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Review

"Basal-like" Breast Carcinomas: Identification by P-cadherin, P63 and EGFR Basal Cytokeratins Expression

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Abstract

Invasive breast carcinomas constitute a heterogeneous group of tumours, with different clinical behaviour and response to chemotherapy. These lesions, as determined morphologically, are thought to arise exclusively from the inner, luminal epithelial cell compartment of the terminal-duct lobular unit of the breast. Irrespective of the true histogenesis of breast carcinomas, it has become increasingly clear that a small proportion of cancers exhibit a basal/myoepithelial phenotype as defined by immunohistochemical positivity for myoepithelial markers, meaning they express molecules normally seen in the basal/myoepithelial compartment of the normal breast. The purpose of this review is to resume the more recent knowledge about the use of a panel of basal molecular markers in "basal-like" breast carcinomas classification and characterization. This subtype characterization has a great importance, since it requires a more focused investigation of putative therapeutic targets. The existing therapies against estrogen receptor (ER) or HER-2 oncogene amplification would not be expected to be effective against basal breast carcinomas, since these tumours express neither of these proteins. In contrast, basal breast carcinomas usually express basal cell cytokeratins (like CK5/6), P-cadherin adhesion molecule, p53 family member p63, and the transmembrane tyrosine kinase receptor EGFR (epidermal growth factor receptor), which can be used as excellent markers for this line of mammary carcinogenesis, and become interesting therapeutic targets against these highly aggressive lesions.

Key words: Breast Neoplasms; Cytokeratin; P-cadherin; Receptor; Epidermal Growth Factor

Introduction

Human breast carcinomas represent a heterogeneous group of tumours, which are diverse in their natural history, their outcome, and their responsiveness to treatment. Variation in transcriptional programs accounts for much of human cells and tumours biological diversity.[1-4] Additionally, the current pathology classification system is suboptimal, since patients with identical tumour types and stage of disease present different responses to therapy and different overall outcomes.[5,6] These limitations stem from the inability to take into account biological prognostic determinants.[7]

Until recently, the degree of differentiation and functional characteristics of epithelial cells, those giving rise to breast carcinoma, have remained unclear.[8] The advent of microarray technology, with high throughput and parallel analysis of thousands of genes, has allowed linking molecular expression profiles to clinical patient's outcomes and responses to therapy. If the

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predictive value of functional genomics is confirmed, it will be possible to predict accurately which tumours will relapse and to choose therapy accordingly. Another important implication is that genetic profiling may lead to the identification of new targets for therapy,[9-11] and better predictive markers are needed to guide difficult treatment decisions.

Recent cDNA and tissue microarrays studies have showed that breast tumours can be classified into specific subtypes, distinguished by differences in their gene expression patterns, which provide a distinctive molecular portrait for each tumour and the basis for an improved breast cancers molecular taxonomy.[12-14] Variations in growth rate, in the activity of specific signaling pathways, and in the cellular composition of the tumours were all reflected in the variation of the expression of a specific subset of genes and in the prognostic status of patients.[15-18]

As expected, the majority of the studies generally separate the tumour samples into those that are clinically described as estrogen receptor (ER) positive and those that are ER negative. Thus, using unsupervised clustering, they could already distinguish to some extent between "good prognosis" and "poor prognosis" tumours.[19,20] In the end, it is possible to identify three tumour groups that might be related to different molecular features of mammary epithelial cell biology: ER-± positive/luminal-like, ER-± negative/basal-like, and ER-± negative/HER-2 positive. An important implication of this classification is that the clinical designation "estrogen receptor negative" breast carcinoma encompasses at least two biologically distinct subtypes of tumours (basal-like and HER-2 positive), which may need to be treated as distinct diseases.[21,22]

"Basal-like" Breast Carcinomas

Breast cancers, as determined morphologically, are thought to arise exclusively from the inner, luminal epithelial cell compartment of the terminal-duct lobular unit of the breast. Irrespective of the true histogenesis (cell of origin) of breast carcinoma, it has become increasingly clear that a small proportion of cancers exhibit a basal/myoepithelial phenotype as defined by immunohistochemical positivity for myoepithelial markers, meaning they express molecules normally seen in the basal/myoepithelial compartment of the normal breast.[23] For this reason, tumours expressing these molecular markers have been named "basal-like" breast carcinomas.[24]

Basal breast tumours represent one of the most intriguing subtypes, since there is no efficient therapy against these lesions, that are often associated with poor prognosis.[25] Although a definition or comprehensive characterization of basal carcinomas is lacking, there are a number of features reported to be associated with this tumour type.[26] Our results, supported by several other recent publications, showed that cytokeratins (CK) 5, 14 and 17, P-cadherin, p63, and EGFR (epidermal growth factor receptor), which are established as markers of mammary basal/myoepithelial cells, can be used to distinguish a tumour basal phenotype.

