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ORIGINAL ARTICLE

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p63, cytokeratin 5, and P-cadherin: three molecular markers to distinguish basal phenotype in breast carcinomas

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Abstract Human breast carcinomas represent a heterogeneous group of tumors diverse in behavior, outcome, and response to therapy. However, the current system of pathological classification does not take into account biologic determinants of prognosis. The purpose of this study was to classify and characterize breast carcinomas based on variations in protein expression patterns derived from immunohistochemical analyses on tissue microarrays (TMAs). Therefore, 11 TMAs representing 168 invasive breast carcinomas were constructed. Breast tumors were classified into four different subtypes depending on estrogen receptor (ER) and HER2 expression. Basal-type tumors expressed neither of these proteins and represented 7.6% of our series; basal-like HER2-overexpressing tumors did not express ER and represented 17.7%; luminal-type tumors expressed ER and represented 72.8% of this series (luminal A 56.3%, luminal B 16.5%). Moreover, we characterized each subtype based on P-cadherin (P-CD), p63, cytokeratin (CK) 5, BCL2, and Ki67 expression. Basal-type tumors were mostly grade III, more frequently P-CD-, p63-, and CK5-positive, and had a high proliferation rate. Conversely, luminal-type tumors rarely expressed basal markers and had a low grade and proliferation rate. Basal-like HER2-overexpressing tumors showed a basal-type profile similar with a high grade and up-regulation of P-CD and CK5. With this study, we show that P-CD, p63, and CK5 are important molecular markers that can be used to distinguish a basal phenotype. In addition, we also demonstrate the usefulness of TMAs in breast carcinoma immunoprofiling.

Keywords Breast cancer · Basal marker · Tissue microarray · Expression profile

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Introduction

Human breast carcinomas represent a heterogeneous group of tumors with a diverse biologic behavior, outcome, and response to therapy. An important challenge to the study and treatment of breast cancer relies on the resolution of molecular heterogeneity that is not apparent by morphological evaluation. Recent cDNA microarray profiling studies on breast tumors have identified distinct subtypes of breast carcinomas that are associated with different clinical outcomes [21, 22]. Sorlie et al. analyzed the expression profiles of 115 sporadic breast tumor samples and categorized them into five main groups: one basal-like, one HER2-overexpressing, two luminal-like (luminal A and luminal B), and one normal breast-tissue-like. When tumors from *BRCA1*-mutated carriers were similarly analyzed, it was found that this genotype strongly predisposes to the basal-like tumor subtype [22, 25]. The so-called basal tumors expressed cytokeratin (CK)5 and CK17 mRNAs, resembling the pattern found in the basal epithelial cells of the normal breast, whereas the luminal phenotype was based on the expression of CK8/18 and CK19 mRNAs similar to those of luminal epithelial cells [2]. The expression of HER1 and P-cadherin (P-CD) was later associated to the basal subtype [15, 16].

Breast cancer treatments evolved and directed target therapies against the estrogen receptor (ER) and HER2 oncogene. However, these therapies would not be expected to be effective against basal breast carcinomas because these tumors express neither of these proteins [15]. The characterization of this subtype assumes a major importance, leading to a more focused investigation on putative

therapeutic targets. Therefore, in the present study, we confirmed by immunohistochemistry (IHC) on tissue microarrays (TMAs) the association of high levels of P-CD and CK5 expression to the basal subtype. Moreover, we show that p63 is also up-regulated in this subgroup and can help distinguish basal breast carcinomas. p63 is a p53 homolog crucial for the maintenance of a stem cell population in several epithelial tissues and is necessary for the normal development of the human mammary gland [9, 13, 14, 26]. According to previous studies from our group, approximately 9% of the invasive ductal carcinomas of the breast are positive for p63 [18].

In this report, the existence of four different subtypes of breast cancer was confirmed by protein expression patterns assessed by IHC on 11 TMAs containing 168 independent breast carcinoma cases. The definition and characterization of the different subtypes were based on the expression of seven molecular markers (ER, HER2, P-CD, p63, CK5, Ki67, and BCL2).

