Immunohistochemical profiling of node negative breast carcinomas allows prediction of metastatic risk

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Abstract. The aim of this study was to identify a prognostic immunohistochemical signature indicative of risk of early metastasis in node-negative breast carcinomas that would also be relevant to the development of new tailored therapy. Quantitative measurements of the immunohistochemical expression of 64 markers (selected from literature data) using high-throughput densitometry (as a continuous variable) of digitised microscopic micro-array images were correlated with clinical outcome in 667 node-negative breast carcinomas (mean follow-up 102 months). Multivariable fractional polynomials model of logistic regression allowed the selection of the best combination of markers (in terms of sensitivity and specificity) to predict patient outcome without any categorisation using predefined cut-points for individual marker measurements. A highly predictive ten-marker (out of 64) signature was identified comprising PI3K, pmTOR, pMAPKAPK-2, SHARP-2, P21, HIF-1α, Moesin, p4^{E}BP-1, pAKT and P27 that well classified 91.4% of node-negative patients (specificity 90.9%, sensitivity 93.7%, area under ROC curve 0.958) independently of estrogen receptors (ER), and progesterone receptors (PR) and HER-2 status (91.6% well classified patients when ER, PR, HER-2 excluded). It is concluded that quantitative immunoprofiling of node-negative breast carcinomas is helpful in selecting patients who should not receive aggressive adjuvant chemotherapy and provides data for the development of tailored therapy.

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Introduction

The incidence of early-stage breast carcinomas, particularly lymph node-negative cases, has increased with the implementation of breast cancer screening programs in Western countries. Patients with node-negative breast cancer have a fairly good ten-year overall survival with loco-regional treatment alone, only 30-40% developing distant metastases (1). However, most patients are offered chemotherapy according to current guidelines, leading to over-treatment of a large proportion (2,3), since there is no means of clearly identifying those patients who will not relapse and hence do not need adjuvant chemotherapy. Therefore, markers to identify patients not requiring aggressive adjuvant therapy are urgently needed, so as to avoid unnecessary exposure of women to the potential toxicity and side-effects of such treatment, and also to reduce the overall cost of breast cancer management.

The development of high-throughput techniques such as gene expression profiling has significant potential for the identification of prognostic classifiers (4-10). Several studies have reported gene signatures predictive of prognosis in node-negative breast carcinomas (11-13). However, these assays i) usually require frozen tissue, and this sampling can be detrimental to pathological diagnosis in small tumours, and ii) large amount of data provided by gene expression micro-arrays may be somewhat liable to misinterpretation or poorly validated (14-16).

In contrast, immunohistochemical assays require only a small amount of tissue easily obtained from paraffin blocks used for diagnosis, and can be standardised by quantification of immunoprecipitates and automated devices (17-21). Our objective was to identify an immunohistochemical signature of poor prognosis (risk of early distant metastasis), in cases of node-negative breast carcinoma, which would be economically acceptable. For this purpose, we assessed 64 markers (a large panel of known prognostic markers of tumour cell growth and proliferation, invasion and scattering, and angiogenesis, in addition to markers of signaling pathways) by standardised quantitative immunohistochemistry (IHC). We used the

SAMBA/TRIBVN device (22-28) that provides accurate numerical data for statistical analysis of continuous variables, with high-throughput assays using TMAs (29-41) in a large retrospective series (n=667) of node-negative breast carcinomas. We have recently reported our experience with this standardised method for quantifying immunoprecipitates (42,43), which proved to be of potential clinical relevance through identification of immunohistochemical signatures of prognosis. However, methods relying upon dichotomising continuous predictors (42,43), using cut-points in logistic regression are controversial (14-15,44,45). Therefore, in the present study, we correlated the quantified immunohistochemical expression of each marker, then of groups of markers (referred to as immunohistochemical signatures), with the patient outcome (mean follow-up 102 months), in order to identify the combination of markers with the best sensitivity and specificity to predict prognosis in terms of occurrence of early distant metastases, using a multivariable fractional polynomials method (46,47). This approach allowed us the use continuous values of variables (densitometry of markers immunoexpression) so that the correlation between a continuous predictor and the outcome variable could be evaluated in some form of non-linearity.

Materials and methods

Patients. We studied a consecutive series of 667 patients with invasive breast carcinomas who were operated from 1996 to 2002 in the same departments at the Conception and Nord hospitals in Marseille. Surgery was in all cases the first treatment. For this first step of treatment, patient management was handled by the same group of surgeons (PB, LB and XC), and the diagnosis was assessed by three senior pathologists (CC, SG and LA). Conservative treatment and node resection (complete or sentinel) were applied according to current European recommendations. Likewise, radiotherapy, chemotherapy and hormone therapy were applied according to criteria used at that time.