Basal phenotype tumours represent a histologically poorly differentiated estrogen receptor (ER)-negative tumour subtype, with or without HER-2 amplification. The results of some studies have suggested that basal phenotype tumours may express less HER-2 protein than other breast cancer types,[27-29] being supported by the results of gene expression microarrays, which exclusively classify HER-2 expressing and basal phenotype tumours as separate entities.[30-32] However, Birmbaum et al. have reported that HER-2 oncogene amplification would be associated with the basal phenotype breast cancer.[33]

Based on 168 invasive breast carcinomas, our group was able to classify tumours into four different subtypes according to ER and HER-2 expression. Basal-type tumours expressed neither of these proteins and represented 7.6% of our series; basal-like HER-2-overexpressing tumours did not express ER and represented 17.7%; luminal-type tumours expressed ER and represented 72.8% of this series (56.3% HER-2 negative, and 16.5% HER-2 positive). We further characterized each subtype of this series based on cytokeratin (CK) 5, P-cadherin, p63, Bcl-2, and Ki67 expression. Basal-type tumours were mostly grade III, and more frequently CK5-, P-cadherin-, and p63-positive, with a high proliferation rate.

Conversely, luminal-type tumours rarely expressed basal markers and had a low grade and proliferation rate. Basal-like HER-2-overexpressing tumours showed a basal-type profile, with P-cadherin and CK5 up-regulation.[34,35] EGFR and osteonectin expressions were also associated to the basal subtype.[36-38] Nielsen et al. have found that the basal-like breast cancer show high expression levels of CK5, EGFR, and c-KIT, and low to absent gene expression of ER and HER-2.[39]

When these breast carcinoma immunoprofiles were compared to familial and sporadic origin, we could observe that basal tumours were mostly associated with familial cases (83.3%), whereas luminal and basal-like HER-2 overexpressing cases were more frequently sporadic ($p<0.0001$).[40]

Actually, it has been described that tumours from *BRCA1*-mutated carriers share a immunohistochemical profile very similar to that from sporadic basal-type carcinomas (high-grade, ER negative, progesterone receptor (PgR) negative, HER-2 negative), a finding recently confirmed by the analysis of the referred basal molecular markers (CK 5/14 and CK17, P-cadherin, p63, and EGFR) expression.[41,42] These results led to the assumption that this genotype strongly predisposes to the basal-like tumour subtype. Based on these results, Foulkes et al. have hypothesized that the wild-type *BRCA1* key function is to act as a stem-cell regulator, besides promoting the differentiation towards glandular epithelium in the normal breast. In *BRCA1* mutated tumours, this transition has failed or was not completed, and basal-cell phenotype gene expression was retained.[43-45]

Other example is the breast metaplastic carcinoma, an unusual neoplasm, characterized by an admixture of glandular epithelial components, which frequently exhibit features of squamous differentiation, and mesenchymal malignant components.[46,47] Regardless of the presence of myoepithelial features in breast metaplastic carcinomas, no consensus has been achieved up to now concerning their putative histogenesis. Several earlier studies have demonstrated that metaplastic breast carcinomas may have immunohistochemical and ultrastructural characteristics consistent with myoepithelial differentiation, like positive for basal CKs, vimentin, •-SMA, P-cadherin, p63, and maspin, and negative for steroid receptors and HER-2.[48-50]

The purpose of this review is to resume the most recent knowledge about the use of this panel of basal molecular markers in basal-like breast carcinomas classification and characterization. The characterization of this subtype has a major importance, since it requires a more focused investigation of putative therapeutic targets. The existent therapies against ER or HER-2 would not be expected to be effective against basal breast carcinomas, since these tumours express neither of these proteins.