Materials and methods

Patients' selection

One hundred sixty eight tumor samples were obtained from patients treated at the State University of Campinas (UNICAMP), Sao Paulo, Brazil, and at Hospital Sao Joao, Porto, Portugal. Of these, 50 tumors presented clinical features of familial breast cancer according to the Breast Cancer Linkage Consortium [5, 6]: early onset, bilaterality, and multiple cases of breast and ovarian cancer in the family (more than two first-degree relatives involved). The remaining 118 cases were from patients without any clinical familial feature (sporadic). All sporadic cases had ER IHC information available for whole tissue sections. From these, 62 tumors also had previous information for P-CD expression, and 73, for fluorescence in situ hybridization (FISH) analysis.

Tissue microarray construction

Representative areas of different lesions were carefully selected on hematoxylin and eosin (H & E)-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each selected specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder ab1802, Abcam, Cambridge, UK). Eleven TMA blocks were constructed, each containing 24 tissue cores, arranged in a 4×6 sector. In each TMA block, nonneoplastic breast tissue cores were also included as controls. After construction, 2-μm tissue sections were cut and adhered to Superfrost Plus glass slides. An H & E-stained 4-μm section from each block was reviewed to confirm the presence of morphological representative areas of the original lesions.

Immunohistochemistry

Immunohistochemical staining was performed using the streptavidin–biotin–peroxidase technique (Lab Vision Corporation, Fremont, CA, USA) in each set of 11 glass slides comprising the TMAs. Antigen retrieval was done by incubating TMA sections in an antigen-unmasking solution, pH 6.0 (Vector Laboratories Inc., Burlingame, CA, USA), or in ethylenediaminetetraacetic acid (EDTA), pH 8 (Lab Vision) at 98°C. The antigen retrieval times, antibodies, dilutions, and suppliers are listed in Table 1. After washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked by incubation of the slides in a 3% hydrogen peroxide solution in methanol (Merck, Germany). The slides were incubated with a blocking serum (Lab Vision) for 10 min and then incubated with the specific antibody. Immunostaining was performed overnight at 4°C (P-CD and CK5) or for 1 h at room temperature (HER2, ER, p63, BCL2, and Ki67). After washes, the slides were incubated with biotinylated secondary antibody, followed by streptavidin-conjugated peroxidase (Lab Vision). Diaminobenzidine was used as a chromogen. Tissues were then counterstained with hematoxylin and coverslipped using a permanent mounting solution (Zymed, San Francisco, CA, USA).

Immunoreactivities were classified by estimating the percentage of tumor cells showing characteristic staining. In nonneoplastic breast tissues, p63 showed nuclear positivity in myoepithelial cells. P-CD presented a distinctive membranous and occasionally cytoplasmic immunoreactivity in nonneoplastic myoepithelial cells. CK5 stained the myoepithelial cells of breast lobules and ducts. Two pathologists (F.S. and R.D.) evaluated the IHC staining. Because nonneoplastic mammary secretory cells do not express P-CD, either membranous or cytoplasmic immu-

Table 1 Antibodies used in the IHC study

Molecular marker	Antibody	Origin	Clone	Dilution	Antigenic retrieval (min)
BCL2	Mmab	Novocastra, UK	NCL-L-Bcl2	1:10	30
CK5	Mmab	Neomarkers, USA	XM26	1:80	20
ERα	Rmab	Neomarkers, USA	SP-1	1:20	30
HER2	Mmab	Novocastra, UK	NCL-L-CB11	1:60	30
Ki67	Rmab	Neomarkers, USA	SP6	1:300	20
P-CD	Mmab	BD Transduction, KY, USA	56	1:50	20
p63	Mmab	Neomarkers, USA	4A4	1:150	30

Mmab Mouse monoclonal antibody, *Rmab* rabbit monoclonal antibody

noreactivity was considered positive when more than 10% of the neoplastic cells expressed this marker [19]. Similarly, we adopted the same cutoff value for nuclear p63 and ER reactivity and for cytoplasmic CK5 and BCL2 reactivity. Four categories were defined for Ki67: 0, 0–5%, 5–25%, and >25% of stained nuclei. To evaluate HER2, the percentage of cells with membranous staining and intensity was assessed. HER2 was evaluated according to the four-category system (0–3+) and considered positive when 3+ was attributed. We compared our HER2 results with previously obtained FISH information. Of 73 tumor cases, 27 were simultaneously positive, and 39 were simultaneously negative. Only seven tumors presented discordant information: five were HER2-positive and FISH-negative, while the remaining two cases were HER2-negative and HER2-positive for the FISH analysis. For those discordant cases, we assumed FISH results.