Due to technical difficulties in performing IHC tests on many serial paraffin sections of a TMA to evaluate the 64 different markers, complete data for all markers were eventually obtained for only 572 patients out of the initial series of 667. The 2007 follow-up data in clinical records (mean follow-up 102 months from 1996 to 2007) showed that 111 of 572 patients had distant metastases. The age of patients at diagnosis ranged from 40 to 65 (mean 57) years. Briefly, tumours were pT1b (32%) and pT1c (68%). All were invasive ductal carcinomas not otherwise specified and 29% were grade 1, 59% grade 2 and 12% grade 3 (Ellis and Elston grading method).

Our study focused mainly on correlation of quantitative immunohistochemical data with the patient outcome, independently of pT and tumour grade. Therefore, current histoprognostic data were not retained for statistical analysis, mainly to limit the burden of data and also to focus on the statistical analysis on continuous variables homogeneously obtained by (numerical) densitometric measurements of immunoprecipitates obtained with the image analyser.

Tissue. Tissue samples were all taken from surgical specimens after formalin fixation. Attention was paid to optimal and consistent tissue-handling procedures, including fast immersion in buffered formalin in an appropriate container by pathologists or by nurses trained in the procedure. Tumour samples were large and thick enough to allow subsequent TMA construction. Duration of fixation was 24 h for smaller resections (<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 with currently available automated devices of the same brand. All paraffin blocks were stored in the same room, where the temperature was maintained at 20˚C prior to TMA construction.

TMA construction. The procedure for construction of TMAs was as previously described (22,27,28,42,43). Briefly, cores were punched from the selected 667 paraffin blocks, and distributed in three new blocks including two cores of 0.6 mm diameter for each tumour (about 220 cases per block). All the TMAs blocks were stored at 4˚C.

Immunohistochemistry. Serial tissue sections were prepared 24 h before immunohistochemical processing and stored at 4˚C, as previously reported (22-28,42,43). The immunoperoxidase procedure was performed using an automated Ventana Benchmark XT device which allowed similar well controlled antigen retrieval for all tumours, and Ventana kits. Markers were detected using 64 commercially available and documented antibodies (except anti-HIF-1α kindly provided by Dr R. Pouyssegur, Nice, France). Antibodies are listed in Table I. Dilutions of each antibody were determined by prescreening on the full 4 μm thick sections before use on TMA sections.

Image analysis. Densitometric measurements of immunoprecipitates in cores were assessed for each marker antibody in each individually identified core after digitization and cropping of the slide images, as previously reported (27,28,40) with a SAMBA 2050 automated device (SAMBA/TRIBVN Technologies, France).

Statistical analysis. Immunohistochemical expression of each marker was first correlated with disease-free survival using NCSS and Statistica statistical software (available on line). When significant differences in mean quantitative scores of expression were identified between patients with or without distant metastasis or death (Mann-Whitney tests), the prognostic relevance was re-evaluated for each marker using log-rank test (Kaplan-Meier curves). The distribution and relationship of prognostic markers were then documented through supervised hierarchical clusters and dendrograms, as previously reported (27,28,42,43).

Importantly we used logistic regression (and ROC curves) to identify the combination of markers (referred to as 'signature') with the best sensitivity and specificity for prediction of prognosis. The method of multivariable fractional polynomials was used to correlate the outcome variable with the quantitative IHC expression of markers as continuous variables without predetermined cut-points (44,46,47).