The existence of this panel of markers is extremely important, since it is proved that using a single basal marker (like, for example, CK5/6), although successful in identifying a subset of patients with poor outcomes, misses approximately half of basal-like tumours. In addition, reliance on the lack of staining for ER and HER-2 alone to identify basal-like breast cancers risks misassignment because of technical failures and/or biological heterogeneity.[51]

Basal Cytokeratins (CK5/6, CK14 and CK17)

Most investigators have addressed breast carcinoma precursors by analysing cytokeratins expression as differentiation markers, since their expression is thought to remain stable throughout carcinogenesis.[52-54] However, modulations can occur within a certain range of possibilities during carcinomas development and progression.[55]

Keratins are proteins from intermediate filaments encoded by KRT genes mostly clustered on paralogous regions of 12q (*KRT1-8*) and 17q (*KRT9-25*) chromosome arms. Two types of CK are distinguished, either based on type and isoelectric point, which means, type I acidic (CK 10-20), and type II basic (CK1-9), or on molecular mass: low or high (CK8 and 18).[56]

Normal breast epithelium is complex and is known to have three populations of cells, defined by their cytokeratin immunoprofile. The luminal layer is composed by a dual population, one of inner luminal and glandular-type epithelial cells associated with simple-epithelium keratins (CK7, CK8, CK18, CK19), and one of basal-type intermediary epithelial cells expressing only basal-

type keratins (CK5, CK6, CK14, CK17), but not alpha-smooth muscle actin (•-SMA). The outer basal layer is composed by myoepithelial cells expressing basal-type keratins and •-SMA.[57] In both the luminal/glandular and myoepithelial lineages, cells seem to exist at intermediate stages of maturation, expressing various combinations of markers.[58]

This distribution led recently to the assumption that mammary CK5/14-positive cells were phenotypically and behaviourally the progenitor (or committed adult stem) cells of human breast epithelium, which could gradually differentiate towards glandular and myoepithelial lineages.[59-62] As a matter of fact, Boecker et al. have proposed five distinct cell populations in normal breast tissue, differentiated by their CK expression: committed stem cells (CK5+), glandular precursor cells (CK5+, and CK8/18/19+), glandular end cells (CK8/18/19+), myoepithelial precursor cells (CK5+, and •-SMA+) and myoepithelial end cells (•-SMA+).[63] However, this model was contested by Clarke et al., which have found no evidence to define a stem-cell phenotype based only on CK5/6 staining in paraffin wax sections,[64] and by Birnbaum et al., which claim that the different steps of differentiation have not been precisely delineated in the mammary gland.[65]

CK Expression and the Basal Phenotype in Breast Carcinomas

As already stated, non-malignant proliferations, pre-malignant lesions and breast carcinomas were always thought to arise from luminal differentiated epithelial cells, as evidenced by strong expression of glandular CK8, CK18 and CK19, similar to cells lining the lumen of normal breast ducts.[66] However, in benign lesions, CK5/6 was found to be strongly expressed in usual ductal hyperplasia, indicating that most cells in this lesion have a true basal-cell phenotype.[67,68] Later on, a small fraction (2% to 18%) of ductal carcinomas were reported to express basal CK5/6, together with its major partners CK14 and CK17, normally found in the basal/myoepithelial cell layer of the mammary duct. This has raised the attention from pathologists, since these were high-grade tumours that presented an aggressive metastatic pattern and poor patient prognosis.[69,70]

Indeed, some studies have demonstrated that CK5/6, CK14 and/or CK17 expression in breast cancer was associated with poor clinical outcome.[71,72] In fact, in node-negative breast carcinomas, the expression of these cytokeratins has been considered a prognostic factor independent of tumour size and grade.[73] Abd El-Rehim et al. examined basal and luminal cell cytokeratins expression in a series of invasive breast carcinomas and found that basal phenotype, defined by CK5/6 and CK14 expression, was related to poor prognosis, ER negativity and younger patient age. In addition, multivariate analysis showed CK5/6 to be an independent indicator for relapse-free interval (not affected by grade, lymph node stage and tumour size).[74]

Recent publications have classified breast cancers based on cytokeratin-5 and -17 expression at the RNA and protein levels, and demonstrated the importance of these markers in defining poor prognosis sporadic tumours. These important observations using different technology platforms produce a new functional classification of breast carcinoma.[24]

