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Traditional and new prognosticators in breast cancer

Nottingham index, Mib-1 and estrogen receptor signaling remain the best predictors of relapse and survival in a series of 289 cases

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Key words: breast cancer, relapse, survival, histological subtypes, prognosticators, Mib-1, estrogen receptor

Abbreviations: OS, overall survival; DFS, disease-free survival; R, incidence of relapses; ER, estrogen receptors; PR, progesteron receptors; DAB, 3,3'-diaminobenzidine tetrahydrochloride; LVI, lymphovascular invasion; T, tumor size; TDLU, termino-ductular-lobular unit; TMA, tissue microarrays; TBS, tris buffer saline; NPI, Nottingham prognostic index

Histopathological and immunohistochemical findings on tissue microarrays, overall survival (OS), disease-free survival (DFS) and incidence of relapses (R) were recorded and statistically analyzed in 289 breast cancers. A higher R and a shorter DFS were significantly related to larger tumors, lymph node invasion, higher tumor grade, absence of estrogen receptors (ER), triple negative tumors and presence of lymphovascular invasion (LVI). Longer OS was observed to be significantly associated with smaller tumor size (T), lymph node negativity, lower tumor grade, absence of LVI, lower Mib-1 expression and with the presence of ER. At multivariate analysis, only T for DFS and lymph node status and triple negativity either for DFS or OS had independent prognostic value.

In the 194 lymph node-negative women DFS and OS were inversely related to tumor grade, absence of ER, Mib-1 expression in more than 15% of neoplastic cells and, only for DFS, presence of LVI. In the 95 lymph node-positive the number of involved nodes was the most discriminating parameter either for DFS or OS; T, Her-2 status and presence of LVI were significantly related to DFS. ER negativity was related to higher grade, progesterone receptors (PR) negativity, Her-2 negativity, hence to triple negativity, to basal-like type, Mib-1expression over 15% of neoplastic cells. Her-2 positivity was related to higher grade, ER positivity and PR positivity. Basal-like type was not an independent prognosticator, while triple negative type has a significant relation to shorter OS. The Nottingham prognostic index accurately identifies prognostic groupings and Mib-1 expression and ER signaling are the key biological predictors even in single cases.

Introduction

Breast cancers are heterogeneous tumors, the vast majority of which originate from the termino-ductular-lobular unit (TDLU) and not from the ductal tree.[1] Nevertheless, they are mainly subdivided into ductal and lobular tumors. Tumors with apparently homogeneous morphology have different genetic profiles. The best characterized of these have been called luminal A, luminal B, HER-2 and basal-like, differing with regard to gene expression, clinical features, response to treatment and prognosis.[2] According to a new cell biology concept, based on gene expression, stem cells (so-called committed progenitor cells) in the human breast can proliferate and give rise to end luminal and myoepithelial lineages.[2] Glandular end cells and cancers originating from them express cytokeratin 8/18, ER, genes associated with ER and PR activation, BCL-2 and GATA 3. These markers are more intensely expressed in some cases, the so-called luminal A, than in other cases, the so-called luminal B variant,[3,4] the latter being more proliferating. The basal compartment and its malignant counterparts, the so-called basal-like cancers, are cells and cancer subtypes mimicking basal epithelial progenitor cells and myoepithelial cells. The majority of them do not express ER, PR and related genes while they strongly express basal cytokeratins,[5,6] proliferation related genes and TP53 mutations, and sometimes BRCA1 mutations.[3,7,8] As expected of progenies coming from progenitor cells, there are intermediate cancer cases (CK 5^+, CK8/18^+, ER^+ and/or $Her2^+$) towards the glandular end cells.[6] According to various authors,[6,9-11] the co-expression of simple (7, 8, 18 and 19) and stratified (4, 5, 14 and 17)

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Table 1. Tumor parameters

Histological tumor type	Number of cases (%)
Ductal NOS	215 (74.4)
Classic lobular	22 (7.6)
Mucinous	18 (6.2)
Apocrine	10 (3.5)
Other	24 (8.3)
Tumor size (cm)	
T1a	14 (4.8)
T1b	47 (16.3)
T1c	137 (47.4)
T2	84 (29.1)
T4b	7 (2.4)
Nodal status	
N0	194 (67.1)
N1a	52 (18)
N2a	28 (9.7)
N3a	15 (5.2)
Grade	
1	49 (17)
2	160 (55.3)
3	80 (27.7)
Lymphovascular invasion	
Absent	198 (68.5)
Present	72 (24.9)
Missing	19 (6.6)

cytokeratins in the same tumors is frequent (from 27–62%), particularly in high-grade tumors. This is not surprising since the basal cytokeratins are also expressed in luminal cells in the TDLU.[11]

