Quantitative immunocytochemical profile to predict early outcome of disease in triple-negative breast carcinomas

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Abstract. We aimed in this study at identifying prognostic immunohistochemical molecular signatures indicative of disease outcome, also relevant for development of new specific therapies, in triple-negative (ER, PR, c-erbB2-negative) breast carcinoma subtypes. We evaluated 42 markers in tissue micro-arrays from a series of 924 breast carcinomas including 184 triple-negative tumors using standardized quantitative immunocytochemical assays and correlated the data with patients' outcome (mean follow-up of 79 months). When 27/42 markers including basal-like markers first found to be individually significant for prognosis in a univariate analysis (log-rank test) in 924 tumors, were secondly evaluated in the triple-negative tumor subtype (184/924), eleven including maspin, P21, P27, PTEN, caveolin, EGFR, FAK, P38, pMAPK, STAT1 and CD10 were 89.2% predictive of disease outcome in logistic regression. When markers reported in the literature as expressed in basal-like subtype were evaluated in the 924 series, only eight (EGFR, CK14, moesin, caveolin, cMet, ckit, CD44v6, C10) were prognosis predictive in univariate analysis (log-rank test) and in logistic regression were predictive of disease outcome in 66.3% independently of ER, PR and c-erbB2 expression and in 72% in triple-negative tumor subset. The results suggest that the category of 'triple-negative' breast carcinomas does not exactly overlap the basal-like subtype, and that immuno-profiling of triple-negative tumors (not similar to that of basal-like tumors) may be helpful to select patients for more aggressive treatment and provides a basis for development of tailored therapy.

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Introduction

Previous studies have reported immunohistochemical profiles of breast carcinomas of various types according the new taxonomic classification based on DNA array profiling, including luminal A and B, Her-2, normal and basal-like carcinoma subtypes. The lack of tailored therapies for the basal-like subtype has led to research interest and clinico-pathological studies (1-15).

Likewise, the main concern in routine practice, closely related to academic issues, is the identification of molecules, potentially blockable by new therapies that specifically target them and also indicators of poor prognosis within tumors requiring more aggressive treatment that would be useless in patients lacking such an immunocytochemical profile of poor prognosis, despite being categorized in the same subgroup by clinicopathological criteria (such as small node-negative grade 2 tumors).

In view of the high cost of diagnostic genomic assays and of therapy of breast carcinomas which represent a major public health concern, simpler methods would be desirable to identify proteomic signatures *in situ* that are predictive of disease outcome. Detection of these signatures by immuno-histochemistry would allow selection of tumors to be treated by specific and more aggressive therapies, as an alternative method to the molecular biological approach that is more appropriate to basic and academic purposes. However, such immunocytochemical procedures need to be standardized as far as possible before they can be recommended for routine clinical practice (16-22), preferably using quantification of immunoprecipitates with automated computer-assisted devices based essentially on densitometry, as we (16-22) and others have previously done.

Published data show some small variations in immuno-histochemical phenotypes in breast carcinomas (as with DNA assays), probably due to heterogeneity in procedures. Moreover, although molecular and immunohistochemical assays may be dependent on methodological quality (1-15), variations in results can also result from the statistical methods used for data analysis, leading to variable interpretation of biological data (23).

In this study, we aimed, first, to investigate expression of markers reported in the literature in triple-negative tumors

when evaluated with high-throughput standardized assays using tissue microarrays (TMAs) (10,13,17,22) after validation a large retrospective series (n=1200) of breast carcinomas; second, to quantify immunohistochemical precipitates within digitized microscopic TMA images using an automated computer-assisted device; and third, to correlate the quantified immunohistochemical expression of each marker and of groups of markers, with patients' outcome (mean follow-up 79 months). The overall goal was to identify the best group of markers, in terms of sensitivity and specificity, to predict prognosis in triple-negative tumors within 48 h on tissue sections, that would be suitable in clinical practice for individual patients at the time of diagnosis, simultaneously with pathological reporting.

Materials and methods

Patients. The subjects were a consecutive series of 1200 patients with ductal invasive breast carcinoma who were operated on from 1995 to 2002 (mean follow-up 79 months) in the same department (Hôpital Conception, Marseille). Surgery was in all cases the first treatment (PB). For this first step of treatment, patient management was handled by the same group of surgeons and senior pathologists (C.C., S.G. and L.A.). Conservative treatment, mastectomy and node resection (complete or sentinel) were applied according to current European recommendations. Likewise, radiotherapy, chemotherapy and hormone therapy were applied according to criteria currently used at that time.

Analysis of the distribution of the series by age, histological type and grade, and nodal status before TMA construction revealed the usual distribution of breast carcinomas and no bias in tumor selection as previously reported (10,13,22) and as compared to literature data (1-15).

Due to technical difficulties in performing immunocytochemical tests on many serial paraffin sections of a TMA to evaluate the 42 different markers, complete data for all markers were finally obtained for only 924 patients out of the initial series of 1200.

The 2005 follow-up data in clinical records showed that 181 out of the 924 tumors were metastatic and 32 patients deceased.

Our study focused mainly on correlation of immunohistochemical data with patients' outcome. Current histoprognostic criteria were not further considered for statistical analysis, mainly to limit the burden of data and also to focus the statistical analysis on continuous variables homogeneously obtained by (numerical) densitometric measurements of immunoprecipitates with the image analyser.