As already described, hereditary forms of breast cancer with *BRCA1* mutations have a distinct, ER-negative, poorly differentiated phenotype of basal-like tumours, which is recognizable both clinically and by gene expression profiling. Therefore, it was thought that the high proportion of medullary carcinomas that have been shown to carry *BRCA1* mutations could be cases with a CK 5/6 positive basal phenotype.[75] Recently, some authors have demonstrated the importance of this marker in defining BRCA1-related breast cancers.[24,76,77] Foulkes et al. associated CK5 expression to BRCA1-derived tumours, since 40% of CK5-positive tumours came from BRCA1-mutated carriers.[78] Laasko et al. studied basal and luminal cytokeratin expression in a large population-based cohort of sporadic invasive ductal breast cancers, as well as in tumours from a separate cohort of *BRCA1* and *BRCA2* germline mutation carriers.[79] They found that 9% of the sporadic tumours were positive for CK5/14, which were essentially of histologic grade III, ER and PgR negative and without *HER-2* oncogene amplification. The majority (78%) of BRCA1-associated tumours were positive for CK5/14 and displayed less

intense CK8/18 staining, including some truly CK5/14-positive CK8/18-negative cases. Only one of 15 BRCA2-associated tumours was CK5/14-positive.[80]

Prognostic analyses have even demonstrated that CK14 and CK17 presence is associated with short overall and disease-free survival in subgroups comprising high-grade, ER negative and vimentin-negative breast tumours.[81] However, new markers are needed to identify, purify, and characterize pure populations of the different cell types and to refine the model.[82]

In a recent study, our group detected CK 5/6 positivity in 48 from 56 cases (85,7%) of metaplastic breast carcinomas, reinforcing the idea that this group of tumours is basal-like breast cancers (data not published).

P-cadherin

P-cadherin, or placental cadherin, was the third classical cadherin to be identified and characterized, using a mouse visceral endoderm cell line.[83,84] Despite its name, P-cadherin is not expressed in human placenta;[85] indeed its name comes from the fact that this molecule was originally isolated from mouse placenta.[86,87] The gene encoding this protein (CDH3) is far less characterized than CDH1 (which codifies E-cadherin), although they share a 67% homology. It also maps to chromosome 16q22.1, a region containing a cluster of several cadherin genes,[88] just 32kb upstream of the human E-cadherin gene.[89] The mature P-cadherin protein has a molecular weight of 118kD, with 829 amino acids, and its structure is highly similar to that of E-cadherin.

Analogously, P-cadherin is mainly expressed at epithelial tissues cell-to-cell borders, but restricted to the basal proliferative cells of stratified epithelia, co-localizing partially with E-cadherin. This protein may be correlated with the maintenance of the proliferative compartment of certain epithelia, due to its restricted distribution, while E-cadherin plays a main role in the formation and maintenance of epithelial tissues due to its broad distribution.[90] In a recent study, mutations in CDH3 gene were found to cause congenital hypotrichosis associated with juvenile macular dystrophy (HJMD), an autosomal recessive disorder, characterized by hair loss heralding progressive macular degeneration and early blindness.[91-93]

In normal non-lactating breasts, E-cadherin is expressed by the luminal epithelial and myoepithelial cells and P-cadherin is only expressed by myoepithelial cells, underlying the luminal epithelium, and by cap cells, which are considered to be the breast stem-cell population.[94] This protein is still expressed in the lactating mammary gland tissue, and high levels of an 80kD soluble P-cadherin in human milk and in semen have been found.[95,96]

As already discussed, grade III invasive ductal carcinomas contain a subset of tumours presenting a specific molecular cytogenetic profile similar to the more conventional myoepithelial or basal carcinomas (CK5 and CK14 positive), and with a worse prognosis for patients.[97] P-cadherin is one of the markers expressed by these tumours, and has been described as a possible prognostic factor for breast cancer.[98-100] This fact raised our interest about the importance of this molecule in carcinogenesis and the progression of breast cancer.

P-cadherin expression and the basal phenotype in breast carcinomas

P-cadherin was detected in about 30% of mammary carcinoma cell lines, suggesting that this cadherin can be expressed by breast epithelial cells.[101] Based on this, several studies have investigated P-cadherin expression in large series of breast tumours. In an early study, P-cadherin was not detected in patients with ductal carcinoma.[102] In contrast, a later study found P-cadherin in some cases of infiltrating ductal carcinoma (20%), where it was associated with reduced E-cadherin and advanced histological grade.[103] More recently, it was shown that approximately half of ductal carcinomas express P-cadherin (35%-50%), whereas it was not detected in lobular carcinomas. Most importantly, these studies showed that P-cadherin expression was significantly associated with poor survival and could constitute an independent prognostic factor for breast cancer.[104-110]