There is much agreement on the fact that the basal-like subtype carries a poor prognosis[5,12] and an increased propensity for visceral metastases to the brain and lung[13] although not for locoregional relapse.[14] Her-2$^+$ tumors, independently of being basal-like or not, also have a poor prognosis,[15] as do triple negative cancers (ER$^-$, PR$^-$, Her-2$^-$), which again can be basal-like or not.[16]

The main focus of the present study is to help to morphophenotypically characterize the subtypes of breast cancers exhibiting a worse behaviour in a series of 289 patients. There are at least two reasons to do this: first, to find elements to predict the response to currently available treatments, in particular cytotoxic chemotherapy and to try to focus on tumor targets for more appropriate therapy; second, to identify patients at low risk of relapse and poor survival when lymph node-negative, so to have more elements to decide whether chemotherapy can be withheld.[17] In short, the aim is to assist in identifying the correct therapeutic approach in single cases, since the combination of tumor size, lymph node status and tumor grade, i.e., the Nottingham index (NPI) identifies patients with good prognosis, moderate prognosis or poor prognosis.[18]

Results

Forty-two out of a total of 289 women with breast cancer died of the disease within 10 y from the diagnosis; the actuarial global OS was 98.5% at 5 y and 84.8% at 10 y. During the study period we recorded 64 relapses of disease, with a median time to progression of 53 mo (range 8–110); the actuarial DFS for the whole population was 87.3% at 5 y and 77.1% at 10 y.

A higher R and a shorter actuarial DFS were significantly related to larger tumors ($p < 0.001$), lymph node invasion ($p < 0.001$), higher tumor grade ($p < 0.05$), absence of ER ($p < 0.05$), triple negative type (<0.05) and presence of LVI ($p < 0.001$). The presence of Mib-1 expression in more than 15% of neoplastic cells resulted in a borderline significance for either higher incidence of R or reduced DFS. Longer OS was observed to be significantly associated with smaller T, absence of lymph node invasion, lower G, absence of LVI, Mib-1 expression in less than 15% of neoplastic cells as well as with the presence of ER. The OS was shorter in triple negative cancers ($p < 0.05$) (**Tables 5 and 6**). No significant correlation was found between DFS, OS and R and PR, Her-2, EGFR, E-cadherin, VEGF, p53, vimentin, BRCA1 and basal-like histotype (**Table 5**).

At multivariate analysis, only T for DFS and lymph node status and triple negativity either for DFS or OS had independent prognostic value. In triple negative cases, ER negativity was the most discriminating value for worse prognosis, followed by PR negativity and Her-2 negativity.

In the 194 women with non-invaded lymph nodes, 18 of whom died of the disease, DFS and OS were inversely related to tumor G, absence of ER, values of Mib-1 expression in more than 15% of neoplastic cells and, only for DFS, presence of LVI. T, PR and Her-2 status, triple negativity, basal-like histotype, as well as all the other markers considered were not discriminating parameters (**Table 7**).

In the 95 patients with lymph node invasion, the number of involved nodes was the most discriminating parameter either for DFS or OS; T, Her-2 status and presence of LVI were significantly related to DFS but not to OS. Triple negativity was very close ($p = 0.06$) but did not reach the level of significance in the correlation to shorter DFS, probably due to a delayed rapid fall of the slope starting from 60 mo of follow-up; however, it had a significant relation to shorter OS ($p < 0.05$). In this group of patients, basal-like tumors had a better prognosis than luminal B cancers ($p < 0.05$).

ER negativity (40 cases) was directly related to higher G ($p < 0.001$), PR negativity ($p < 0.001$), Her-2 negativity ($p < 0.005$), hence to triple negativity ($p < 0.001$), to basal-like histotype ($p < 0.001$), Mib-1 expression over 15% ($p < 0.001$), EGFR negativity ($p < 0.001$), p53 positivity ($p < 0.05$) and vimentin positivity ($p < 0.05$). It was not significantly related to T, lymph node status, LVI, E-cadherin, VEGF and BRCA1.