Tissues. Tissue samples were all taken from surgical specimens after formalin fixation. Attention was paid to optimal homogeneous tissue handling, including fast immersion in buffered formalin in appropriate containers in the operation room by pathologists or by nurses trained in the procedure. Tumor fragments were large and thick enough to allow further TMA construction. Duration of fixation was 24 h for smaller samples (<5 cm) and 48 h for larger ones, to improve formalin penetration, before specimen dissection at room temperature. After fixation, paraffin pre-embedding and embedding were performed in currently available automated devices of the same brand.

All paraffin blocks were stored in the same room where temperature was maintained at 20˚C prior to TMA construction.

TMA construction. The procedure for TMA construction was as previously described (10,13,17,22). Briefly, cores were punched from the selected 1200 paraffin blocks (from 1200 patients), distributed in new blocks including two cores for each tumor (200 cases per block, a total of 2400 cores, measuring 0.6 mm in diameter). All the new blocks were also stored at 4˚C before sections 4-μm thick were prepared for each marker to be examined by immunohistochemical assay.

Immunohistochemistry. Serial tissue sections were assessed and stored at 4˚C 24 h before immunohistochemical processing, as previously reported (16-22). The immunoperoxidase procedure was performed using an automated Ventana Benchmark XT device and Ventana kits.

Markers were detected using commercially available documented antibodies (Table I). Dilutions of antibodies were determined by prescreening on the usual full 4-μm thick sections prior to use on TMA sections.

Image analysis. Automated densitometric measurements of immunoprecipitates in cores were assessed for each marker antibody in each core individually identified after digitization and image cropping of the slides, as previously reported (10,13,22). Briefly, TMA analysis with a SAMBA 2050 automated device (SAMBA Technologies, TRIBVN, France) was performed according to the following protocol.

First, an image of the entire slide was built up using a low power magnification (x2, pixel dimension 3.7 μm). This image was made of a mosaic of images acquired along a rectangular grid with contiguous fields. Second, the area of the slide containing the TMA cores was automatically delineated and scanned at higher magnification (x10, pixel dimension 7.4 μm). Third, after autofocusing, the images were acquired with an overlap greater than the largest mechanical positioning error. Using the image contents, a matching algorithm determined precisely the relative position of each image with respect to its neighbors. Calculated overlap was removed from images to produce a new set of higher-magnification images (x20), thus covering precisely the cores of interest. A specially developed tool referred to as TMA crop then allowed superimposition of the TMA grid onto the reduced image and precise alignment of each node of the grid with the core location within the image. The final step was performed automatically using the core image contents to ensure pixel precision of the match. From the images acquired with x20 magnification, a new set of images was next computed, one for each core. After color analysis of the core images, the SAMBA 'immuno' software was applied as previously reported (16-22) in the usual full tissue sections.

In the present study, we correlated the patients' follow-up parameters with a quantitative score combining the surface stained and the intensity of staining computed by the Samba 'immuno' software.

Table I. The antibodies used in this study.

	Antibody	Supplier	Source[a]	Clone
1	FGFR-1 Flg (C-15)	Santa Cruz	Rpab	FGFR-1 Flg (C-15)
2	Maspin	BD Pharmingen	Mmab	G167-70
3	P-Cadherin	Novocastra	Mmab	56C1
4	Ezrin (p81, 80 k, cytovillin)	Neomarkers	Mmab	3C12
5	CD 44v6	Novocastra	Mmab	VFF-7
6	Moesin 1	Biomeda	Mmab	38/87
7	Cytokeratins 8 & 18	Zymed	Mmab	Zym5,2(UCD/PR-10,11)
8	Cytokeratin 17	Dako	Mmab	E3
9	Cytokeratin 14	Novocastra	Mmab	LL002
10	Phospho-STAT3	Cell Signaling	Mmab	Tyr 705 D3A7
11	CD 10	Novocastra	Mmab	56C6
12	CD 34	Dako	Mmab	QBEnd-10
13	Vimentin	Immunotech	Mmab	V9
14	Cytokeratin 19	Dako	Mmab	BA17
15	Phospho-MAPKAPK-2	Cell Signaling	Rmab	(Thr334)
16	EGFR	Ventana	Mmab	3C6
17	STAT-1	Cell Signaling	Mmab	9H2
18	FAK	Cell Signaling	Rpab	
19	p38 MAP kinase	Cell Signaling	Rpab	
20	P27 Kip1	Cell Signaling	Rpab	
21	P21Waf1-Cip1	Cell Signaling	Mmab	DCS60
22	SHARP 2	Abcam	Rpab	
23	FYN	Abcam	Mmab	1S
24	P63	Dako	Mmab	4A4
25	Cytokeratin 903	Dako	Mmab	34BE12
26	CA IX	Abcam	Rpab	
27	E-Cadherin	Zymed	Mmab	4A2C7
28	CD 117 (c-Kit)	Dako	Rpab	
29	Cytokeratins 5-6	Dako	Mmab	D5/16B4
30	PTEN	Cell Signaling	Mmab	26H9
31	PI3 kinase	Cell Signaling	Rpab	
32	JAK 1	Cell Signaling	Rpab	
33	c-Met	Chemicon/Abcys	Mmab	4AT44
34	Caveolin 1	Santa Cruz	Rpab	
35	CD 105	Dako	Mmab	SN6h
36	CD 146	Novocastra	Mmab	N1238
37	Bcl2	Dako	Mmab	124
38	P53	Dako	Mmab	DO-7
39	P16	Neomarkers	Mmab	Ab7(16PO7)
40	c-erbB2	Novocastra	Mmab	CB11
41	PR	Ventana	Mmab	1E2
42	ER	Ventana	Mmab	6F11

[a]Mouse monoclonal antibody; rabbit polyclonal antibody.