The aberrant P-cadherin expression in breast tumours was usually accompanied by loss of E-cadherin expression, suggesting the existence

of the so-called cadherin switch, a mechanism implied in carcinogenesis, possibly by assigning cells different adhesive properties, by activating distinct signaling pathways and conferring them different morphological and behavioural characteristics.[111,112] In fact, P-cadherin expression seems to be a better indicator of clinical outcome in breast cancer than alterations in the expression of E-cadherin or catenins.[113] Unfortunately, in a study designed to determine if the level of soluble P-cadherin in serum might be elevated in patients with P-cadherin-positive tumours, no correlation was found.[114]

P-cadherin expression also correlated significantly with hormonal receptor content (the majority of the cases were ER and PgR negative).[107,115,116] and EGFR presence,[117] but no apparent relationship was found between its expression and tumour size and axillary lymph node metastases.[118-120] Furthermore, we have previously found that P-cadherin aberrant expression results from a lack of ER-• signaling and induces in vitro cell invasion in a juxtamembrane domain-dependent manner.[121]

It is not clear what might activate P-cadherin expression in a tumour cell whose progenitor does not normally express it. However, it is likely that growth factors and hormones present in the tumour environment might stimulate the expression of an inappropriate cadherin and that changes in the promoter regions of their codifying genes can be involved. For example, changes in DNA methylation or acetylation in tumour cells might trigger inappropriate cadherin expression.[122] Cytosine methylation of this region occurs in P-cadherin-negative prostate cancer cell lines but not in cell lines expressing this protein.[123] Recently, we have found that aberrant P-cadherin expression in breast cancer might be regulated by gene-promoter hypomethylation.[124]

Arnes et al. undertook a detailed evaluation of the relationship between P-cadherin, prognostic markers in breast cancer, and outcome. P-cadherin was present in 31% of breast cancers cases and was more frequent in tumors with a basal epithelial phenotype (i.e., high-grade, ER- and p27-negative tumors, with cytokeratin 5/6, cyclin E, TP53 expression, and the presence of BRCA1 mutations and vascular nests (all $P < 0.001$). P-cadherin expression was associated with a relative risk of death from breast cancer at a 10-year follow-up of 2.9 (95% confidence interval, 1.8-4.7; $p< 0.0001$) and was a predictor of poor univariate survival in both lymph node-negative and -positive breast cancers. In a multivariate analysis, P-cadherin levels effect was found to be dependent of other basal-related markers. Multivariable interaction modelling showed P-cadherin positivity to be highly predictive of a poor prognosis in small, node-negative breast cancers (relative risk, 7.1; $p = 0.006$).[125] Other studies have recently confirmed this strong correlation between P-cadherin and BRCA1–derived tumours, demonstrating the usefulness of this protein for the evaluation of immunophenotypic features of hereditary breast cancer.[126,127]

Based on this, P-cadherin is frequently identified in medullary carcinomas, but also in metaplastic carcinomas, suggesting a basal-cell histogenetic origin or line of differentiation for these tumours.[128,129] Han et al. reported P-cadherin expression in all cases of sarcomatoid metaplastic carcinomas (spindle cell) and carcinosarcoma (with heterologous elements).[130] Our study supported these results, because two out of three spindle cell metaplastic carcinomas and two out of three carcinosarcomas were P-cadherin-positive.[131]

One can conclude that P-cadherin is a marker for basal-like breast cancers, including metaplastic breast carcinomas, and is strongly associated with the presence of BRCA1 mutations. It is an adverse prognostic factor, particularly in small, node-negative breast cancers.

P63

P63 is a member of p53 family proteins, with the codifying gene located on chromosome 3q27,132,133 the identification of which has opened a new chapter in developmental and cancer biology. P63 gene exhibits a high sequential and structural homology to p53, leading to the early speculation that p63 proteins would function as tumour suppressors, similarly to p53.[134] In spite of the extensive homology between p63 and its more ancient related gene, there is a prominent difference concerning p63 gene ability to produce two different classes of proteins, with at least six

distinct isoforms.[135,136] One class contains a region that is similar in size and primary aminoacid sequence to p53 transactivation domain and is referred to as the TA class (TAp63). The second class creates a gene product that lacks this NH2-terminal domain and is referred to as the •N class (•Np63).[137-140] Due to the numerous variants that can be generated from the p63 gene, exhaustive studies have focused on determining not only which variants are expressed in certain tissues but also the signalling pathways regulated by those different protein isoforms and whether p63 activates or represses gene transcription.[141] Studies have shown that various p63 isoforms are capable of regulating p53 reporter genes and can either promote or oppose p53-induced apoptosis in a manner that correlates with the presence or absence of NH2-terminal TA region.[142]

