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P-cadherin, Vimentin and CK14 for identification of basal-like phenotype in breast carcinomas: an immunohistochemical study

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Summary. Introduction: The most suitable immunohistochemical criterion to identify basal-like breast carcinomas (BLBC), a molecular subgroup of breast cancer associated with poor prognosis, is the triple negative phenotype along with CK5 and/or EGFR immunoreactivity. However, several putative basal markers have been suggested as alternatives to identify BLBC with more accuracy. Experimental Design: The expression of CK5, EGFR, P-cadherin, CK14, Vimentin and p63 were evaluated in 462 invasive breast carcinomas to determine their sensitivity and specificity for BLBC identification. Results: P-cadherin and CK5 showed higher sensitivity values, while EGFR, Vimentin and CK14 were the most specific markers. The combination of CK5 with P-cadherin, Vimentin or CK14 proved to be a reliable option for distinguishing the basal phenotype, compared to the "gold standard" pair CK5/EGFR. Furthermore, P-cadherin was still able to recognize a large number of putative BLBC among the "unclassified" group (ER-/PR-/HER2-/CK5-/EGFR-). Conclusions: P-cadherin, Vimentin and CK14 can recognize BLBC already identified in triple negative/ CK5 and/or EGFR+ tumors, and due to P-cadherin sensitivity for BLBC identification this marker can reliably recruit a large number of breast carcinomas with basal phenotype among immunohistochemistry triple negative/ CK5 and/or EGFR - pool of tumors. Although they need GEP validation, our results can introduce the idea of these markers as additional options in the daily workup of breast pathology laboratories to identify BLBC.

Key words: Basal-like breast cancer, P-cadherin, CK14, Vimentin

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Introduction

In the European Union, breast cancer is the most incident form of cancer in women, with an estimated 429.900 cases diagnosed per year (28.9% of all incident cases in women) (Ferlay et al., 2007; Milanezi et al., 2008). Breast cancer is frequently designated as a heterogeneous disease with divergent biological behaviors. cDNA microarray studies have provided an improvement in cellular and molecular understanding of breast cancer, identifying distinct subtypes of breast carcinomas with different molecular signatures and clinical outcomes (Perou et al., 2000; Sorlie et al., 2001, 2003; Rakha et al., 2006a,b). The basal-like subtype has definitely drawn the attention of the scientific community. These tumors are characterized by a triple negative (TN) phenotype, lacking the expression of hormone receptors (HR) [estrogen and progesterone receptors (ER and PR, respectively)] and HER2. Basal-like breast carcinomas (BLBC) are associated with

aggressive tumor behavior and shorter overall survival when compared to the luminal and HER2-overexpressing subtypes and there is an enthusiastic search for molecular markers expressed in BLBC that could be used as targets to therapy (Nielsen et al., 2004). Histologically, they are poorly differentiated carcinomas, present high nuclear and histological grade and frequently show medullary and metaplastic features (Tsuda et al., 2000; Fulford et al., 2006; Livasy et al., 2006; Rakha et al., 2006a,b). A distinct pattern of metastasis to brain and lungs, known to be associated with poor prognosis, and less significant involvement of axillary lymph nodes, has also been described in BLBC (Tsuda et al., 2000; Banerjee et al., 2006; Fulford et al., 2007). Nowadays, gene expression profiles (GEP) or cDNA microarrays studies are currently considered the "gold standard" methods for the identification of breast carcinomas with basal phenotype, since these technologies were the first to identify BLBC as a distinct subgroup with a specific molecular signature (Perou et al., 2000) and clinical identity (Sorlie et al., 2001, 2003; van't Veer et al., 2002). However, GEP are expensive, not easily applicable as a routine laboratory diagnostic tool in large scale clinical-pathological analysis and have limited value in retrospective studies using formalin-fixed paraffin-embedded (FFPE) tissues (Cheang et al., 2008; Reis-Filho and Tutt, 2008). Thus, the idea of developing an immunohistochemical (IHC)-based assay for the identification of BLBC is appealing. The variation in the transcriptional and translational programs of cells that accounts for the different molecular identities of breast carcinomas also reinforces the interest in creating an IHC-based assay for BLBC definition. The characteristic protein expression of tumors would be a useful surrogate of GEP, and the IHC profile would help to standardize investigations and uniformly identify a group of tumors with a basal-like transcriptional program (Reis-Filho and Tutt, 2008).

However, the most appropriate panel of antibodies to be used, in order to identify breast carcinomas with basal phenotype, has not reached a consensus yet. In 2008, Tang et al. (2008) compared the different IHC classifications that have been used to define basal-like and non basal-like breast carcinomas; interestingly, they showed that in high grade breast carcinomas, which is a common feature of basal phenotype, the rates of BLBC ranged between 19% and 76%, indicating the need for a more consensual strategy between laboratories.