Statistical analysis. Immunohistochemical expression of each marker was first correlated to patients' disease-free survival using NCSS and Statistica statistical software.

When significant differences in mean expression were identified between patients with and without disease-free survival, the prognostic significance was determined by log-rank tests (Kaplan-Meier curves). The appropriate threshold of prognostic significance for a given marker was determined as recommended (24) and as previously assessed in large tissue sections (16-21) and in TMAs (10,13,22).

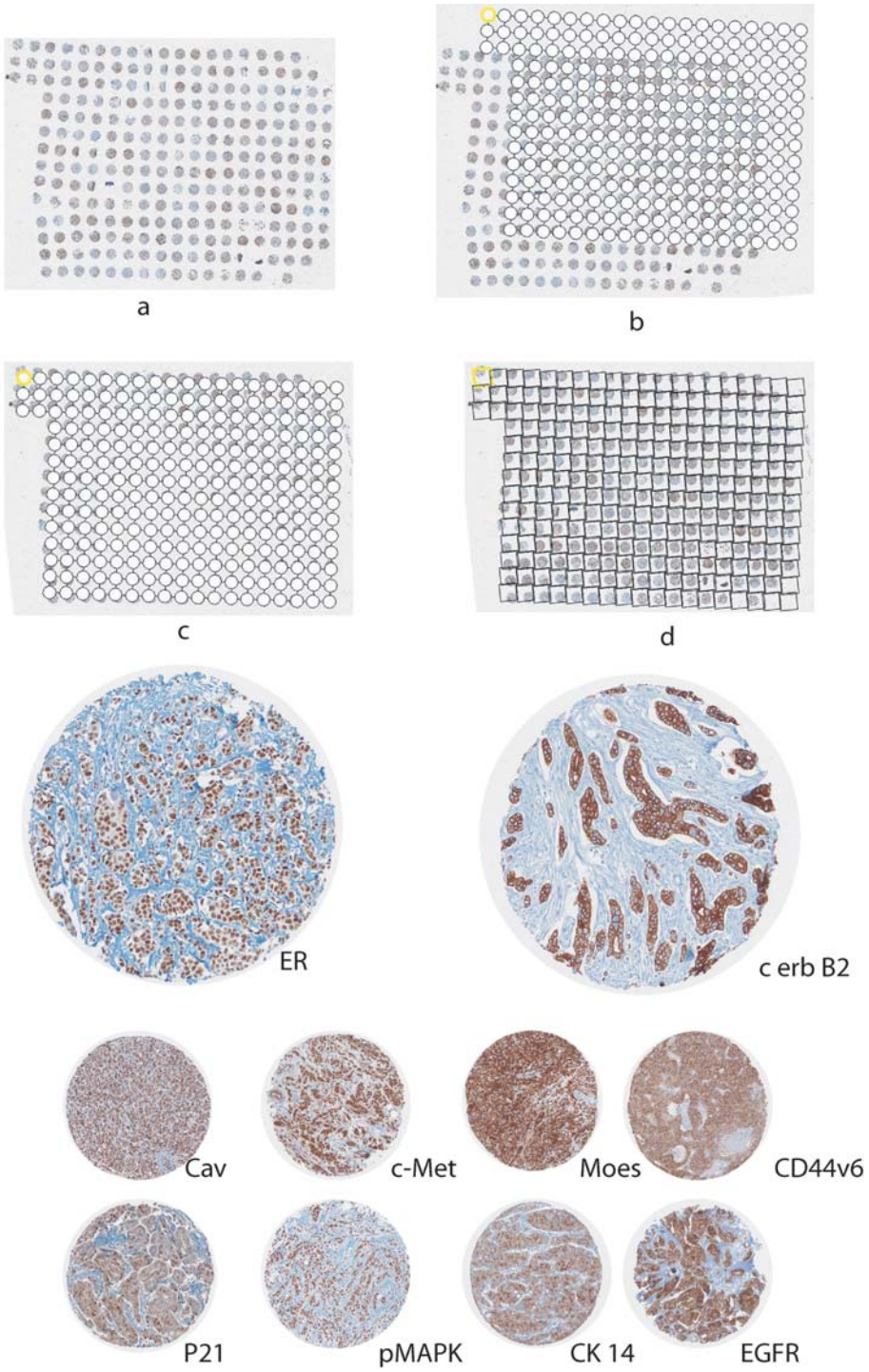

Figure 1. TMA enclosing 268 cores (0.6 μm in diameter) from breast carcinomas: An example of immunohistochemical test and 'cropping' procedure (a, b, c and d) consisting in superimposing a standard grid on the TMA image by a modifying grid shape in order to separate each spot prior to densitometry measurement.

Logistic regression (with ROC curves) was then used to identify the combination of markers with the best sensitivity and specificity of a proteomic signature indicative of prognosis.

Finally, unsupervised hierarchical clustering of significant prognostic indicators in the overall series enabled the relationship of the markers providing qualitative data to be compared with previously reported research results on the role played by these molecules in the process of cancer metastasis.

Results

Immunostaining in TMAs is illustrated in Fig. 1. Table II shows that 27 markers were prognostically significant in univariate analysis after determination of the cut-off value according to Altman *et al* (24) (Fig. 2), in 924 tumors for which complete immunocytochemical data could be obtained.

Logistic regression was performed for those markers with prognostic significance in the univariate log-rank analysis in

Table II. Optimal thresholds of quantitative scores for 27 markers of prognostic significance in univariate analysis log-rank test, as determined according to the method of Altman *et al* (24) for 924 patients with breast cancer.