Immunohistochemistry studies indicated that p63 expression is restricted to the basal cell layers of stratified epithelium.[143,144] The generation of p63-null mice confirmed p63 crucial role in maintaining the surface epithelium, since these mice are born with a lack of skin stratification.[145,146] These data were very important because they involve a specific role for p63 in maintaining keratinocyte stem-cell populations. In a very recent paper, Koster et al. elegantly demonstrated that p63 plays a dual role in embryogenesis and in epithelial differentiation, showing that this protein is capable of initiating epithelial stratification during development and to maintain basal cells proliferative potential in mature epidermis. Further, the authors demonstrated that TAp63 isoforms are the first to be expressed during embryogenesis and are required for initiation of epithelial stratification. In addition, these isoforms inhibit terminal differentiation, suggesting that they must be counterbalanced by •Np63 isoforms to allow cells to respond to signals required for maturation of embryonic epidermis [147]. In addition to its manifest importance to development, p63 is hypothesized to play an important role in maintaining the epidermal stem-cell population. As referred, p63 localizes in the basal/progenitor cells of several epithelial tissues such as the epidermis, the sweat glands, the tongue, the esophagus, the prostate and mammary gland, being •Np63• the predominant, if not the only, variant expressed.[148-151]

Although p63 function is not fully understood, the striking epithelial defects throughout the body seen in p63 knock-out mice suggest that this gene plays a key role in maintaining basal, progenitor cell populations of epithelia and provide evidence that besides its role in maintaining the replicative potential of basal cells, p63 may contribute to the maintenance of a multipotent phenotype.[152]

Several cancer studies have analysed the sequence of p63, isolated from various human tumours and numerous human cancer cell lines, and found p63 to be rarely if ever mutated.[153,154] However, some other works about primary human tumours and cell lines are giving a strong contribution to the corroboration of the possible role of p63 in epithelial tumours growth and development.[155-169] Some of those studies found deregulated p63 expression, sometimes in conjunction with amplification of its genomic region at 3q27-28, to be a frequent occurrence in a subset of human epithelial tumours.[170-174] According to Yang and colleagues, the initial findings concerning p63 isoforms role in cancer point out that •Np63• is the primary p63 variant expressed in squamous epithelial tissues and, more importantly, determined that this isoform can act antagonistically toward p53.[175] Recently, Reis-Filho et al. published a study where p63 expression was analysed in 51 normal and 400 neoplastic human tissues samples using a multi-tumour tissue microarray (TARP). No detectable p63 expression was identified in mesenchymal, neural, endothelial, and smooth muscle or adipose cells, a result consistent with restricted p63 expression in squamous and basal epithelial tissues. However, p63 was expressed in 93% of squamous cell carcinomas (SCC) of the lung, 10% of ductal carcinomas of the breast and 25% of endometrioid carcinomas of the ovary. The strong p63 expression in SCC has been in fact a target of numerous studies some of them suggesting that p63 and CK5 should be used together to identify 70-80% of all poorly differentiated SCC and to discriminate them from other poorly differentiated and undifferentiated carcinomas.[176]

Together with the paucity of mutation in cancer, there is a growing consensus that p63 actually may act more like an oncogene than as a tumour suppressor gene.[177] However, because different p63 isoforms have different activities,

it has become important to identify individual p63 proteins in order to determine their respective functions in normal and neoplastic tissues and in the carcinogenesis process.

P63 Expression and the Basal Phenotype in Breast Carcinomas

P63 is consistently expressed in basal cells of several types of multilayered epithelia (e.g., skin, cervix, prostate and salivary glands) and also in myoepithelial cells of the breast and sweat glands.[178-182] It has been shown that p63 decorates myoepithelial cells nuclei in normal breast ducts and lobules, as well as in several types of myoepithelial derived tumors.[183-186] One of the first classical studies demonstrating p63 expression in normal breast and neoplastic breast disease was published by Barbareshi and colleagues (2001), who described p63 immunoreactivity in the myoepithelial component of fibroadenomas, adenomyoepitheliomas, adenoidcystic carcinomas, and in 4.6% of ductal carcinomas of the breast.[187]