The TN phenotype criterion is used by some authors who assume that Triple Negative tumors and BLBC are synonymous (Kreike et al., 2007; Spitale et al., 2008). In fact, this criterion is quite convenient, since it includes standard biomarkers already used in the clinical management of breast cancer. However, relying on negative results to perform a diagnostic interpretation may be risky due to technical failures leading to a decrease in specificity. Other authors use high molecular weight cytokeratins alone (CK5/6, CK14 or CK17) to identify BLBC, claiming that BLBC and triple negative tumors are different identities (van de Rijn et al., 2002; Abd El-Rehim et al., 2004; Fulford et al., 2007; Rakha et al., 2007b). In addition, since basal-like breast carcinomas express proteins that are characteristic from the basal/myoepihelial outer layer of the mammary gland, such as EGFR, p63, P-cadherin, calponin, CD10, S100 and α-smooth-muscle actin (α-SMA) (Jones et al., 2001; Reis-Filho et al., 2003; Nielsen et al., 2004; Livasy et al., 2006), some definitions of BLBC associate the lack of expression of ER, PR and HER2 with the immunoreactivity for some of these basal markers that were already correlated with basal phenotype and poor prognosis (Nielsen et al., 2004; Matos et al., 2005; Laakso et al., 2006). Our group has previously demonstrated that using a panel of antibodies for ER, PR, HER2, CK5/6 and/or EGFR and/or P-cadherin and/or p63 it is possible to distinguish invasive (Matos et al., 2005) and i*n situ* (Paredes et al., 2007b) BLBC. However, Nielsen et al. (2004) found that expression of CK5/6 and EGFR together with negativity for ER and HER2 would be the immunoprofile that identifies the same basal-like carcinomas found by cDNA microarrays, with a sensitivity of 76% and a specificity of 100%. This criterion is, therefore, considered the "gold standard" immunoprofile to classify BLBC.

In this study, we aim to refine the immunohistochemical criterion to identify BLBC by analyzing the sensitivity and the specificity of the main basal markers that have been described, namely CK5, EGFR, P-cadherin, CK14, Vimentin and p63 and suggest possible additional markers for BLBC identification, especially in CK5 and EGFR negative breast carcinomas.

Materials and methods

Breast tumour samples

Formalin-fixed, paraffin-embedded tissues of 462 invasive breast carcinomas were consecutively retrieved from the histopathology files of three Departments of Pathology: University Hospital of the Federal University of Santa Catarina (Florianópolis, Brazil), Hospital Divino Espírito Santo (HDES), (Ponta Delgada, São Miguel, Portugal), and a private Laboratory of Pathology in Araçatuba, Brazil. All cases were reviewed by three pathologists (FM, FS and LV) on haematoxylin and eosin-stained (H&E) sections.

TMA construction

Representative areas of the invasive breast carcinomas were carefully selected on the H&E-stained sections and marked on individual paraffin blocks. Two tissue cores (2 mm in diameter) were obtained from each specimen and precisely deposited into a recipient paraffin block using a TMA workstation (TMA builder 20010.02, Histopatholoy Ltd, Hungary). Forty seven TMA blocks were constructed, each one containing 24 tissue cores, arranged in a 4x6 sector. In each TMA

block, normal breast and testicular tissue were included as controls. After construction, 2 μm tissue sections were cut and adhered to glass slides (Polysine*TM*, Menzel-Glasser, Germany) for the immunohistochemical studies and a H&E-stained section from each TMA block was reviewed in order to confirm the presence of morphological representative areas of the original lesions.

Immunohistochemistry

All the immunohistochemical assays were performed with specific monoclonal antibodies. Details about primary antibodies, antigen retrieval and IHC detection systems are described in Table1. Except for EGFR, in which epitope retrieval was performed by proteolytic enzyme digestion for 20 minutes (pepsin A, 4 g/l; Sigma-Aldrich, USA) at 37°C, all epitope retrieval was heat-induced at 98ºC in a water-bath during 30 minutes, using a commercially available citrate buffer solution (Vector Laboratories, USA), 1:100, pH=6.0, or an ethylenediaminetetraacetic (EDTA) solution (Novocastra, UK), 1:10, pH=9.0, as antigen unmasking solutions. After the respective antigen retrieval and washes in a phosphate buffer solution (PBS), endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution (Panreac, Spain) in methanol (Sigma-Aldrich, USA) for 10 minutes. The slides were incubated in a blocking serum (LabVision, USA) for 15 min and then incubated with the respective primary monoclonal antibodies. Immunoassays were performed using the streptavidin-biotin-peroxidase technique (SABC), (LabVision Corporation, Fremont, CA, USA) or the HRP labeled polymer (DakoCytomation, USA) detection system, according to manufacturer's instructions. All reactions were revealed with diamino-benzidine (DAB) chromogen (DakoCytomation). Tissues were then counterstained with Mayer's haematoxylin, dehydrated and coverslipped using a permanent mounting solution (Mounting Medium, Richard Allan Scientific, USA). Positive and negative controls were included in every set of reactions for each antibody used. Normal breast ducts and lobules present in many of the selected areas were also used as internal controls, as well as the non-neoplastic breast tissue cores included in each array. The evaluation of immunohistochemistry results was performed by three pathologists as follows: ER, PR and p63 were considered positive whenever more than 10% of the neoplastic cells showed nuclear staining; similarly, the same cutoff was used for CK5, CK14 and Vimentin cytoplasmic staining, as well as for P-cadherin membrane staining. Membrane expression for HER2 and EGFR was evaluated according to the DakoCytomation HercepTest® scoring system (Reis-Filho et al., 2005). Breast carcinomas were considered HER2-overexpressing whenever the immunohistochemical reaction was classified as 3+ or when gene amplification was confirmed by Chromogenic *In Situ* hybridization (CISH) in the 2+ cases, as described in other works (Ricardo et al., 2007). For EGFR, the cases were considered positive whenever the immunostaining was 2+ or 3+.