		P-value (log-rank)	Quantitative score threshold
1	SHARP 2	<0.001	5.6
2	PR	<0.0001	7.9
3	ER	<0.01	9.3
4	STAT1	<0.0001	10.7
5	PTEN	<0.0001	2.7
6	pSTAT-3	<0.01	4.4
7	pMAPK	<0.001	12.5
8	P38	<0.001	1.1
9	P27	<0.01	5.3
10	P21	<0.001	10.3
11	P16	<0.01	7.5
12	Moesin	<0.0001	16.4
13	Maspin	<0.001	7.3
14	CK14	<0.001	8.3
15	FYN	<0.01	2.7
16	FGF-R	<0.01	23.0
17	FAK	<0.001	2.0
18	Ezrin	<0.001	3.9
19	c-Kit	<0.001	12.9
20	c-Met 2	<0.01	17.0
21	EGFR	<0.01	9.4
22	CK19	<0.01	4.6
23	c-erbB2	<0.001	28.0
24	CD 44 v6	<0.001	11.9
25	CD 146	<0.001	2.3
26	CD 10	<0.01	4.0
27	Caveolin	<0.01	29.0

patients with ER-, PR- and c-erbB2-negative tumors (n=184/924), of which 168/181 were disease-free and 16/181 metastatic.

The first-step regression showed that with 24 markers (Table III) (Fig. 2a and b), 89.7% of the patients were well classified (15/16 metastatic patients with the 24-marker signature and 18/168 disease-free patients with the 24-marker signature), with 93.8% sensitivity and 89.3% specificity. In a second-step regression using an 11-marker signature including EGFR, caveolin, FAK, maspin, P21, P27, PTEN, P38, pMAPK, STAT-1 and CD10 (Table IV and Fig. 3), 89.1% of patients were well classified.

Unsupervised hierarchical clustering of the 24 prognostic markers is shown in Fig. 4 (independently of ER, PR and c-erbB2 status) (Fig. 4).

Eight markers of the basal-like subtype (c-Met, caveolin, moesin, CD44v6, CK14, EGFR, c-Kit and CD10) reported in the literature (1-15) and predictive of disease outcome on log-rank univariate analysis in the series of 924 patients were evaluated by logistic regression (Table V, Fig. 3) independently

Table III. Logistic regression of 24 markers of prognostic significance in triple-negative tumors (n=184/924) in univariate log-rank test.

	Immunocytochemical marker	P-value
1	c-Kit	0.068
2	CD10	0.0001
3	STAT-1	0.0003
4	SHARP2	0.44297
5	PTEN	0.0238
6	pSTAT3	0.56922
7	pMAPK	0.00224
8	P38	0.04678
9	P27	0.0077
10	P21	0.00015
11	P16	0.1621
12	Moes	0.0559
13	Maspin	0.00251
14	CK14	0.11288
15	FYN	0.40538
16	FgFR1	0.99544
17	FAK	0.00974
18	EGFR	0.0782
19	c-Met	0.56346
20	CK19	0.26917
21	CD44v6	0.54556
22	CD146	0.39627
23	CD10	0.0088
24	Caveolin	0.01813

Table IV. Logistic regression in triple-negative (n=184/924) tumors with 11 markers.

	Immunocytochemical marker	P-value
1	STAT1	0.024
2	PTEN	0.0422
3	pMAPK	0.02568
4	P38	0.04002
5	P27	0.00747
6	P21	0.00022
7	Maspin	0.00543
8	CD10	0.00061
9	FAK	0.01148
10	EGFR	0.0018
11	Caveolin	0.04868

of ER, PR and c-erbB2 status. The first-step regression showed that 66.3% (69.6% sensitivity and 65.5% specificity) of the patients were well classified, including 126/181 metastatic patients (with the eight-marker signature) and 218/743 disease-free patients (without the eight-marker

