteins key regulators of apoptosis or programmed cell death. The tumorigenic potential of inappropriate BCL2 protein expression associated with adverse outcome was first described in subsets of non-Hodgkin's lymphoma as a result of the chromosomal translocation [t(14,18)][54,63].

Overexpression of BCL2 protein has been identified in a variety of solid organ malignancies, but in contrast to non-Hodgkin's lymphoma, BCL2 protein expression in breast cancer is associated with a nonaggressive phenotype of low-grade, slowly proliferative ER+ breast tumours. [55,56,63]. This favourable prognostic effect of BCL2 in breast cancer is explained by its non-apoptotic functions[57,63]. BCL2 is expressed in normal breast glandular epithelium and is upregulated by oestrogen, possibly as a direct result of transcriptional induction[58,63]. Its amplification or copy number gain is a rare condition and correlation between transcript and protein levels in breast cancer is unlinear, involving a post-transcriptional regulation [59,63]. Moreover, many studies had demostrated that expression of BCL2 was associated with improved survival in breast cancer, but this was attributed to its correlation with ER status[60-63]. If we count patients with ER+/BCL2− disease from those with ER−/BCL2+ disease, the first habe been found to have a worse prognosis than last ones[60,61].

The prognostic value of BCL2 was present across molecular subtypes, and was independent of some parameters such as stage, grade and tumour size. BCL2 could be used to separate patients with unnecessary cytotoxic therapy and it provides additional prognostic information[63].

The other marker, p53 is well studied in cancers, but its value in breast cancer in predicting clinical outcome remains debatable. The p53 gene is located on the short arm of chromosome 17 and encodes a 375 amino acid nuclear phosphoprotein that prevents propagation of genetically modified cells[64]. Wild type p53 is a tumour suppressor protein playing an essential role in regulating genomic stability by controlling the cell cycle and inducing apoptosis when cell damage cannot be repaired [65-67]. In normal cells, p53 has a very short half-life due to ubiquitylation and proteasome degradation[68,69].IHC is more proper as a method, because wild-type p53 protein is rapidly degraded, while *TP53* mutations (18%–25% of primary breast carcinomas) are often associated with the production of a stable protein. In addition, sequencing of the *p53* gene in all breast cancers would be expensive and time consuming for routine practice[70,71,73]. A higher tumour grade, negative estrogen and progesterone receptor status, and the more aggressive basal subtypewere associatedwithabnormal p53 immunohistochemical expression or p53-positive status [70,73]. Regarding early breast cancers, some scientists have reported that a p53 mutation has no influence on the outcome and therefore, the value is too weak for p53 status to be recommended as a routine marker in clinical practice[74].

**ANGIOGENESIS MARKERS**

Tumor growth and metastasis dependent on tumour angiogenesis and this complex process involves a delicate balance between angiogenic and antiangiogenic factors. There are numerous studies investigating the relationship between tumour angiogenesis and prognostic and response to antiangiogenic drugs. Analysis of these factors in tumour or serum of breast cancer patients by IHC or multiplex protein assay (FASTQuant® Microspot Assays) can improve diagnosis and prognosis of the disease. There is a large list regarding angiogenesis markers: Angiogenin, Ang2, keratinocyte growth factor (KGF), fibroblast growth factor basic, intercellular adhesion molecule (ICAM)-1, platelet-derived growth factor-BB and the family of vascular endothelial growth factor. Regarding these, it has been observed that breast cancer tumours exhibited high levels, as well in serum and they were compared to the benign breast diseases patients. When some of they have been evaluated either in tumour either serum in breast cancer patients, they exhibited association with standard clinical parameters, ER status as well as intratumoural microvessel density of tumours[75].

The commonly used method to appreciate angiogenesis is light microscopy counting of intratumoral blood vessels (MVD) stained with against factor VIII related antigen or anti CD31 or CD34. The main difficulty is the great variability in density between different areas of tumor and among observers. Counting of newly formed microvessels stained is a useful tool in the early detection of metastatic potential and in the selection of patients for whom anti-angiogenesis drugs might be beneficial. The reactivity level of CD34 antigen was assessed by immunohistochemistry in all types of invasive ductal breast cancer and its level seems to be a useful predictor for the development of local lymph node metastasis and indicate the benefit of antiangiogenic treatment[76,77].

  Anti-angiogenic drugs have been approved recently for the therapy of advanced cancers, including breast cancers. These drugs, alone or in combination with chemotherapy, have shown to be able to improve overall or progression-free survival in cancer patients. Unfortunately, the lack of validated biomarkers capable of selecting the patients who are most likely to beneﬁt from targeted drugs such as bevacizumab, sunitinib, sorafenib and pazopanib in fact, limits a rational use of these drugs and the ability to determine optimal dose and scheduling of these drugs [78].