Hormone receptor (ER and PR) positive tumors were considered luminal A and B whether or not they overexpressed HER2, respectively (Sotiriou et al., 2003; Matos et al., 2005; Paredes et al., 2007b; Spitale et al., 2008; Tamimi et al., 2008). Cases lacking ER/PR with overexpression of HER2 were classified as HER2 overexpressing tumors. ER-/PR-/HER2- cases with immunoreactivity for EGFR and/or CK5 were considered BLBC according to the gold standard Nielsen's criterion and cases without expression of the five biomarkers were considered unclassified. When the immunoreactivity for the additional basal markers, namely P-cadherin, CK14 and Vimentin are used, the positive cases for at least one of these markers were considered as BLBC (P-cad and/or CK14 and/or Vim). Since for some markers the immunohistochemical result was not interpretable, the statistical analyses were performed using only 387 breast tumors cases which were classified for all the biomarkers tested.

Table 1. Conditions of the immunohistochemical reactions performed in this study.

	Primary antibodies				Antigen retrieval buffer	Detection method
Antigen	Clone	Origin	Incubation time (min)	Dilution		
ER	SP1	Neomarkers, USA	30	1:150	Citrate	SABC*
PR	SP2	Neomarkers, USA	30	1:300	Citrate	HRP-Polymer **
HER2	SP3	Neomarkers, USA	30	1:80	Citrate	SABC*
CK5	XM26	Neomarkers, USA	60	1:50	Tris-EDTA	SABC*
EGFR	31G7	Zymed	60	1:100	Pepsin	HRP-Polymer **
P-cadherin	56	BD Transduction	60	1:50	Tris-EDTA	HRP-Polymer **
CK14	LL002	Novocastra, UK	60	1:400	Tris-EDTA	HRP-Polymer **
Vimentin	V9	Dako, USA	30	1:150	Citrate	SABC*
p63	4A4	Neomarkers, USA	60	1:150	Citrate	SABC*

* SABC: streptavidin-avidin-biotin-complex; **: HRP-Polymer (horseradish peroxidase - polymer).

Statistical analysis

Statistical analysis was performed by SPSS statistics 17.0 (SPSS Inc., Chicago, IL, USA) software program. χ^2 contingency test was used to determine associations between groups and the results were considered statistically significant if the p value was lower than 0.05. In order to determine which were the most sensitive and specific biomarkers to identify BLBC, the sensitivity and the specificity of the antibodies used were calculated. Sensitivity measurement was defined by the quotient between the true positive (TrueP) cases and the sum of the true positive and the false negative (FalseN) cases [sensitivity = TrueP/(TrueP+FalseN)]. Specificity was measured in a similar way, by the quotient between the true negative (TrueN) cases with the sum of the true negatives and the false positives (FalseP) [specificity = TrueN/(TrueN+FalseP)]. PPV (Positive Predictive Value) and NPV (Negative Predictive Value) were calculated as follows: PPV = TrueP/(TrueP+FalseP) and PNV = TrueN/(TrueN+FalseN). As described before, ER/PR/HER2 negative tumors that express CK5/6 and/or EGFR were considered BLBC. Consequently, TrueP and TrueN cases were the BLBC tumors that were positive or negative, respectively, to the marker or pair of markers in analysis. Inversely, FalseP and FalseN were non BLBC positive or negative to the basal markers in study.

Follow-up information was available for 282 of the 387 cases and a maximum cutoff of 77 months was considered. Survival curves were estimated by the Kaplan-Meier method using log-rank test to assess significant differences for overall survival.

Results

In this series of 387 breast carcinomas, 223/387 (57.6%) and 144/387 (37.2%) cases were ER and PR positive, respectively, and 65/387(16.8%) overexpressed HER2. Using the ER/PR/HER2- (TN) criterion, this series comprises 109 (28.2%) triple negative and 278 (71.8%) non-Triple Negative tumors. Considering the molecular subtypes of breast cancer, 213 (55%) cases were luminal A, 13 (3.4%) luminal B and 52 (13.4%) HER2-overexpressing tumors. According to Nielsen's criterion, 37 (9.6%) cases presented a basal-like phenotype and 72 (18.6%) were considered "unclassified" by this criterion. We analyzed the associations between CK5, EGFR, P-cadherin, CK14, p63 and Vimentin and the BLBC versus non BLBC (Table 2). As expected, the markers were significantly associated with the basal phenotype ($p<0.0001$), with the exception for p63 ($p=0.5403$). Fig. 1 shows the immunohistochemical staining for CK5, EGFR, P-cadherin, Vimentin and CK14 in BLBC.