In the last five years, our group has been describing p63 distribution in breast epithelium as a reliable myoepithelial and stem-cell marker, as well as in neoplastic cells nuclei of metaplastic carcinomas of the breast (MCB).[188-194]

The presence of myoepithelial cells in breast lesions is an important feature that aids differential diagnosis. However, it is often not easy to identify myoepithelial cells by morphologic examination alone.[195] Currently, several immunohistochemical markers able to recognize a variety of cytoplasmic smooth muscle-related antigens are commonly used for demonstrating myoepithelial cells.[196] The examination of breast ductal carcinomas in situ (DCIS) showed a remarkable nuclear reactivity for p63 in myoepithelial cells nuclei around in situ carcinomas, helping in the differential diagnosis between breast invasive or in situ carcinomas.

Another field where p63 has been extensively studied by our group concerns its particular expression in metaplastic carcinomas of the breast.[197,198] Since MCB shows mesenchymal-like spindle-shape cells or metaplastic elements, including bone, cartilage, and squamous cells, and may be genetically related to the basal and myoepithelial cell pattern, it has been tempting to evaluate p63, as well as P-cadherin and maspin, expression in these lesions, since these are molecules consistently expressed by breast myoepithelial and basal (stem) cells. In fact, p63 and other myoepithelial cell markers have been recently described in matrix-producing and metaplastic carcinomas of the breast, suggesting that these tumours share a myoepithelial cell differentiation.[199] In a recent study, we showed that 44 out of 56 cases (78,5%) of metaplastic breast carcinomas were p63-positive (data not published).

In non-metaplastic breast carcinomas, p63 has been also proposed as one of the three molecular markers that distinguish basal phenotype (together with CK5 and P-cadherin expression).[200,201] A small fraction of breast cancers expresses CK5 together with CK14, which are normally found in the basal cell layer of the mammary duct. However, in addition to CKs, p63 expression has also been found in some breast tumours, where it was associated with high grade, large tumour size, nodal metastasis and ER negativity.[185,202,203]

In a recent study our group conducted, breast carcinomas were classified and characterized according to variations in protein expression patterns derived from immunohistochemical analyses on tissue microarrays, confirming not only the association between high levels of P-cadherin and CK5 expression and the basal phenotype, but also that p63 is up-regulated in this subgroup and can help to distinguish basal breast carcinomas. Although in that work the percentage of tumours positive as regards p63 expression (20%) was higher than in other studies, that refer an expression between 4% and 12%,[185,204-206] when we analysed p63 expression independently within the basal type subgroup, we found that 55.5% of these tumours expressed this protein. Additionally, we observed that the majority of the tumours classified as luminal were simultaneously p63, P-cadherin and CK5 negative,[207] reinforcing the particular phenotype of the considered breast basal tumours. Concerning clinicopathological correlations to p63, we must emphasize the observation that more than 15% of basal HER-2-

overexpressing tumours were also positive regarding that basal marker, results similar to those of the study of Laasko et al.[208,209]

It is also important to highlight that basal-type tumours that were positive for P-cadherin, p63 and CK5 were mostly grade III and had a high proliferation rate, in opposition to the luminal type.[210]

The biochemical and biological activities attributed to p63, as well as its regulation expression in human tissues, have been diverse and complex. However, a better understanding of p63 role in breast carcinogenesis requires the determination of which variant is expressed in the various mammary tumour types.

Epidermal Growth Factor Receptor (EGFR)

The receptor tyrosine kinase (RTK) superfamily subclass I is formed by ErbB or epidermal growth factor (EGF) receptors and includes four members: EGFR/ErbB1/HER1, ErbB2/Neu/HER2, ErbB3/HER3, and ErbB4/HER4.211

All members have an extracellular ligand-binding domain, a single membrane-spanning region and a cytoplasmic tyrosine kinase domain. They are expressed in various tissues of epithelial, mesenchymal and neuronal origin,[212] where they play a fundamental role in processes such as development, proliferation and differentiation.