Statistical analysis

The χ^2 contingency test was used for categorical variables to determine differences between phenotypes. A p value of <0.05 was considered to reflect a significant association. The StatView 5.0 (SAS Institute Inc., Cary, NC, USA) program was used for this analysis.

Results

Tissue microarray analysis and validation data

To validate the use of TMA for immunoprofiling, we compared, from whole tissue sections, available information on protein expression levels of ER and P-CD with those obtained by IHC on TMA. Of 118 breast carcinomas, 88 were simultaneously ER-positive, whereas 25 were simultaneously negative. Only three cases presented divergent information, and four were not interpretable. Overall, the concordance was 97.4%. In the same way, P-CD expression was also validated. Of 62 sporadic tumors, 12 were simultaneously positive and 48 were simultaneously negative, whereas only 2 cases were discordant. For this basal marker, we observed an overall concordance of 96.8%. This high concordance between IHC on whole tissue sections and on TMAs justified the further use of TMAs.

Immunohistochemistry profiles in breast tumors

We performed IHC on each set of 11 TMA slides for ERα, HER2, P-CD, p63, CK5, Ki67, and BCL2. The results are shown in Table 2 and Fig. 1. Cases positive for ER, p63, and Ki67 showed the characteristic nuclear staining, whereas BCL2 and CK5 showed a cytoplasmic pattern on neoplastic cells. HER2- and P-CD-positive tumors showed a membranous staining. We observed that 72.9% of breast carcinomas in our series were ER-positive, whereas 34.5% were HER2-positive (3+). Less than 31.7% of the tumors analyzed presented expression of a basal marker (P-CD, p63, and CK5).

Like Nielsen et al. [15], we also classified each tumor in a practical way based on its ER and HER2 expression. A total of 158 cases were immunohistochemically interpretable to allow sample characterization into one of five groups (Table 3). If a tumor was ER-positive, it would be classified as luminal; however, we distinguish luminals A and B on the basis of HER2 overexpression. If a tumor was ER-positive and HER2-negative (0, 1, or 2+), it would be classified as luminal A (ER+/HER2−); however, if it was ER- and HER2-positive, it would be classified as luminal B (ER+/HER2+). If a tumor was ER-negative and HER2-positive (ER−/HER2+), it would be classified as basal-like HER2-overexpressing, and if it was both ER- and HER2-negative but positive for at least one basal marker (P-CD and/or p63 and/or CK5), it would be classified as basal (ER−/HER2−). If a tumor did not show expression for any of these markers, it would be classified as negative and would not be considered in the remaining analyses. Using this definition, we observed that basal type comprised 7.6% of all tumors, whereas luminals A and B comprised 56.3 and 16.5%, respectively. Basal-like HER2-overexpressing tumors represented 17.7% of the series, and null phenotype, 1.9% (Table 3). When these immunoprofiles were compared in relation to familial and sporadic origin (Table 4), we observed that basal tumors were mostly associated to familial cases (83.3%), whereas luminal and basal-like HER2-overexpressing cases were more frequently sporadic (69.7% of luminal A, 92.3% of luminal B, and 71.4% of basal-like HER2-overexpressing tumors; $p<0.0001$).

The protein expression profiles clearly differed in luminal A, luminal B, basal, and basal-like HER2-overexpressing tumors concerning P-CD, p63, CK5, Ki67, and BCL2 (Table 4). Statistically strong significant differences between the four groups were observed in this study. Basal and basal-like HER2-overexpressing tumors presented a high histological grade (75 and 81.5% grade III, respectively, $p=0.0001$), had a higher proliferative rate, and demonstrated a clearly higher frequency of P-CD (83.3 and 60.9%, $p<0.0001$) and CK5 (66.7 and 37%, $p<0.0001$) expression (Table 4). Luminal pattern was associated with an increasing frequency of tumors expressing BCL2 (69.1% luminal A and 56.5% luminal B, $p=0.0006$) and with a lower expression of basal markers. Actually, less than 20%

Table 2 Results of IHC stainings on TMA

Molecular marker	Interpretable cores	Positive staining (%)[a]	Negative staining (%)
ER	166	121 (72.9)	45 (27.1)
HER2	162	56 (34.5)	106 (65.5)
P-CD	145	46 (31.7)	99 (68.3)
p63	154	31 (20.1)	123 (79.9)
CK5	149	33 (22.1)	116 (77.9)
BCL2	138	78 (56.5)	60 (43.5)
Ki67	138	33 (23.9)	105 (76.1)