Afterwards, the sensitivity, specificity, PPV and NPV of each biomarker for the identification of BLBC were calculated (Table 3), except for p63 which was not even related with basal phenotype. CK5 was the most sensitive biomarker (91.9%), followed by P-cadherin (67.6%). CK14 and EGFR were the most specific markers, presenting 98.6% and 97.1% of specificity, respectively, and vimentin was also shown to be very specific (86.9%).

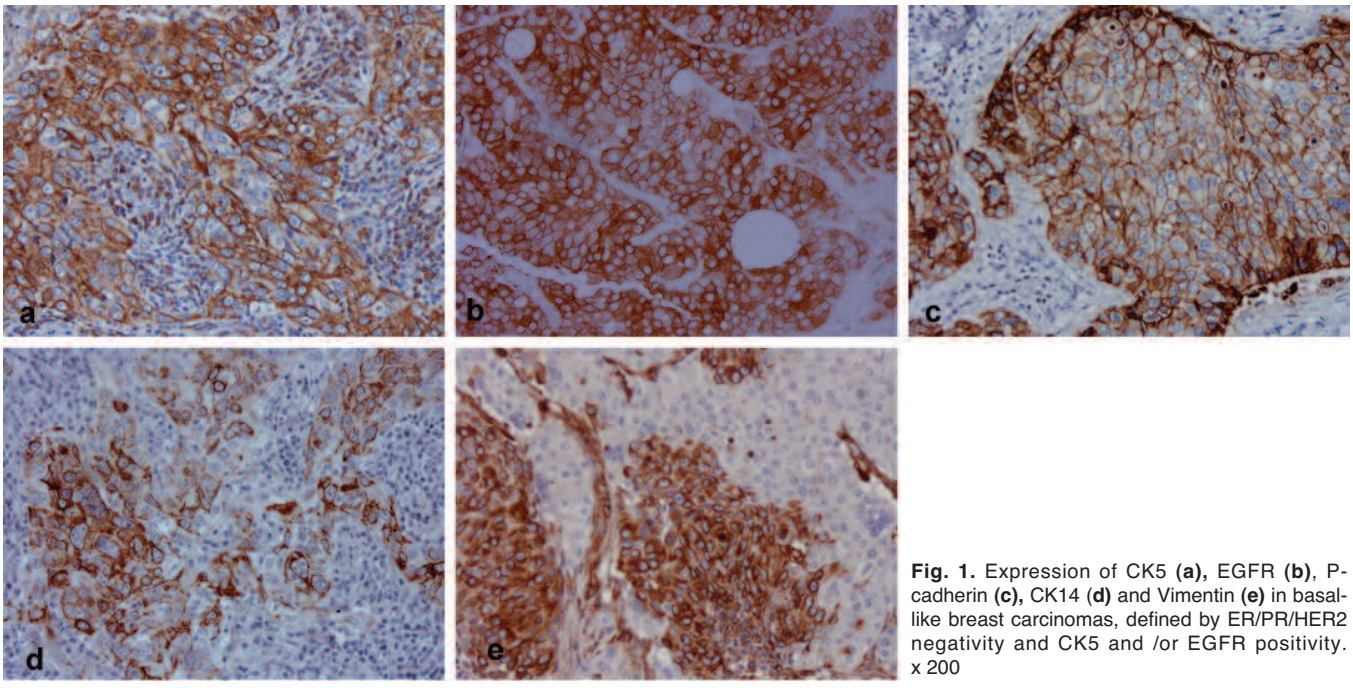

Fig. 1. Expression of CK5 **(a),** EGFR **(b)**, P-cadherin **(c),** CK14 **(d)** and Vimentin **(e)** in basal-like breast carcinomas, defined by ER/PR/HER2 negativity and CK5 and /or EGFR positivity. x 200

In order to find the best combination of basal markers with the ability to identify BLBC, we evaluated the most sensitive and the most specific markers in pairs (CK5, P-cadherin with CK14, EGFR or Vimentin). Since P-cadherin presented good sensitivity and specificity values, we also evaluated its association with CK5 (Table 4). The statistical associations considered cases that were positive for both markers (+/+), positive for at least one marker (+/- or -/+) or negative for both (-/-). Table 5 shows the percentages of sensitivity, specificity, PPV and NPV for the several pairs of markers. In these analyses, we considered as true positive the cases that were +/+ and positive for at least one of the markers in the subgroup of BLBC previously distinguished by Nielsen's criterion, and as false positive the cases that

Table 2. Association between the expression of CK5, EGFR, P-cadherin, CK14, p63 and vimentin with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	P
	387	37(9.6%)	350(90.4%)	
CK5				<0.0001
+	89	34(91.9%)	55(15.7%)	
-	298	3(8.1%)	295(84.3%)	
EGFR				<0.0001
+	21	11(29.7%)	10(2.9%)	
-	366	26(70.3%)	340(97.1%)	
P-cadherin				<0.0001
+	123	25(67.6%)	98(28%)	
-	264	12(32.4%)	252(72%)	
CK14				<0.0001
+	17	12(32.4%)	5(1.4%)	
-	370	25(67.6%)	345(98.6%)	
p63				0.5403
+	14	2(5.4%)	12(3.4%)	
-	373	35(94.6%)	338(96.6%)	
Vimentin				<0.0001
+	63	17(45.9%)	46(13.1%)	
-	324	20(54.1%)	304(86.9%)	