A family of ligands, the EGF-related peptide growth factors, binds the extracellular domain of ErbB receptors, leading to the formation of either homo or heterodimers. Consequently, dimerization stimulates the receptors intrinsic tyrosine kinase activity and triggers autophosphorylation of specific tyrosine residues within the cytoplasmic domain. These phosphorylated residues serve as docking sites for signalling molecules involved in the regulation of intracellular signalling cascades.[213] Two of the main signalling pathways activated by ErbB receptors are the mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3 kinase (PI3K)-AKT.[214,215] These pathways activation causes a series of events such as cellular proliferation, apoptosis, angiogenesis, adhesion and cellular motility.[216]

The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane tyrosine kinase activated by several ligands. It is translated from two mRNA transcripts of 6 and 10 Kb encoded by a gene located on chromosome 7q21. Its expression in normal and neoplastic breast has been extensively studied, since EGFR is required for normal mammary development and lactation and is frequently aberrantly expressed in breast tumours.[217]

EGFR is expressed throughout embryogenesis in the blastocyst and in all three germ-cell layers of the embryo.[218] Together with its ligands, it also shares a broad tissue distribution in adult tissues[219,220] and has an important role in breast development.[221] EGFR act at puberty, late pregnancy and lactation, being preferentially expressed in lactating ducts and alveoli.[222]

In mice mammary epithelial cells, EGF stimulates proliferation and is necessary for normal murine mammary development, playing also an important role in lactation as shown by the major retardation of mammary development and consequent loss of milk production in mice with surgically resected salivary glands (where EGF is produced).[223] Recent studies have showed EGFR to be frequently expressed in the basal cell layer.[224]

EGFR Expression and the Basal Phenotype in Breast Carcinomas

Several authors suggest that EGFR can help to differentiate basal cell tumours, since CK5/6 positive tumours are found to be associated with EGFR expression.[225,226]

Nielsen et al.[227] found by means of gene expression data followed by immunohistochemical validation that basal-like breast tumors are ER and HER2 negative and CK5/6 and/or EGFR positive. EGFR expression was present in 44.1% of cancers positive for a basal cytokeratins and was significantly less common among basal cytokeratin negative cases (7,9%). Lakhani et al.[228] showed that BRCA1 mutation carriers are also frequently positive for EGFR staining – 67% of BRCA1 tumours positive for this marker versus 21% in the control group –, corroborating the idea that in fact EGFR is a basal cell marker. Another example is the

demonstration that metaplastic carcinomas of the breast that consistently express basal/myoepithelial markers[229-235] are also EGFR positive. Reis-Filho et al.[236] found EGFR expression in 19 out of 25 cases (76%), and also demonstrated that in 7 out of 19 (37%) cases this overexpression was due to EGFR gene amplification. Leibl & Moinfar[237] demonstrated in a series of 20 metaplastic carcinomas, EGFR positivity in 14 out of the 20 cases (70%). However, these authors also included weakly and moderately positive cases. If we consider only strong positivity cases, EGFR expression is found in 40% of the cases (8/20). The differences observed between the studies might be due to different antibodies used for immunohistochemistry or even to the different antigen retrieval methods used.

In recent studies, Jacquemier et al.[238] showed that typical medullary breast carcinomas present a basal/myoepithelial phenotype by associating these tumours with several already described markers. Interestingly, several medullary breast carcinomas have also EGFR expression, confirming the idea that EGFR can help to characterize further the basal/myoepithelial phenotype.

Although EGFR expression alone is not a basal-like breast cancer specific marker, nevertheless, as it is expressed in basal like breast cancers, when combined with other markers it greatly assists the immunohistochemical identification of these tumours.

Nowadays, EGFR is a valid target in cancer therapy in both colon cancers and non-small cell lung carcinomas; however, its use as a prognostic or as a predictive marker is still questionable. EGFR expression in this subset of basal/myoepithelial breast tumours raises the possibility of using specific anti-EGFR therapies.[239] Still, there is a need to select optimally patients for therapy since, for example, not all lung cancer patients respond to therapy. It is feasible that anti-EGFR therapy might be useful in breast cancer not only as a monotherapy but also as part of a combined therapy.

Conclusion

Basal-like breast carcinomas are a distinct clinical and pathological entity characterized by basal cytokeratins (CK5/6, CK14), P-cadherin, p63 and EGFR expression. It has been observed that the expression of these markers is highly associated with a more aggressive clinical course of tumours; however, they are not being routinely used in the standard histological diagnosis of breast cancer. Since there are no other prognostic markers to identify this group of basal-like tumours, patients are being treated according to the current classification, which considers basal-like and non-basal-like tumours the same entity. With this review, we suggest that basal markers should be used in clinical practice in order to identify breast cancer patients that will have a shorter disease-free and overall survival and cases where new options of treatment should be investigated.

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