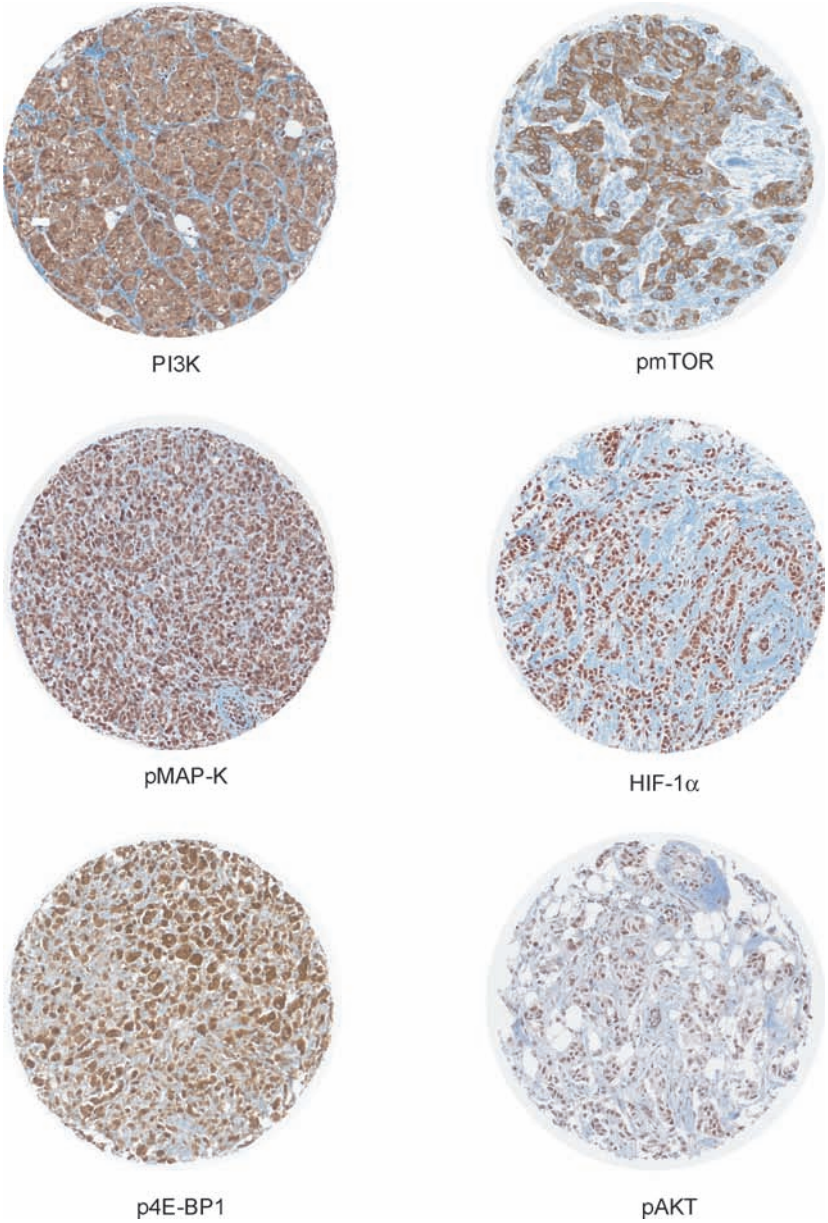

Figure 1. Individual core immunostaining in TMA of node negative breast carcinomas illustrating the immunoreactions with 6 of the 10 markers included (PI3K, pmTOR, pMAPKAPK-2, HIF-1α, p4^{E}-BP-1, pAKT) in the prognostic signature.

Results

Screening of potential markers of prognosis. TMAs containing tumour samples from patients with (n=111) or without distant metastasis (n=461) were screened for immunoexpression of markers (Table I, Fig. 1). Complete data for all 64 markers were available for 572 tumours (loss of some cores in TMAs occurred for 95 tumours). The prognostic significance of markers, individually evaluated by a univariate log-rank test (Fig. 2) as previously described (22-28,42,43,52,55), served for hierarchical clustering as shown in Fig. 3.

Logistic regression (ROC curves). The relationship between groups of predictive markers and the outcome variable was then evaluated by the multivariable fractional polynomials method (44,46,47), without any cut-point but keeping marker quantitative score values as continuous variables. The optimal combination computered from the image analysis data bank for the 64 markers in 572 node-negative breast carcinomas is shown in Table II and Fig. 4. A ten-marker signature comprising PI3K, pmTOR, pMAPKAPK-2, SHARP-2, P21, HIF-1α, moesin, p4^{E}BP-1, p AKT and P27 was found to well classify 91.4% of the patients in the category of either unfavorable or favorable prognosis with 93.7% sensitivity (well classified 104/111) and 90.9% specificity (well classified 419/461) (Fig. 4).

When the logistic regression was re-assessed (Table II) with exclusion of ER, PR and HER-2 (61 markers instead of 64), the same signature correctly classified patients in 91.61% of the cases, suggesting that the above set of 10 markers constitutes a significant indicator of prognosis independently of hormone receptor status and HER-2 amplification. When the multivariable fractional polynomials method was used on this series of 572 tumours, the estimated logistic regression

Table I. Antibodies used in 667 node-negative breast carcinomas (Ventana Benchmark XT automated device, immunoperoxidase).