[a]Ki67 positives include all positive categories defined

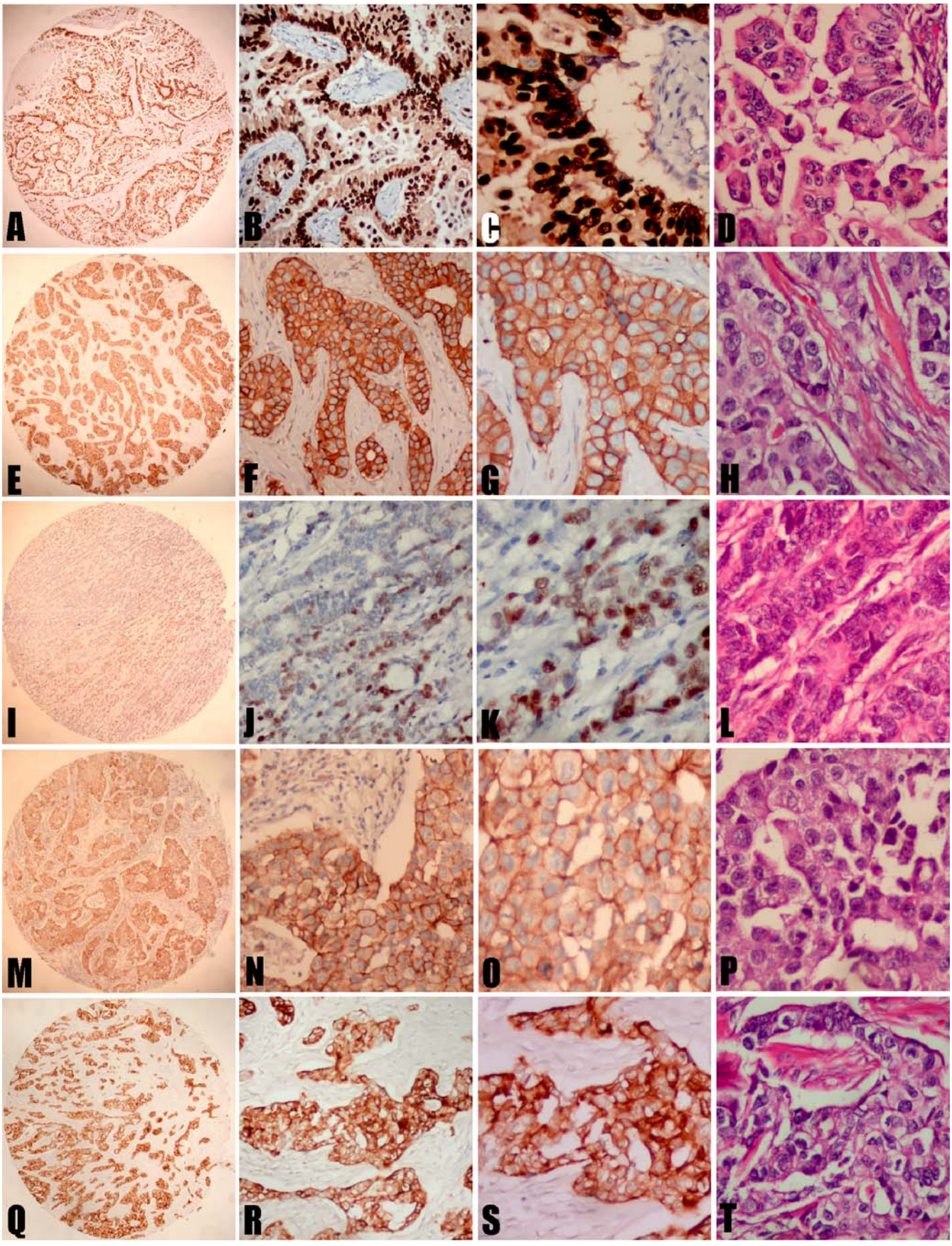

Fig. 1 Expression of proteins studied by IHC on TMAs. **a**–**c** ER staining. **e**–**g** HER2 staining. **i**–**k** p63 staining. **m**–**o** P-CD staining. **q**–**s** CK5 staining. All TMA cores represent neoplastic tissue with a strong staining pattern. **d**, **h**, **l**, **p**, **t** H & E staining of the core represented in each line [original magnification×50 (**a**, **e**, **i**, **m**, **q**), ×200 (**b**, **f**, **j**, **n**, **r**), and ×400 (**c**, **d**, **g**, **h**, **k**, **l**, **o**, **p**, **s**, **t**)]