Table 3. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the IHC method for the basal-markers studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5	91.9	84.3	38.2	99.0
EGFR	29.7	97.1	52.4	92.9
P-cadherin	67.6	72.0	20.3	95.5
CK14	32.4	98.6	70.6	93.2
Vimentin	45.9	86.9	27.0	93.8

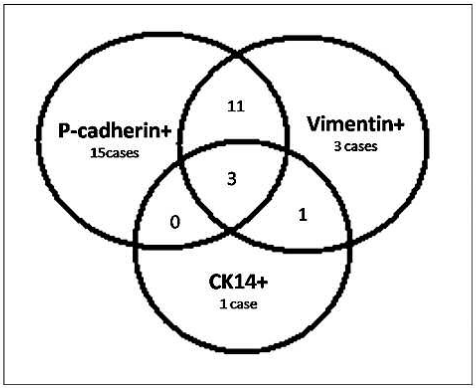

Fig. 2. Distribution of P-cadherin, vimentin and CK14 expression in triple negative tumors that were negative for CK5 and EGFR.

Table 4. Association between the expression of pairs of basal markers with basal-like and non basal-like breast carcinomas.

	n	Basal n (%)	Non basal n(%)	p
CK5/EGFR				<0.0001
+/+	11	8(21.6%)	3(0.8%)	
At least one +	88	29(78.4%)	59(16.9%)	
-/-	288	0(0%)	288(82.3%)	
CK5/CK14				<0.0001
+/+	11	11(29.7%)	0(0%)	
At least one +	83	23(62.2%)	60(17.1%)	
-/-	293	3(8.1%)	290(82.9%)	
CK5/Vim				<0.0001
+/+	24	16(43.2%)	8(2.3%)	
At least one +	104	19(51.4%)	85(24.3%)	
-/-	259	2(5.4%)	257(73.4%)	
P-cadherin/EGFR				<0.0001
+/+	13	8(21.6%)	5(1.4%)	
At least one +	118	20(54.1%)	98(28%)	
-/-	256	9(24.3%)	247(70.6%)	
P-cadherin/CK14				<0.0001
+/+	12	9(24.3%)	3(0.9%)	
At least one +	116	19(51.4%)	97(27.7%)	
-/-	259	9(24.3%)	250(71.4%)	
P-cadherin/Vim				<0.0001
+/+	41	11(29.7%)	30(8.6%)	
At least one +	104	20(54.1%)	84(24%)	
-/-	242	6(16.2%)	236(67.4%)	
P-cadherin/CK5				<0.0001
+/+	38	23(62.2%)	15(4.3%)	
At least one +	136	13(35.1%)	123(35.1%)	
-/-	213	1(2.7%)	212(60.6%)	

Table 5. Sensitivity, specificity, PPV and NPV of the IHC method for the pairs of basal-markers antibodies studied to discriminate a basal-like carcinoma.

	Sensitivity (%)	Specificity (%)	PPV (%)	PNV (%)
CK5/EGFR	100	82.3	11.4	100
CK5/CK14	91.9	82.9	10.5	99
CK5/Vim	94.6	73.4	12.0	99.2
P-cadherin/EGFR	75.7	70.6	10.2	96.5
P-cadherin/CK14	75.7	71.4	10.1	96.5
P-cadherin/Vim	83.8	67.4	11.6	97.5
P-cadherin/CK5	97.3	60.6	14.5	99.5

were positive for the two markers and the ones expressing at least one marker in non basal-like tumors. True negative and false negative were the -/- cases in non basal-like and in BLBC, respectively. All the associations were statistically significant ($p<0.0001$). The pair CK5/EGFR presented, as expected, the highest values of sensitivity and specificity, 100% and 82.3%, respectively. However, concerning sensitivity, the pairs CK5/CK14, P-cadherin/CK5 and CK5/Vimentin showed similar values to the "gold standard" CK5/EGFR pair, with 91.9%, 97.3% and 94.6% of sensitivity, respectively. The specificity of CK5/CK14 combination (82.9%) was approximately equal to the one presented by CK5/EGFR (82.3%).

In the BLBC group, when analyzing the number of cases that were +/+ and positive for at least one of the markers of the pair, against the -/- cases (Table 6), it is possible to observe that only one basal-like breast carcinoma was negative for both markers in P-cadherin/CK5 pair. The CK5/Vimentin pair missed the expression in 2 cases, while CK5/CK14 did not stain three BLBC. All the other pairs were positive in BLBC for the two markers, or for at least one of them, in at least 75.7% of breast carcinomas with basal phenotype.

More importantly, given the sensitivity of P-cadherin and the specificity of CK14 and Vimentin, we also analyzed their expression among the TN/CK5 and EGFR

Table 6. Analyzes of the distribution of expression of the pairs of markers in BLBC.