	Antibodies	Supplier	Source[a]	Clone
1	MMP7	Abcam	Rpab	
2	MMP11	Abcam	Rmab	EP1259Y
3	Elf4E	Cell signaling	Rmab	C46H6
4	P70 S6 Kinase	Cell signaling	Rmab	49D7
5	FOXO3a	Cell signaling	Rpab	
6	P 42-MAP-Kinase (ERK-2)	Cell signaling	Rpab	
7	AF6	BD Biosciences	Rmab	35
8	YB1	Abcam	Mpab	
9	Phospho-mTOR (Ser2448)	Cell signaling	Rmab	49F9
10	PTEN	Signet COVANCE	Mmab	6H2.1
11	VEGF	R&D Systems	Mmab	26503
12	Phospho-4^{E}-BP-1(Thr37/46)	Cell signaling	Rmab	236B4
13	HIF 1α	Gift[b]	Mmab	729T3
14	MDR	Abcam	Mmab	JSB1
15	Topoisomerase IIα	Dako	Mmab	Ki-S1
16	ß-Catenin	Novocastra	Mmab	17C2
17	GATA-3	Santa Cruz	Mmab	HG3-31
18	FGFR-1 Flg (C-15)	Santa Cruz	Rpab	
19	Maspin	BD Pharmingen	Mmab	G167-70
20	MET	Chemicon/Abcys	Mmab	4AT44
21	P-Cadherin	Novocastra	Mmab	56C1
22	Ezrin (p81, 80k, cytovillin)	Neomarkers	Mmab	3C12
23	phospho-AKT (Ser473)	Cell Signaling	Rmab	587F11
24	CD 44v6	Novocastra	Mmab	VFF-7
25	CD44 (HCAM)	Novocastra	Mmab	F10-44-2
26	Moesin	Biomeda	Mmab	38/87
27	Moesin	Neomarkers	Mmab	38/87
28	Cytokeratins 8 - 18	Zymed	Mmab	(UCD/PR-10,11)
29	Cytokeratin 17	Dako	Mmab	E3
30	Cytokeratin 14	Novocastra	Mmab	LL002
31	phospho-STAT-3(Tyr705)	Cell Signaling	Rmab	D3A7
32	Melan A	Dako	Rmab	A103
33	CD 10	Novocastra	Mmab	56C6
34	CD 34	Dako	Mmab	QBEnd-10
35	Vimentin	Immunotech	Mmab	V9
36	Cytokeratin 19	Dako	Mmab	BA17
37	phospho-MAPKAPK-2	Cell Signaling	Rmab	(Thr334)
38	EGFR	Ventana	Mmab	3C6
39	STAT-1	Cell Signaling	Mmab	9H2
40	FAK	Cell Signaling	Rpab	
41	P38 MAP-Kinase	Cell Signaling	Rpab	
42	P27 Kip1	Cell Signaling	Rpab	
43	P21Waf1-Cip1	Cell Signaling	Mmab	DCS60
44	SHARP 2	Abcam	Rpab	
45	FYN	Abcam	Mmab	1S
46	P63	Dako	Mmab	4A4
47	Cytokeratin 903	Dako	Mmab	34BE12
48	Carbonic anhydrase IX	Abcam	Rpab	
49	E-Cadherin	Zymed	Mmab	4A2C7
50	CD 117 / KIT	Dako	Rpab	
51	Cytokeratins 5-6	Dako	Mmab	D5/16B4
52	PTEN	Cell Signaling	Mmab	26H9
53	PI3 Kinase	Cell Signaling	Rpab	

Table I. Continued

	Antibodies	Supplier	Source[a]	Clone
54	JAK 1	Cell Signaling	Rpab	
55	MET	Novocastra	Mmab	8F11
56	Caveolin 1	Santa Cruz	Rpab	
57	CD-105	Dako	Mmab	SN6h
58	CD-146	Novocastra	Mmab	N1238
59	BCL-2	Dako	Mmab	124
60	P53	Dako	Mmab	DO-7
61	P16	Neomarkers	Mmab	Ab7(16PO7)
62	HER-2	Novocastra	Mmab	CB11
63	PR	Ventana	Mmab	1E2
64	ER	Ventana	Mmab	6F11

[a]Mmab, mouse monoclonal antibody; Rpab, rabbit polyclonal antibody. [b]Kindly provided by Pouyssegur *et al* (51).