Table 3 Frequencies of immunohistochemically defined subtypes of breast carcinomas in 158 informative tumors for the tested markers using TMA

Subtype	ER	HER2	P-CD and/or p63 and/or CK5	Frequency (%)
Luminal A	Positive	Negative	Positive/negative	89 (56.3)
Luminal B	Positive	Positive	Positive/negative	26 (16.5)
Basal	Negative	Negative	Positive	12 (7.6)
Basal-like	Negative	Positive	Positive/negative	28 (17.7)
HER2-overexpressing				
Negative	Negative	Negative	Negative	3 (1.9)

of luminal tumors presented a basal marker expression (Table 4). p63 expression was strongly associated to basal tumors (55.5%, p=0.0255).

Finally, we analyzed whether each basal marker was expressed exclusively by the tumors or if its expression was associated with another basal marker (Table 5). We observed that the majority of the luminal tumors (60.3% luminal A and 73.8% luminal B) were simultaneously negative to P-CD, CK5, and p63. This percentage was much lower for basal-like HER2-overexpressing tumors (36.4%) and absent for basal ones. Within basal and basal-like HER2-overexpressing subtypes, p63 was only positive when coexpressed with other basal markers. The same occurred for CK5 within a basal subtype. Simultaneous expression of P-CD, p63, and CK5 was rare in luminal A tumors (4.1%), being completely absent within the luminal B subtype. Interestingly, we observed that 18.2% of basal tumors and 13.7% of basal-like HER2-overexpressing tumors were simultaneously positive for all basal markers (p=0.0016).

Discussion

Gene expression profiling has refined the classification of sporadic breast tumors into distinct subtypes [17, 21, 22, 25]. Basal breast tumors represent one of the most important subtypes since there is no efficient therapy against it, and it is often associated with a poor prognosis [1, 21, 22, 24, 25]. Gene expression profiling approaches have also allowed the observation that familial *BRCA1* mutant tumors segregate strongly with basal-type sporadic cancers, which indicate that basal-type sporadic tumors and familial *BRCA1* tumors can have similar etiologies [23]. *BRCA1* tumors commonly express basal cytokeratins, but not ER, and are of a higher histological grade, indicating a

Table 4 IHC profiles in relation to pathological and clinical variables

	Luminal A [*n* (%)]	Luminal B [*n* (%)]	Basal [*n* (%)]	Basal-like HER2-overexpressing [*n* (%)]	*p*
Clustering					
Familial	27 (30.3)	2 (7.7)	10 (83.3)	8 (28.6)	<0.0001
Sporadic	62 (69.7)	24 (92.3)	2 (16.7)	20 (71.4)	
Grade					
I	18 (21.2)	5 (20)	0	2 (7.4)	0.0001
II	42 (49.4)	11 (44)	2 (25)	3 (11.1)	
III	25 (29.4)	9 (36)	6 (75)	22 (81.5)	
P-CD					
Negative	64 (80)	21 (84)	2 (16.7)	9 (39.1)	<0.0001
Positive	16 (20)	4 (16)	10 (83.3)	14 (60.9)	
p63					
Negative	70 (80.5)	21 (87.5)	5 (45.5)	22 (84.6)	0.0255
Positive	17 (19.5)	3 (12.5)	6 (55.5)	4 (15.4)	
CK5					
Negative	69 (84.1)	22 (91.7)	4 (33.3)	17 (63)	<0.0001
Positive	13 (15.9)	2 (8.3)	8 (66.7)	10 (37)	
Ki67					
0	67 (83.8)	16 (69.6)	5 (45.5)	15 (68.2)	0.0230
>0–5%	5 (6.2)	3 (13)	2 (18.1)	2 (9.1)	
>5–25%	8 (10)	4 (17.4)	3 (27.3)	5 (22.7)	
>25%	0	0	1 (9.1)	0	
BCL2					
Negative	25 (30.9)	10 (43.5)	7 (63.6)	17 (77.3)	0.0006
Positive	56 (69.1)	13 (56.5)	4 (36.4)	5 (22.7)	