Most of the biological and clinical activity of the anti-angiogenic drugs currently approved for cancer therapy is against the VEGF-related pathways. The VEGF system is part of the platelet-derived growth factor gene family, and interacts with its specific receptors; VEGFR-1 (flt-1) and VEGFR-2 (flt-1)for VEGF-A, a very potent angiogenic growth factor. VEGF-B, interacting with VEGFR-1, is seems to have an important role in the maintenance of existing vessels, but this protein is not so well studied. VEGF A and B, their receptors VEGFR-1 and 2 are expressed in a variety of normal cells, but overexpression in malignant tumors has been described[79-82].

There are different techniques applied to assess VEGF-A, the immunohistochemistry being the most convenient in routine diagnosis as well as research, as it allows single cell analysis combined with morphology. The results are currently based on visual examination of IHC-stained tissue slides and several different scoring systems have been used[79,83,84]. A part of these evaluate the intensity of the immunoreactivity, while others combine the examination of intensity score with the percentage of cells staining resulting in a semiquantitative scoring system. Unfortunatelly, a such scoring is defined by subjectivity and debatable intra- and interobserver reproducibility. Therefore, using the same tumour sections, some laboratory also introduced an automated method for analyzing VEGF expression and they obtained AI score. Methods for computer-assisted image analysis of VEGF-A improved reproducibility by reducing some of the variation between measurements [79,85,86].

The prognostic importance of VEGF in invasive breast cancer is associated with tumour stage, estrogen receptor status and inversely correlated with tumour grade and measurement of tumour VEGF, as an indicator of angiogenesis, is more reliable prognostically than measurement of microvessel density or serum VEGF[87,88]. Also, Tamoxifen treatment was associated with higher circulating and platelet-derived VEGF levels[88]*.*

**CONCLUSION**

IHC has become an integral part of the pathology laboratory. It’s a more mature technology and accessible to the majority of pathology laboratories. Some of the uses of IHC include diagnostic issues or are useful for estimating prognosis or predicting the therapy response. The best approach to the use of immunohistochemical markers is to combine them with standard hematoxylin-eosin slides examination and to use panels of markers. Once the potential value of a new immunohistochemical test method is appreciated, the burden will be to ensure the standardization of the testing protocol to maintain conformity of the test and minimize interlaboratory variation. Results may vary widely depending on the choice of fixative, choice of antibody manufacturer, and the type of immunostaining methods. A scoring system of test results should be regularly adopted and properly reported. IHC testing would be cost saving and economical per test, and it can be done in parallel with other tests. In the event of equivocal results, a back-up test method by multigene assays or others methods should be made available. Once these parameters are standardized, immunohistochemistry will assume a better and well-defined role in management of patients with cancer.

It is clear that the role of immunohistochemistry in detecting biomarker expression in pathology depends largely on research studies that demonstrate differential immunohistochemical expression and other studies that show good correlation between positive expression and response to new therapy. Although gene study is a sensitive technique, it lacks specificity to distinguish among different cells, and it may be contaminated by other cells. In addition, gene profile analysis is complex and inconvenient for routine clinical use.

Regarding subtypes, while a majority of scientists still pled against multi-gene expression array profiling being required for subtype definition, about half of the panel of St. Gallen 2013 opted for use of a clinic-pathologic definition as sufficient for subtype definition. It is concluded that only ER, PR, Ki-67 and Her-2/neu are recommended for clinical use.

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**Table 1 Diagnostic markers**

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| --- | --- | --- |
| **Marker** | **Staining pattern** | **Useful for** |
| Smooth muscle actin (SMA) | Cytoplasmic staining | Myoepithelial differentiation |
| Calponin | Cytoplasmic staining | Myoepithelial differentiation |
| p63 | Nuclear | Myoepithelial differentiation |
| Smooth muscle myosin heavy chain (SMMHC) | Cytoplasmic | Myoepithelial differentiation |
| CD10 | Membranous staining | Myoepithelial differentiation |
| S100 | Cytoplasmic | Myoepithelial differentiation |
| High-molecular-weight cytokeratins (14 and 5/6) | Cytoplasmic | To distinguish invasive carcinoma from benign proliferations; expressed by lobular carcinomas |
| Cytokeratin 8 | Peripheral cytoplasmic | Ductal carcinoma cells |
| Cytokeratin 8 | Perinuclear staining | Lobular carcinoma cells |
| CK 7 and 20 |  | Mammary origin of a metastatic carcinoma |
| E-cadherin | Membranous staining | Usual ductal carcinomas |
| Hormone receptors ER, PR | Nuclear | Identified subtypes, mammary origin |
| HER2 neu | Membranous staining | Identified subtypes |
| Gross cystic disease ﬂuid protein 15 | Cytoplasmic | Mammary origin of a metastatic carcinoma |
| Mammoglobin A | Cytoplasmic | Mammary origin of a metastatic carcinoma |
| Carcinoembryonic antigen, CEAD-14 clone | Cytoplasmic | Evaluation of metastatic mammary carcinoma |