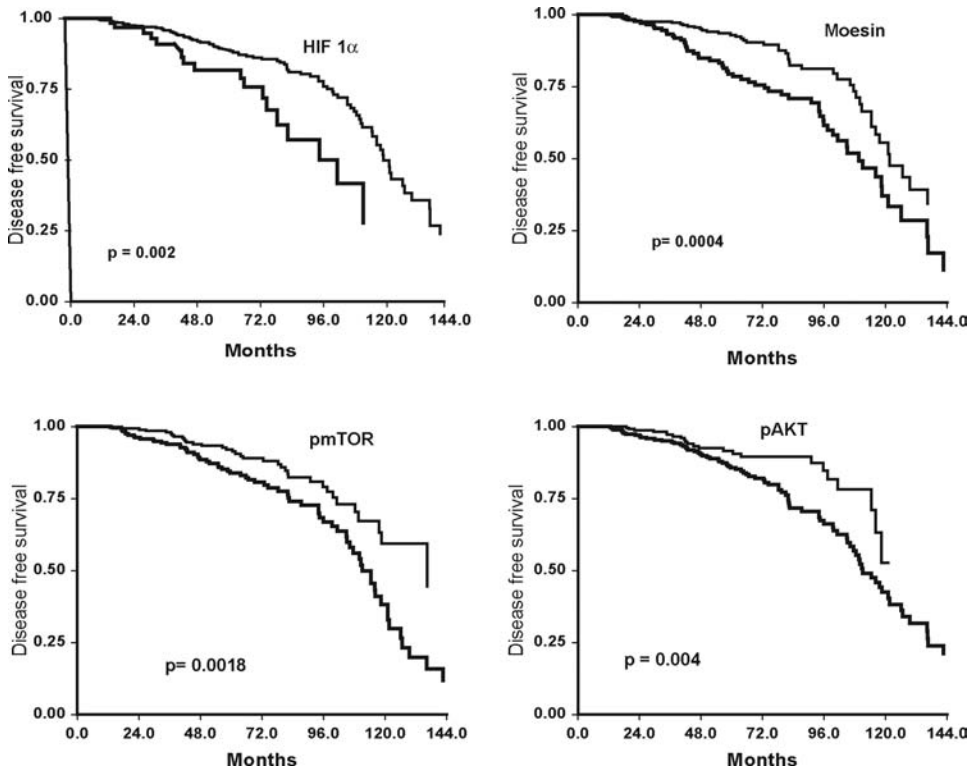

Figure 2. Kaplan-Meier curves (log-rank test) determining individual prognostic significance of markers (as shown for HIF-1α, Moesin, pmTOR and pAKT) quantitative scores in 572 node-negative breast carcinomas (TMA, quantitative immunochemical assays).

model for adverse outcome (logit) for the 10-marker signature (quantitative score/QS of individual 64 markers) was:

Logit: -0.62602 + 3.2469E-02 x (p4E-BP1 QS) + 0.19086 x (HIF-1α QS) + 3.9232E-02 x (Moesin QS) + 2.9267E-02 x (P21 QS) + 7.1781E-02 x (P27 QS) + 8.30815E-02 x (pAKT QS) + 7.7037E-02 x (PI3K QS) + 7.4231E-02 x (pMAPKAPK QS) + 0.31909 x (pmTOR QS) + 4.973E-02 x (SHARP-2 QS).

This model estimates 'B' for a specific group (in the present analysis the group of patients with metastases), where logit (Y) = XB and (X) = densitometric quantitative score for each marker. To calculate the probability of classifying in the correct category of outcome, the logit is transformed using Prob = exp (-logit) / [1 + exp (-logit)] or Prob = exp (- XB)/ [1 + exp (-XB)].

Likewise for the ten-marker signature (quantitative scores of 61 markers, that is all 64 minus ER, PR and HER-2): Logit : -0.50965 + 3.2708E-02 x (p4E-BP1 QS) + 0.18267 x (HIF-1α QS) + 3.7484E-02 x (Moesin QS) + 4.1069E-02 x (P21 QS) + 8.4261E-02 x (pAKT QS) + 7.8838E-02 x (PI3K