		Basal n (%)
CK5/EGFR	+/+ and at least one + -/-	37(100%) 0(0%)
CK5/CK14	+/+ and at least one + -/-	34(91.9%) 3(9.1%)
CK5/Vim	+/+ and at least one + -/-	35(94.6%) 2(5.4%)
P-cadherin/EGFR	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/CK14	+/+ and at least one + -/-	28(75.7%) 9(24.3%)
P-cadherin/Vim	+/+ and at least one + -/-	31(83.8%) 6(16.2%)
P-cadherin/CK5	+/+ and at least one + -/-	36(97.3%) 1(2.7%)

Table 7. Expression of P-cadherin, vimentin and CK14 in the 72 TN tumors also negative for CK5 and EGFR.

		TN/CK5 and EGFR- n=72
P-cadherin	+ -	29(40.3%) 43(59.7%)
Vimentin	+ -	18(25%) 54(75%)
CK14	+ -	5(6.9%) 67(93.1%)

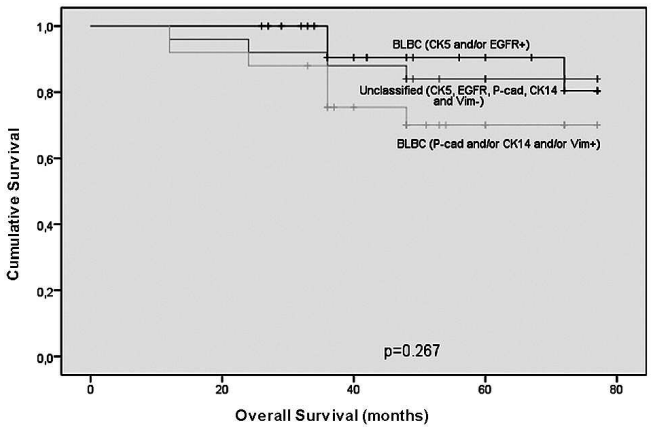

Fig. 3. Kaplan-Meier survival curves for overall survival (OS) of triple negative breast carcinoma patient's cohort, with a 77 months cut-off. BLBC defined by TN/CK5 and/or EGFR+ [BLBC (CK5 and/or EGFR+)], BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin [BLBC (P-cad and/or CK14 and/or Vim)] and tumors that were negative for all the basal markers in study were analyzed, p=0.267 (not statistically significant).

Table 8. Distribution of histological grade among triple negative breast carcinomas of the studied series.

	Histological grade		
Triple negative tumors (n=103*)	I	II	III
BLBC (CK5 and/or EGFR+) (n=34)	3 (9%)	12 (35%)	19 (56%)
BLBC (P-cadherin and/or CK14 and/or Vimentin+) (n=32)	2 (6%)	15 (47%)	15 (47%)
Unclassified (TN,CK5, EGFR, P-cad, CK14 and Vim-) (n=37)	17 (46%)	15 (40%)	5 (14%)

BLBC (CK5 and/or EGFR+) are the TN tumors that were positive for CK5 and/or EGFR and BLBC (P-cadherin and/or CK14 and/or Vimentin+) are the TN/CK5 and EGFR- tumors immunoreactive for one of the additional markers in study: P-cadherin, CK14 and vimentin. *: Histological grade of some cases could not be assessed because the patients were submitted to preoperative chemotherapy.

negative tumors ("unclassified" by Nielsen's criterion). In 38/72 (52.8%) cases, none of the biomarkers were expressed; however, in the other 34/72 cases (47.2%), there was the expression of, at least, one of the biomarkers. P-cadherin was present in 29 (40.3%), Vimentin in 18 (25%) and CK14 in 5 (6.9%) of these tumors (Table 7). In a more detailed analysis, 15 cases were positive only for P-cadherin, while only one and three cases were positive for CK14 and for Vimentin alone, respectively (Fig. 2).

Interestingly, if we consider as BLBC these TN/CK5 and EGFR- "unclassified" cases that presented immunoreactivity for P-cadherin, CK14 and/or Vimentin [BLBC (Pcad and/or CK14 and/or Vimentin+)], this series presents 71/387 (18%) of BLBC. BLBC defined by TN/CK5 and/or EGFR+ and BLBC defined as ER/PR/HER2-, CK5/EGFR- and immunoreactivity for P-cadherin and/or CK14 and/or Vimentin were analyzed separately. These two differently defined BLBC presented a similar percentage of high histological grade tumors [56% and 47% in BLBC (CK5 and/or EGFR+) and in BLBC (Pcad and/or CK14 and/or Vimentin+), respectively], (Table 8). The overall survival was similar for the two groups as we can see in Figure 3.