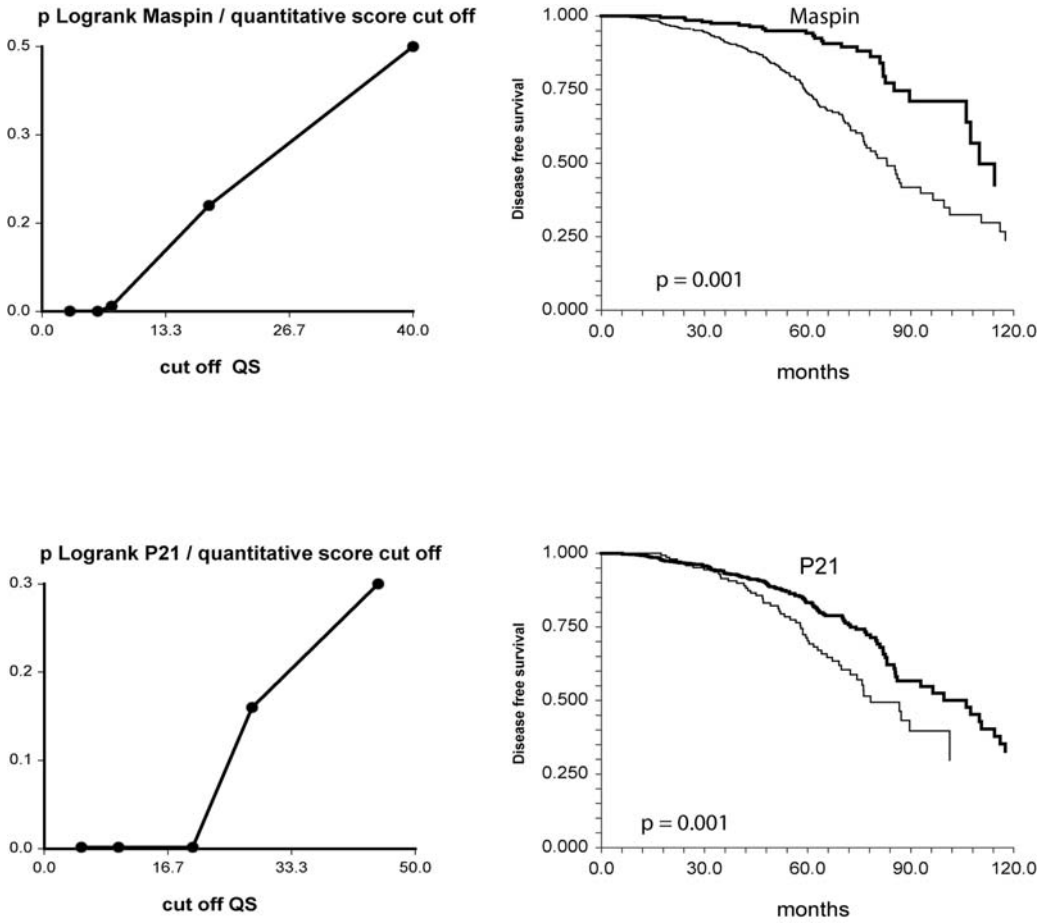

Figure 2. Kaplan-Meier curves (log-rank test) and p-value curves [according to Altman *et al* (24)] used to determine the threshold of quantitative scores for prognostic significance of markers; in this example, P21 and maspin in 924 breast carcinomas (TMA, quantitative immunochemical assays).

Table V. Logistic regression of eight basal-like markers predictive of prognosis in log-rank univariate analysis in 924 breast carcinomas.

	Immunocytochemical marker	P-value
1	EGFR	0.09417
2	CK-14	0.00119
3	Moesin	0.00001
4	Caveolin	< 0.0001
5	c-Met	0.00169
6	cKIT	0.00118
7	CD44v6	0.00187
8	CD10	0.00577

signature). In a second-step regression with seven markers (excluding EGFR), a similar 64.8% of patients were well classified (Table V, Fig. 3).

When markers of basal-like carcinomas were considered in triple-negative tumors, the first step of logistic regression with 11 markers showed that 72% of patients were well classified and in a second step regression with 10 markers, 71.6% were well classified (Tables VI and VII and Fig. 3).

Table VI. Logistic regression with basal-like markers that were prognostically predictive in univariate log rank test, when ER, PR and c-erbB2 are included in the regression in 924 breast carcinomas.

	Immunocytochemical markers	P-value
1	Caveolin	0.0002
2	CD44v6	0.03555
3	c-erbB2	0.0002
4	c-KIT	0.00101
5	c-Met	0.00012
6	EGFR	0.04559
7	CK14	0.00204
8	Moesin	0.00002
9	CD10	0.00388
10	ER	0.00888
11	PR	< 0.0001

Discussion

Immunocytochemical signatures of triple-negative and basal-like tumors. Several published studies (1-15) have