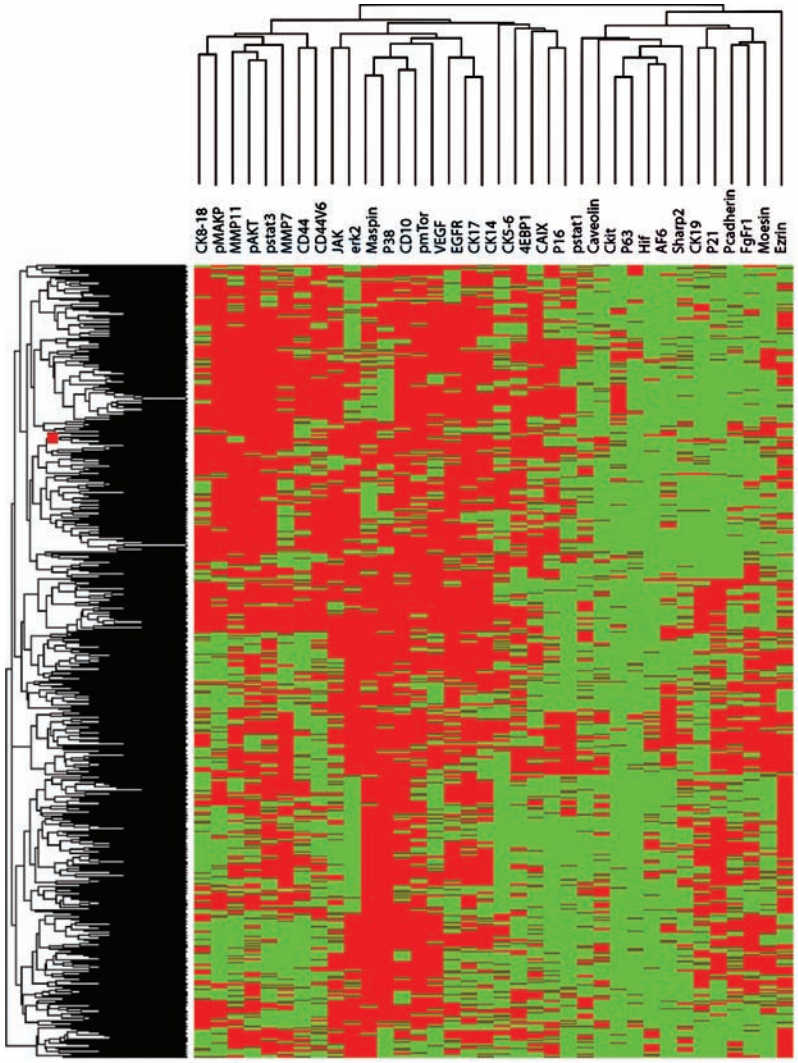

Figure 3. Supervised hierarchical clustering of the markers (excluding ER, PR and HER-2) with prognostic significance in the log-rank test, established with quantitative densitometry of immunohistochemical assays on TMA (n=572 patients with node-negative breast carcinomas) and quantitative score cut-points for each marker, as determined by log-rank test.

Table II. Prognostic predictive value of optimal combination of markers and groups of markers determined by logistic regression, evaluated by quantitative IHC in 572 node negative breast carcinomas.

Signatures	Immunohistochemical markers	Logistic regression	
		% ([a])	Surface under ROC curve
Optimal signature (Independent of ER, PR, HER-2)[b]	PI3K, pmTOR, pMAPKAPK-2, SHARP-2, P21, HIF-1α, Moesin, $p4^{E}$-BP-1, pAKT, P27	91.6	0.95
Angiogenesis	HIF-1α, VEGF, CD146, CD34, FGFR-1, CA-IX	65	0.74
Angiogenesis + signaling pathways[c]	PI3K, pAKT, pMAPKAPK-2, pmTOR, P38 MAPKinase, $p4^{E}$-BP-1, ElF4E, FOXO3a, FAK, JAK, FYN, STAT-1, STAT-3, SHARP-2, P42 MAPKinase, HIF-1α, VEGF, CD146, CD34, FGFR-1, CA-IX	90.03	0.94

[a]Percentages of well classified patients (series of 572 node negative patients with breast carcinoma, 102 months mean follow up); [b]n=10; [c]n=24.