Table 5 Basal marker expression in relation to different subtypes

	Luminal A (n=73) (%)	Luminal B (n=23) (%)	Basal (n=11) (%)	Basal-like HER2-overexpressing (n=22) (%)	p
Simultaneously negative	44 (60.3)	17 (73.8)	0	8 (36.4)	0.0016
Exclusively P-CD+	7 (9.6)	1 (4.4)	2 (18.2)	5 (22.7)	
Exclusively p63+	7 (9.6)	2 (8.6)	0	0	
Exclusively CK5+	4 (5.5)	1 (4.4)	0	1 (4.5)	
P-CD+ and p63+	2 (2.7)	1 (4.4)	2 (18.2)	1 (4.5)	
P-CD+ and CK5+	3 (4.1)	1 (4.4)	3 (27.2)	4 (18.2)	
CK5+ and p63+	3 (4.1)	0	2 (18.2)	0	
P-CD+ and p63+ and CK5+	3 (4.1)	0	2 (18.2)	3 (13.7)	

more rapidly dividing tumor [10, 16]. In the present study, we observed that the majority of basal tumors had a familial clustering, were the most proliferative ones, and were more frequently grade III. Foulkes et al. [10] associated CK5 expression to *BRCA1*-derived tumors. In their study, 40% of CK5-positive tumors were from *BRCA1*-mutated carriers. Palacios et al. [16] have also demonstrated a strong correlation between P-CD- and *BRCA1*-derived tumors. In the present study, we show that P-CD and CK5 were up-regulated in the basal subtype. This evidence suggests that these patients are at a high risk of having *BRCA1* mutations; however, additional studies are needed to investigate this issue further. Basal tumors were also characterized by p63 overexpression. This molecular marker, expressed in the basal cell layer, is thought to play an essential role in the differentiation and growth control in stratified epithelia [13].

Besides the identification of a basal-like subtype, we also distinguish luminal A, luminal B, and basal-like HER2-overexpressing subtypes. For this purpose, we used TMA technology since it allows the screening of a large series of samples at the same time without losing tumor representativity [7, 11, 12, 20].

The basal-like HER2-overexpressing subtype was characterized by a high grade and up-regulation of P-CD and CK5. In fact, Birnbaum et al. [2] have recently suggested that HER2-overexpressing tumors should be included in a bona fide basal-like subclass. Luminal-like tumors were distinguished on the basis of HER2 expression. The luminal A subtype did not express HER2 and was strongly associated with low proliferation and with high levels of BCL2 expression. This is not surprising since it is well known that BCL2 is up-regulated by ER [7]. The luminal B subtype was characterized by HER2 overexpression. These tumors were mostly sporadic and rarely expressed a basal marker.

Finally, we studied whether each basal marker was expressed exclusively by the tumors, or if its expression was associated with another basal marker. p63, P-CD, and CK5 are proteins that are expressed early in epithelial differentiation and may contribute to a committed stem cell and/or progenitor phenotype [3, 4, 9]. In this study, we thus confirmed the existence of heterogeneity among breast tumor cells since we observed different patterns of basal marker expression within luminal A, luminal B, basal-like HER2-overexpressing, and basal subtypes. Luminal A and luminal B subtypes were characterized by tumors that expressed exclusively one or two molecular markers, the majority being simultaneously negative. In contrast, the basal subtype rarely expressed just one basal marker but frequently expressed them simultaneously, which suggests a more undifferentiated profile. These pieces of evidence are in accordance with a hierarchy model of breast cancer oncogenesis that predicts functional heterogeneity among the cells that comprise the tumor [2, 8]. Malignant cells may have been derived from stem cells that were either able to differentiate to a mature estrogen-responsive stage (luminal tumors) or, due to a block in differentiation, stopped earlier in an immature stage (basal tumors). A basal-like HER2-overexpressing profile may represent an intermediary phenotype which failed the terminal differentiation of cells toward the luminal lineage due to a constitutive activation of HER2, an epidermal growth factor receptor (EGFR) family member [2].

With this work, we demonstrated that TMAs are an efficient and reliable platform for subclassifying breast cancers into relevant subtypes, using only a limited number of markers. Moreover, we showed that ER, HER2, P-CD, p63, and CK5 were important to characterize and distinguish basal-like breast tumors.

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