Discussion

The need for a more precise diagnosis of breast cancer that converges with the clinical outcome and the choice of the most appropriate therapy has motivated studies in different areas of breast cancer research. The cDNA microarray technology is a "gold standard" method for the recognition of the basal phenotype, but from a practical point of view, we need to translate these results to an accessible method. It is undeniable that the BLBC immunohistochemistry definition requires cDNA microarray validation, since these tumors were first identified by this technique (Perou et al., 2000; Livasy et al., 2006). However, from the pathologists and oncologists point of view, the lack of molecular targets for therapy in this subgroup of patients indicates the urgent need for an easier and less expensive way to identify BLBC patients. Based on this, there is an attempt to establish an immunohistochemical surrogate panel, easily applied on FFPE samples, which identifies a pool of breast cancer patients who may require more aggressive systemic therapy and that would be the most appropriate subjects for clinical trials, specifically targeting this molecular subgroup of breast cancer. However, there is still no consensual definition about the ideal IHC panel of biomarkers to distinguish the basal phenotype. In fact, many different panels have been used, in which CK5, EGFR, P-cadherin, CK14 and Vimentin are included. Due to this diversity of criteria, a wide range of percentages of BLBC are described in the several studied series (van de Rijn et al., 2002; Foulkes et al., 2004; Jones et al., 2004; Abd El-Rehim et al., 2005; Arnes et al., 2005; Collett et al., 2005; Kusinska et al., 2005; Laakso et al., 2005; Potemski et al., 2005; Banerjee et al., 2006; Fulford et al., 2006, 2007; Kim et al., 2006; Rakha et al., 2006a,b, 2007a,b,c; Rodriguez-Pinilla et al., 2006, 2007; Siziopikou and Cobleigh, 2007). Nielsen et al. (2004) demonstrated that CK5 and EGFR could reliably discriminate BLBC that were identified by GEP, considering these two basal markers the "gold standard" immunohistochemical panel of antibodies to the BLBC identification, together with ER and HER2 lack of expression. Recently, Cheang et al. (2008) compared two BLBC immuno-panels and concluded that the ER-/PR-/HER2- and expression of CK5 and/or EGFR provides the more accurate definition of BLBC and can better predict breast cancer patient's survival.

However, we cannot assure which are the best antibodies to be included in a daily practice panel for the recognition of the basal phenotype in breast carcinomas: should we look for the most sensitive or the most specific ones? None of these markers are actually pathognomonic of a basal phenotype, since they are variably expressed in the other subgroups of breast carcinomas, which support the search for "ideal" biomarkers to be used in the anatomic pathology workup and with clinical relevance.

We demonstrate herein that P-cadherin, Vimentin or CK14 may possibly be useful biomarkers to include in IHC panels for distinguishing BLBC. P-cadherin reveals consistent values of sensitivity and specificity, while Vimentin and CK14 presented high specificity values. The three markers were able to reliably recognize the basal phenotype, especially when associated to CK5.

The presence of P-cadherin, an adhesion molecule expressed in myoepithelial cells of the normal mammary gland, was already described in invasive and in *in situ* breast carcinomas with worst prognosis, namely in those with high histological grade and basal phenotype (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005, 2007b). The role of P-cadherin in breast carcinogenesis has been one of the main fields of our research group's interest and we have observed that this molecule presents an inverse correlation with HR (Peralta Soler et al., 1999; Gamallo et al., 2001; Kovacs and Walker, 2003; Paredes et al., 2005) and a direct correlation with EGFR (Kovacs and Walker, 2003), HER2 and high proliferation rates, strengthening the value of P-cadherin as a poor prognostic indicator in breast cancer (Palacios et al., 1995; Peralta Soler et al., 1999; Gamallo et al., 2001; Paredes et al., 2005). The expression of P-cadherin in neoplastic cells has already been related to a histogenetic origin in cap cells or to the acquisition of a stem cell-like phenotype, suggesting that P-cadherin-expressing tumors could be associated to a stem cell origin (Peralta Soler et al., 1999, Gamallo et al., 2001, Paredes et al., 2007). Recently, it has been suggested that basal-like breast carcinomas may be genuine stem/early progenitor cell tumors of the mammary gland, relating their origin to a more undifferentiated type of precursor cells (Honeth et al., 2008). Also, Rakha et al. (2009)

demonstrated more evidence of the features of dual-lineage differentiation/stem cell phenotype of BLBC by showing a higher frequency of CK19 expression in this type of tumor.

CK14 does not show a differential presence in breast carcinomas with basal phenotype identified by cDNA microarray technology, but this cytokeratin is frequently associated with poor prognosis (Jones et al., 2004) and with the morphological features observed in BLBC (Tsuda et al., 2000). For this reason, CK14 has been included in the immunopanel used to identify BLBC by several other authors (Laakso et al., 2005, 2006; Rakha et al., 2006a,b; Reis-Filho et al., 2006).

Vimentin is an intermediate filament protein whose expression in normal mammary gland is also restricted to myoepithelial/ basal layer. Its expression has been associated with high histological grade, lack of ER, p53 mutations, high proliferation rates (Raymond and Leong, 1989; Domagala et al., 1990a,b; Koutselini et al., 1995; Santini et al., 1996; Thomas et al., 1999) and expression of CK5/6 and EGFR (Korsching et al., 2005; Reis-Filho, 2005). Vimentin-expressing carcinomas have been observed in association with sporadic and familial BLBC and with a specific pattern of metastasis similar to BLBC (Rodriguez-Pinilla et al., 2007). Like P-cadherin, Vimentin was also described to be differentially expressed by BLBC identified by GEP, being proposed to integrate the panel of antibodies for the identification of BLBC (Livasy et al., 2006).