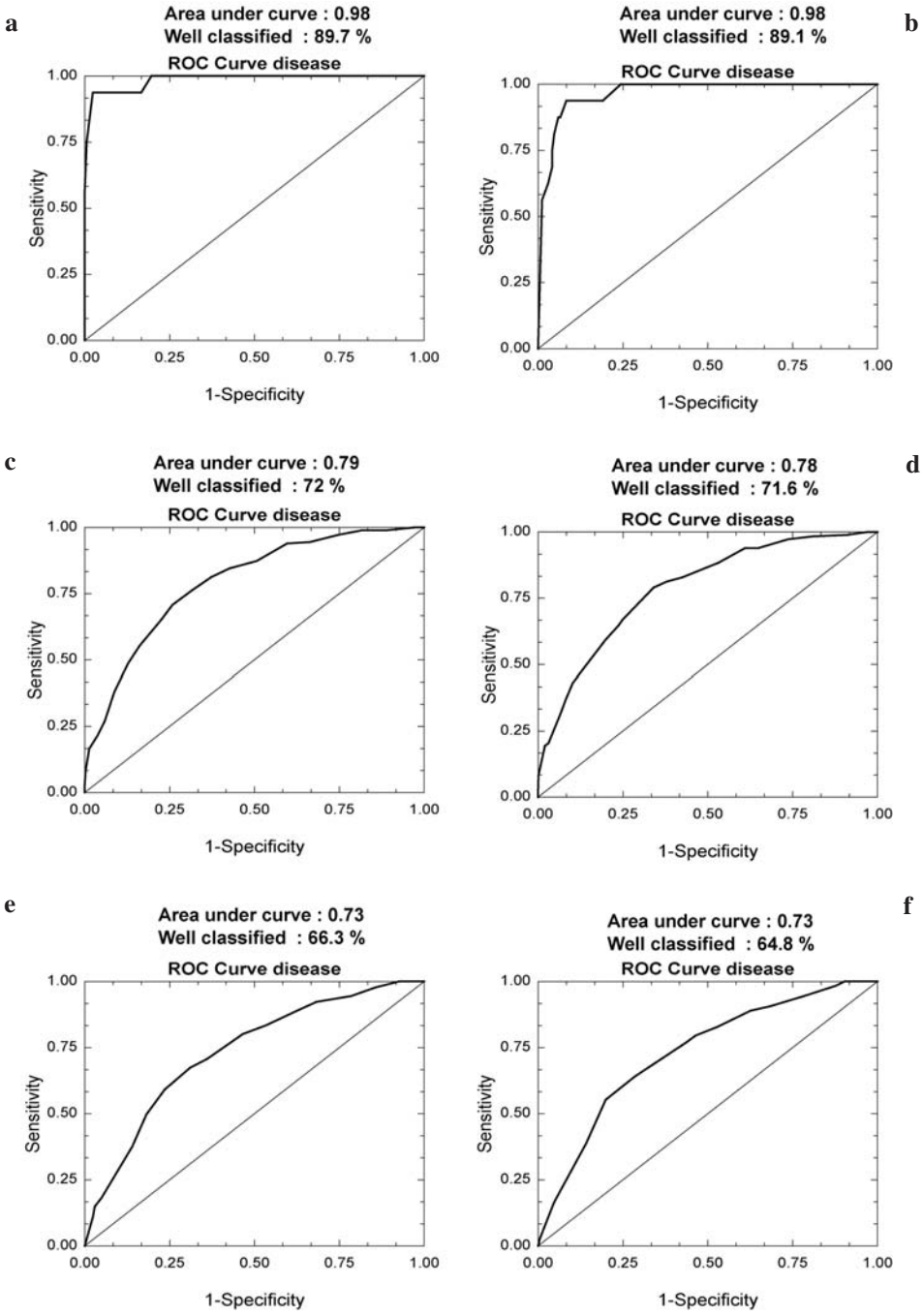

Figure 3. Logistic regression and ROC curves first (a, c, e) and second (b, d, f) steps: (a) with 24 and (b) with 11-marker signatures in triple-negative tumors (n=184) (89.7 and 89.1% well classified patients); (c) with 8 and (d) 7 basal-like markers in triple-negative tumors (72 and 71.6%) well classified patients; and (e) 8 and (f) 7 basal-like markers in the complete 924 series (66.3 and 64.8% well classified patients).

documented basal-like carcinomas that represent a subtype of breast carcinomas lacking tailored therapies, since they most often lack ER and PR receptors and c-erbB2 amplification. However, some recent studies (7,8) have shown that triple-negative tumors may not be entirely equivalent to basal-like carcinomas. Although exhibiting immunoprofiles closely similar to that reported for the basal-like subtype, triple-negative carcinomas respond to anthracyclines but, like BRCA1 tumors, have lower sensitivity to taxans, are very aggressive tumors with early recurrence and short survival (8) and also have epithelial mesenchymal transition or basal myoepithelial profiles (25-27). In fact, these triple-negative tumors lack an internationally accepted definition, which will depend upon the thresholds used for ER- and PR-negativity.

In our study, we used quantitative immunohistochemical assays to identify a signature giving 89.1% prediction of poor prognosis and early recurrence with 11 markers in triple-negative tumors. The ER- and PR-positive status was defined according to the quantitative score threshold for ER and PR prognostic value in a log-rank univariate analysis, after validation of cut-off points using the curve of p-values according to the recommendations of Altman *et al* (24), as shown in Table II and Fig. 2. This 11-marker signature (out of 24 including markers of basal-like subtype and others)