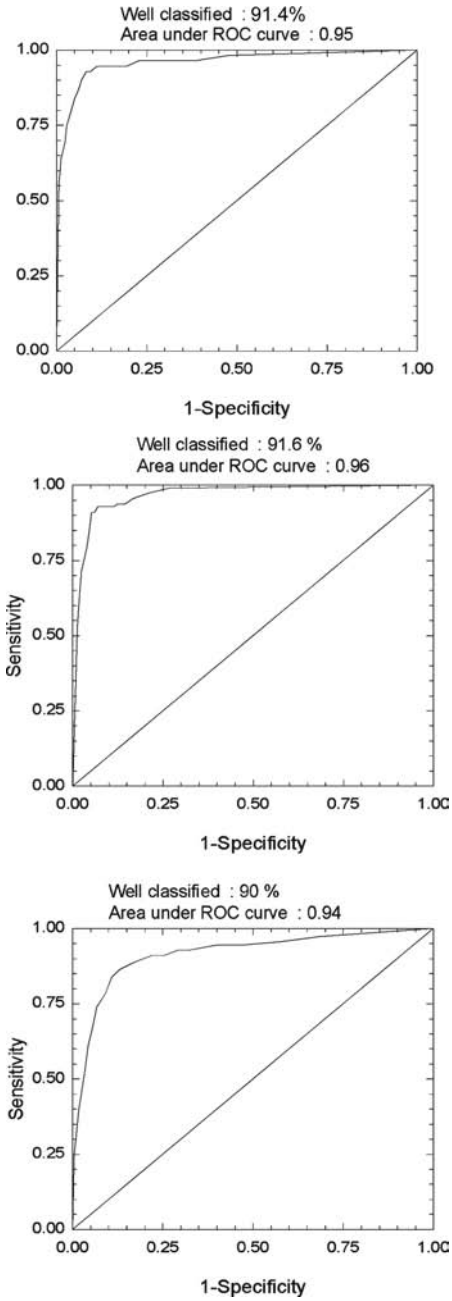

Figure 4. First and second steps of logistic regression and ROC curves determining the signature (PI3K, pmTOR, pMAPKAPK-2, SHARP-2, P21, HIF-1α, Moesin, p4EBP-1, pAKT and P27) that correctly classified node-negative patients (91.4 and 90% top and bottom respectively in the category of favorable and unfavorable prognosis). A second regression assessed independently of ER, PR and HER-2 status also correctly classified 91.6% of the patients (middle). Quantitative immunohistochemical assays on TMA of 572 node-negative breast carcinomas.

QS) + 7.0824E-02 x (pMAPKAPK QS) + 0.3126 x (pmTOR QS) + 4.4381E-02 x (SHARP2 QS) + 5.2845E-02 x (P27 QS). and the probability was computed by the same procedure as indicated above.

When a shortened list of preselected markers was analysed, grouped into subsets according to their specific biological functions based on literature data, two other combinations of markers exhibited a similarly strong relevance for prediction of prognosis. Thus the association of markers of angiogenesis, or main signaling pathways properly classified 90% of the patients (sensitivity 89.19% well classified patients 99/111, specificity 90.24% well classified patients 416/461). But this signature included many more markers (n=24) instead of 10, to obtain a high predictive value (Table II).

Discussion

We used a high-throughput quantitative immunohistochemical procedure as previously reported (42,43) to analyse samples from a large series of patients with node-negative breast carcinoma (n=572), in order to identify an optimal combination of markers allowing the selection of patients who would not benefit from adjuvant chemotherapy.

Marker screening. No previous study has been reported that used this approach of quantitative immunocytochemical tumour profiling for prediction of prognosis in node-negative breast carcinomas, although some quantitative immunohistochemical assays have been reported for smaller sets of markers (17-21) with different study designs. Our selection of markers, mainly including a panel of molecules involved in tumour growth and progression, was based on a literature review and also upon our previous experience of their reliable and discriminative IHC in routine sections from frozen or fixed tissue and TMAs (22-28,40,52,55). The final choice of antibodies, as noted previously (42,43), thus relied on their commercial availability, their suitability for use on paraffin-embedded archival material, their potential clinical relevance for breast cancer management, as reported in recent investigations on gene expression profiles (4-13), and previous IHC studies (29-39). This clearly implies that the signature proposed here may evolve as the number of the markers tested increases, but it is likely that it will be difficult to find a combination with significantly stronger sensitivity and specificity, than those described herein.

Most recent high-throughput immunohistochemical studies have focused on tumours of poor prognosis that currently lack tailored therapy, such as basal-like and triple-negative breast carcinomas, in order to identify new molecular targets for specific therapies (37,38). In contrast, our study was designed to select patients with node-negative tumours having the most favorable prognosis, who would not require aggressive adjuvant chemotherapy. Other studies have similarly assessed gene expression profiling in node-negative tumours (10,12,13), but IHC procedures are much easier to conduct, requiring sections of only 4 microns thick (40 microns for 10 markers) from formalin-fixed tissue in paraffin blocks remaining available after diagnosis. Procedures can be performed in 24-48 h in Pathology departments, and importantly are much more cost-effective than genomic tests (about ten-fold cheaper).