Our results show that P-cadherin, CK14 and Vimentin, together with CK5, can identify almost all BLBC that were classified as such using the most widely accepted IHC panel to classify BLBC: ER/PR/HER2- and CK5 and/or EGFR+.

Triple negative phenotype by IHC is one of the characteristic features of BLBC and several authors claim that basal tumors are almost all TN tumors (Diaz et al., 2007; Kreike et al., 2007). Kreike et al. (2007), in a series of 97 TN cases, observed that 90% of these tumors have a basal phenotype by cDNA microarray analysis. However, the lack of expression of ER, PR and HER2 as the sole criterion to identify these tumors is risky (Rakha et al., 2008) because there are technique limitations when dealing with FFPE tissue samples, which reinforces the need for a more suitable panel.

There is a significant overlapping of features shared by triple negative and BLBC in what concerns, for example, the prevalence of these types of cancer in younger patients, in African-American women (Morris et al., 2007), their presentation as interval cancers, a similar pattern of recurrence (Dent et al., 2007; Tischkowitz et al., 2007), the more aggressive behavior comparing with other types of breast cancer (Reis-Filho and Tutt, 2008) and the biological and clinical similarity between sporadic TN and BLBC with breast carcinomas arising from BRCA1 mutation carriers (Reis-Filho and Tutt, 2008). However, several studies claim that this overlap is not complete (Bertucci et al., 2008, Rakha and Ellis, 2009). It is known that TN carcinomas with basal phenotype have a significant shorter disease-free survival than TN without expression of basal markers (Rakha et al., 2007a; Tischkowitz et al., 2007) and that germline BRCA1 mutation carriers are more probably found in TN tumors expressing CK5/6 and /or EGFR than in TN with no expression of these basal markers (Turner et al., 2007; Rakha et al., 2009). It has also been observed in GEP that triple negative group is composed by other subgroups of tumors with different outcomes, namely the normal breast-like tumors (Perou et al., 2000; Sorlie et al., 2001, 2003; Sotiriou et al., 2003; Fan et al., 2006; Hu et al., 2006; Hennessy et al., 2009) and a recently described subgroup of claudin-low tumors (Herschkowitz et al., 2007; Hennessy et al., 2009). The existence of TN tumors that do not react immunohistochemically with any of the basal markers routinely used has been described, and variably designated as non basal triple negative, unclassified, undetermined, null phenotype (Liu et al., 2008) or TN3BKE- (Triple Negative 3 Basal Keratins and EGFR-) (Rakha et al., 2009). It seems extremely important to distinguish BLBC from the whole triple negative group, reducing the TN heterogeneity, since their biological behavior appears to be different. The lightening of this heterogeneity would enable patients to benefit from their differential recognition (Rakha et al., 2007a, 2008, 2009; Liu et al., 2008; Reis-Filho and Tutt, 2008; Tan et al., 2008; Rakha and Ellis, 2009). This distinction is also important because TN tumors defined by IHC tend to be clinically considered as BLBC and selected for clinical trials (Bertucci et al., 2008), probably misleading the effect of the drugs in the clinical trials.

It is interesting to emphasize that among the analyzed TN/CK5 and EGFR- tumors that were also negative for P-cadherin, CK14 and Vimentin, approximately 50% of these cases presented low histological grade (Table 8). P-cadherin was expressed alone in a higher number (15 cases) of TN/CK5 and EGFR negative tumors, compared with CK14 (1 case) and Vimentin (3 cases). When P-cadherin, CK14 and Vimentin expression are considered along with CK5 and EGFR for the BLBC identification, 34 cases are added to the 37 already identified BLBC (CK5 and/or EGFR+) and the percentage of basal-like tumors in the pool of TN cases of our series rounds the 65% (71/109). This rate is similar to the one identified by Bertucci (Bertucci et al., 2008), where 70% of IHQ TN tumors presented a basal phenotype by GEP. It is worth noticing that using P-cadherin, CK14 and Vimentin to recruit BLBC from the pool of tumors that could not be classified using only CK5 and EGFR as basal makers, these newly identified BLBC are clinically similar to basal-like tumors identified by Nielsen's criterion, since the majority of the cases presented high histological grade and there are no significant differences in what concerns overall survival of the patients.

Although CK5 and EGFR have been consistently used to recognize BLBC, P-cadherin, CK14 and

Vimentin could also be recruited for an immunohistochemical recognition of BLBC (Paredes et al., 2002, 2007a,b; Matos et al., 2005; Livasy et al., 2006; Rodriguez-Pinilla et al., 2007). Our results showed that these three markers can reliably identify the basal phenotype, especially when associated to CK5, and can be alternative options in this setting. We also demonstrate that P-cadherin, due to its high sensitivity, can recognize possible BLBC among the IHC TN tumors, probably identifying patients with poor prognosis that can benefit from this differential recognition. Pathologists have faced continuous changes in the diagnostic approach of breast cancer and, regarding its classification, it is still controversial whether or not the histological classification should be replaced by the "molecular" taxonomy. Therefore, it is essential to move towards a standardized methodology to establish an IHC panel of biomarkers to the most appropriate recognition of basal-like breast carcinomas.

Conflict of interest. The authors declare that they have no conflict of interest.

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