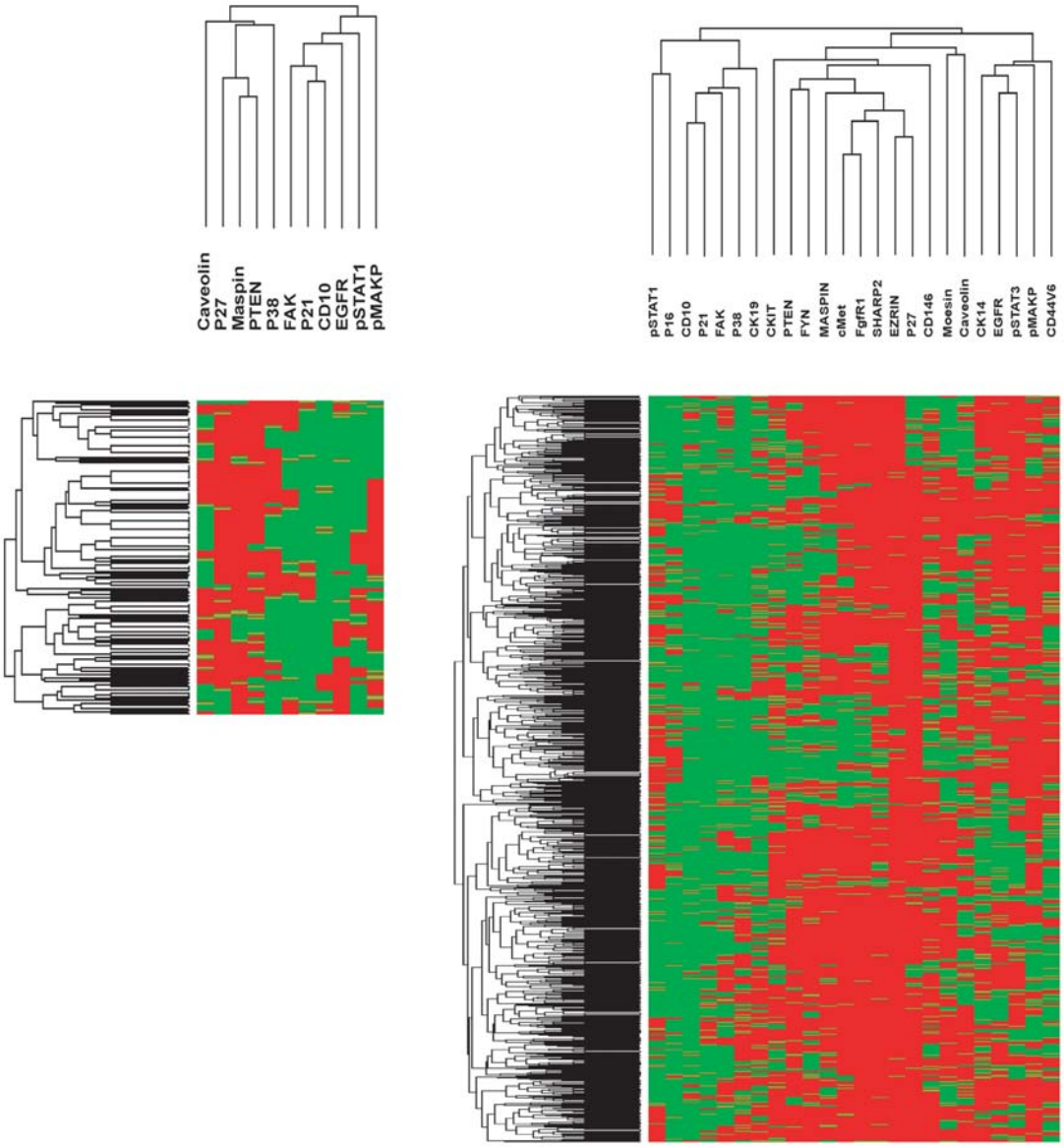

Figure 4. Unsupervised hierarchical clustering of the 24 markers with prognostic significance in the log-rank test, from quantitative densitometry of immunohistochemical assays on TMA in the 924 series of patients with breast carcinoma and of the 11 markers prognostic significant in the 184 triple-negative carcinomas.

Table VII. Summary of logistic regression with immunoprofiling of basal-like tumors in 924 breast carcinomas.

Signatures/Immunocytochemical markers of prognosis	Sensitivity	Specificity	Well classified	Number of patients well classified (n=924)	
	%	%	%	Positive and events	Negative and no events
'Basal-like' without ER, PR, c-erbB2[a] (n=8 markers)	69.6	65.5	66.3	126/181	256/743
Second regression (n=7)	74.6	62.45	64.8	135/181	279/743
'Basal-like'with ER, PR, c-erbB2[a] (n=11 markers)	70.66	74.6	72.0	135/181	218/743
Second regression (n=10)	71.3	71.7	71.6	129/181	210/743

[a]Included or not in the regression.

included caveolin, EGFR, FAK, maspin, P21, P27, PTEN, P38, pMAPK, STAT-1, CD10 (plus negative ER, PR, c-erbB2) (Table IV). When only basal-like markers, selected according to literature data and prognostically significant in univariate analysis of our series, were included in the logistic regression to predict disease outcome, 72% of patients were well classified with 8 markers including EGFR, c-Kit, moesin, caveolin, CD10, c-Met, CD44v6, in ER-, PR- and c-erbB2-negative tumors (Table IV). This result suggests that categories of triple-negative and basal-like tumors do not completely overlap, in terms of the quantitative immunocytochemical assays that we used, as previously shown (8).

Prognostic value of markers in signatures. Marker proteins involved in tumor cell motility and spreading were of particular interest in our study to predict patients' outcome, which is highly dependent on development of metastasis. Some studies have underlined the role of c-Met in tumor spreading (reviewed in ref. 10). We and others have shown the significance of c-Met for poor prognosis in breast cancers (10,21,22) and also that of CD44v6 (28).

Recent studies have shown the synergistic role of CD44v6 and c-Met in several types of tumor cells (28) and the interaction of the EMR (ezrin-moesin-radixin) superfamily and CD44v6 for HGF activation of c-Met to promote the ERK signalling cascade, inducing cell migration. Moreover, EMR components act on cell adhesion (integrin ß2) and the cytoskeleton (29,30). FAK (focal adhesion kinase) is also known to play a pivotal role in the control of integrin-mediated cell functions including cell migration, progression and survival, co-acting with c-Met and EMR (31). Consistent with these findings, CD44v6, c-Met, moesin and FAK are included in the immunocytochemical signature of poor prognosis determined by logistic regression for early (79 months) metastatic disease in basal-like carcinomas, but not in triple-negative tumors.

Cytokeratin 14 expression in association with EGFR in basal-like carcinomas has already been shown to be a strong indicator of poor outcome and particularly predictive in relation to lung and brain metastases. Our results correlate with these previous studies (6,7,26,27).

CK14 is found in metaplastic carcinomas in association with P63 and EGFR (25,26). CK14 was a significant indicator of risk of metastasis in our study in basal-like carcinomas but not in triple-negative tumors. CK5-6 with CD10 was also previously shown to be a prognostic indicator (5).

Caveolins are membrane proteins involved in membrane trafficking, gene regulation, signal transduction and mediation of intracellular processes, as well as in carcinogenesis, being overexpressed in 4 to 57% of invasive breast carcinomas (32,33). Conflicting results on the prognostic significance of overexpression in breast carcinomas have been reported (32). More specifically, caveolin has been reported in basal-like and metaplastic breast carcinomas (26,32). Interestingly, our results show that caveolin in invasive breast carcinomas is definitely associated with poor prognosis in univariate analysis and in triple-negative tumors and along with markers of tumor cell spreading (and increased motility) in basal-like tumors after logistic regression.

High-grade breast tumors with reduced expression of tumor-suppressor genes such as PTEN and P21 and P27, are associated with poor outcome. In our study, reduced expression of P21 and P27 showed prognostic significance in logistic regression in triple-negative tumors and are probably also predictors of responsiveness to chemotherapy.