Statistical analysis. Choosing cut-off points for positive staining of prognostic markers in semi-quantitative analyses is a major issue that must be addressed prior to attempting statistical analysis and correlation with the patient outcome in order for this methodology to be usable in routine clinical practice. In contrast, high-throughput quantification of immunoprecipitates by densitometry, using dedicated computerized devices properly and uniformly calibrated for immuno-

histochemical detection with a large tumour series (TMAs), provides an excellent method for comparison of subsets of tumours in a given cohort. This method yields measurements appropriate for statistical analysis using continuous variables. Cut-point values of immunostaining can be determined according to log-rank tests for determination of prognostic values by splitting patients in a series into two categories or more, those with tumours expressing the markers above or below validated (45) thresholds predictive of disease-free survival. However, the use of cut-points has been criticized and a more sophisticated strategy can be proposed (44-47) that keeps continuous the values of quantitative scores measurements. According to this principle and recommendation, we applied this multivariable model for quantifying the expression of potential predictors of prognosis. Markers were quantitatively evaluated in TMA tissue sections by image analysis, using arbitrary units for densitometric measurements that are suitable for comparative studies of marker expression in variable clinical settings, particularly to correlate properly the expression of markers with the outcome variables. In this regard, using this multivariable fractional polynomial method (44,46,47), we analysed the correlation between marker expression and patient outcome without dichotomising our tumour series into subsets by the use of a cut-point. In other words, all the 64 markers (and subsets of markers) were concomitantly analysed according a non-linear model, in order to obtain the best combination of a limited number of markers with optimal accuracy, sensitivity and specificity for prognosis prediction. In this manner, we found that a ten-marker signature (PI3K, pmTOR, pMAPKAPK-2, SHARP-2, P21, HIF-1α, Moesin, 4^{E}BP-1, pAKT and P27) was sufficient to correctly classify patients in 91.43% of the cases, independently of ER, PR and HER-2 status. However, validation of this signature is now required in prospective studies using routine sections of individual tumours.

Biological relevance. The markers included in the signature include mainly molecules involved in the main signaling pathways within abnormal tumour cells with altered cell machinery. Most of these markers have been individually reported as dysfunctional in tumours (40,42,43) and described as potential targets for specific therapy. Interestingly, among those included in our ten-marker signature, some are involved in the mTOR pathway (PI3K, AKT, 4^{E}-BP-1) and other signaling networks. The mTOR (mammalian Target Of Rapamycin) protein shows aberrant high activity in breast cancers, as well as other gynecological malignancies, which induces increased tumour cell metabolism and growth, and has been proposed as an interesting target for specific therapy. Phase I and II trials have shown that molecules such as the rapamycin analogues tensirolimus (CCI 799), everolimus (RAD CO1) and ARIAD (AP 23573), which specifically inhibit the mTOR protein, have significant antitumour activitya against gynecological cancers (48). Likewise, in breast cancers, molecules blocking mTOR activity increase sensitivity to anthracyclines and taxans (49) and show synergistic action with letrozole (50).

Overexpression of Moesin in breast carcinomas has recently been reported in basal-like carcinomas (35). It is therefore not surprising that Moesin is indicative of prognosis, in association with the other markers of the signature that we have also identified in node-negative tumours that are associated with an unfavorable patient outcome, and so require more aggressive treatment.

HIF-1α is a major transcriptional factor in nutrient stress signaling, and is crucial for the development of anticancer therapy (51). In a previous study (52), we have shown that expression of HIF-1α in usual tissue sections of breast carcinoma was prognostically predictive in univariate analysis in node-negative tumours. HIF-1α has been found to up-regulate mTOR through upstream mTOR activators including PI3K and AKT (53), suggesting that the effects of HIF-1α on tumour cell metabolism and growth can be blocked through mTOR inactivation by PI3K and AKT inhibitors (54). HIF-1α also mediates VEGF angiogenic machinery, acting on tumour neoangiogenesis through PI3K, AKT and other factors including the ras-MAPK pathways (51,55). Blockage of VEGF by monoclonal antibodies or by anti-VEGF-R molecules such as sunitinib, sorafenib or vatalanib may reduce tumour growth due to overexpression of HIF-1α in tumour cells (reviewed in 51,52,55).

In conclusion, using 1) a high-throughput procedure to profile 572 node-negative tumours in TMAs, 2) computer-assisted densitometric quantification, 3) non-linear fractional polynomial logistic regression using continuous values of quantitative immunohistochemical variables to identify the combinations of a limited number of markers without any categorisation through pre-defined cut-points, we found a ten-marker signature that allowed correct classification of 91.6% of the patients, independently of hormone receptor and HER-2 status, and that particularly identified patients with low risk of early metastasis, who consequently should not require adjuvant chemotherapy.

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