Prediction of reponse to therapy and immunocytochemical profiling. New approaches to molecular typing of tumors are relevant to prognosis, but also to prediction of response to therapy. Therefore, markers identified as prognostic indicators can also be regarded as indicators of responsiveness to current chemotherapies or as targets for tailored therapies. For example, caveolins, moesin and CD44v6 have been shown to be indicators of responsiveness to anthracyclines and paxitaxel (8). Also, caveolins have been shown to be predictors of response to platinum salts (reviewed in ref. 8).

Caveolins have recently been shown to be overexpressed and amplified in a subset of basal-like and metaplastic breast carcinomas (3,5,32). Savage *et al* (32) showed that caveolin 1 expression was significantly associated with basal-like subtype and with shorter disease-free and overall survival on univariate analysis. ABI-007 is a novel, biologically interactive, nanometer-sized albumin-bound paclitaxel particle initially developed to avoid toxicity associated with polyethylated castor oil (34). In a phase III study, Gradishar *et al* (34) compared ABI-007 with polyethylated castor oil-based paclitaxel in women with metastatic breast cancer. ABI-007 showed greater efficacy and a favorable safety profile, though no subgroup analysis of molecular phenotypes for differential efficacy of the treatment was performed. After incorporation of ABI-007 with albumin into the blood circulation, ABI-007 is preferentially transported from the blood to the tumor site in two ways. One route is through leaky junctions of endothelial cells that are highly pronounced around the tumor tissue by induction of angiogenesis. The second, perhaps more prominent, way is through receptor-mediated transcytosis of this albumin-bound ABI-007. This second mechanism is mediated by caveolin. Breast cancer patients with higher caveolin 1 expression may therefore show better efficacy and a more favorable safety profile when treated with ABI-007 (35).

Moesin has already been shown to be overexpressed in breast cancer of poor prognosis such as the basal-like subtype (5). Our study has confirmed this finding in a large series, showing in both univariate analysis and logistic regression that moesin has prognostic significance in tumors expressing other basal-like markers, but not in triple-negative ones.

Dasatinib is a small molecule orally active as a kinase inhibitor of both the src and abl proteins. Finn *et al* (36) recently reported that it selectively inhibits growth of basal-like and 'triple-negative' breast cancer cell lines growing *in vitro*. Interestingly, they identified a set of three biologically relevant genes whose elevated expression is associated with dasanitib inhibition, including moesin, caveolins and yes-associated protein, with sensitivity and specificity of 88 and 86%, respectively. Therefore dasatinib may also be an effective treatment option in breast cancer subtypes with caveolin expression (36). Dasananib is also active against PDGF-R and c-Kit and has been shown to be efficient in

leukemia after failure of imatinib therapy (37). However, response to imatinib and dasatinib in breast carcinomas with c-Kit expression remains to be demonstrated. Likewise, sunitinib, that is recommended in gastrointestinal stromal tumors (GIST) expressing c-Kit and in advanced kidney carcinomas, is an inhibitor of angiogenic tyrosine kinase receptors involved in angiogenesis such as PDGR, VEGFR, FLT3 and c-Kit. This suggests that c-Kit in breast carcinomas of poor prognosis could also be targeted by sunitinib.

Antibodies against c-Met and small molecules such as PHA66752 or kerin that target c-Met, or the NK4 molecule that blocks HGF binding to c-Met have been reported (reviewed in ref. 10) to act as specific inhibitors of c-Met and signalling pathways including P38, pMAPK, ERK, FAK and P21 in breast cancer, while other tyrosine kinase inhibitors such as Iressa, Tarceva, Herceptin and Genifinib do not inhibit c-Met activity (38).

EGFR is expressed in tumors of poor prognosis and has been included in the signature for poor prognosis of triple-negative and basal-like carcinomas (1,2-9,11,12,15). Interestingly, we also observed that significantly increased expression of the STAT-3 and STAT-1 signalling pathway, resulting partly from EGFR activation, was associated with poor outcome, like others (reviewed in ref. 8), suggesting that EGFR is activated when immunocytochemically over-expressed.

Malignant EGFR-expressing tumors have been shown to respond to tailored therapies like cetuximab (a monoclonal antibody against EGFR), lapatinib (an EGFR tyrosine kinase receptor inhibitor), or gefitinib or enlotinib, which are inhibitors of cellular kinase activity. Although cetuximab has been shown to reduce death in laryngeal tumors and gefitinib and enlotinib in non-small cell lung cancer (8), no data are available on levels of EGFR expression in breast carcinomas and prediction of tumor responsiveness to specific therapy. But new tools are now available to rapidly detect amplification of the c-erbB2 gene by FISH/CISH/SISH so as to better identify tumors potentially responsive to these specific therapies.

In conclusion, using quantitative standardized immunocytochemical assays, we measured some established markers of basal-like breast carcinomas, such as EGFR, CK14, c-Met, caveolins, moesin, CD44v6, c-Kit, CD10, that individually are predictive of disease outcome in log-rank univariate analysis and together are 66.3% predictive of poor prognosis, but are 72% predictive when associated with ER-, PR- and c-erbB2-negative status. When triple-negative tumors were considered among 24 markers found to be individually prognostically significant in univariate log-rank analysis, the association of 11 markers (EGFR, maspin, P21, P27, PTEN, P38, pMAPK, STAT-1, caveolin, FAK and CD10) was 89.2% predictive of poor prognosis and probably indicative of responsiveness of current chemotherapy. Our results suggest that i) triple-negative and basal-like tumors are not identical types, since their prognostic immunoprofiles do not overlap and ii) the immunomolecular signatures of both of these types may be helpful in current practice to select patients for more aggressive therapy and to develop new, more specific targeted therapies.

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