Her-2 positivity (73 cases) was directly related to higher G ($p < 0.001$), ER positivity ($p < 0.005$) and PR positivity ($p < 0.005$); it tended to be associated with larger tumors although without reaching the significance level ($p = 0.06$).

Her-2$^+$ and Her-2$^-$ cases were allocated in the same percentage in the basal-like group of cancers[36] but, compared with the

luminal A and B groups, Her-2$^+$ tumors were significantly more numerous in the basal-like group ($p < 0.005$). G3 cancers ($p < 0.05$), EGFR$^+$ tumors ($p < 0.001$) and triple negative ($p < 0.001$) cancers were more numerous in the basal-like group, while ER$^+$ tumors were less numerous ($p < 0.001$) than in the luminal A and B groups. Her-2$^-$ cancers had a significantly higher Mib-1 expression ($p < 0.01$), were more frequently EGFR$^-$ ($p < 0.005$) and p53$^+$ ($p < 0.01$), and had a higher G ($p < 0.001$).

Table 2. Biological characteristics of the study population (289 patients)

		Number (%)
Estrogen receptors	Absent	40 (13.8)
	Present	249 (86.2)
Progesteron receptors	Absent	61 (21.1)
	Present	228 (78.9)
Her-2	Negative	216 (74.7)
	Positive	73 (25.3)
Triple negative	No	268 (92.7)
	Yes	21 (7.3)
Immunophenotype	Basal-like	36 (12.4)
	Luminal A	222 (76.8)
	Luminal B	31 (10.8)
Mib-1	≤15%	196 (67.8)
	>15%	74 (25.6)
	Missing	19 (6.6)
CD44	Absent	167 (57.8)
	Present	86 (29.8)
	Missing	36 (12.5)
E-Cadherin	Absent	20 (6.9)
	Present	186 (64.4)
	Missing	83 (28.7)
EGFR	Absent	21 (7.3)
	Present	241 (83.4)
	Missing	27 (9.3)
VGFR	Absent	201 (69.6)
	Present	58 (20.1)
	Missing	30 (10.4)
p53	Absent	203 (70.2)
	Present	23 (8)
	Missing	63 (21.8)
BRCA-1	Absent	205 (70.9)
	Present	8 (2.8)
	Missing	76 (26.3)
Vimentine	Absent	213 (73.7)
	Present	7 (2.4)
	Missing	69 (23.9)

Discussion

In our series of cases, DFS and R were significantly correlated to T, lymph node status, G, LVI as morphological factors and to ER signaling. Longer OS was significantly associated with smaller T, lymph node negativity, lower G, no LVI, and ER positivity and Mib-1 expression in less than 15% of neoplastic cells. In the patients without lymph node invasion, T lost its influence on survival, while lower G, ER positivity and Mib-1 expression in less than 15% of neoplastic cells were associated with longer OS and DFS and with lower R. In patients with lymph node invasion, the most discriminating parameters were the number of involved lymph nodes and the tumor size. In this group of cases, luminal B cancers had a worse prognosis than luminal A and basal-like tumors, while triple negativity had a significant relation to shorter OS. Therefore, we can say that, in agreement with Wirapati et al.[19] NPI[20] accurately identifies prognostic groupings. Proliferation, as tested by Mib-1 expression, and ER signaling were the key biological predictive parameters in our cases. In contrast with Wirapati et al.,[19] these parameters did not include Her-2. In our cases, Her-2 was not discriminating by itself; however, Her-2 negativity added to the discriminating negative power of ER negativity and PR negativity.

In our series, as in others',[14,21,22] triple negative cases as a group had a worse prognosis; however, they were a heterogeneous category including cancers with excellent prognosis (such as the medullary histotype) and cancers with poor prognosis.[23,24] Belonging to this group does not identify the prognosis of single cases.[25]

We also analyzed the so-called basal-like cancer "problem." Basal-like cancers originate from the TDLU and not from myoepithelium since cytokeratins 5, 14 and 17, which decorate basal cells in stratified epithelia, are also expressed by luminal cells. Luminal cells express cytokeratins 8, 18 and 19, which are also expressed by basal-like cancers, even in our cases, but not by myoepithelial cells.[11] Nielsen et al.[12] identify basal-like tumors by gene-expression profiling with four markers (ER negativity, Her-2 negativity, EGFR positivity, CK 5/6 positivity) obtaining a specificity of 100%. Cheang claims[26] that five markers (ER, PR, Her-2, EGFR and CK 5/6) are needed, while Moinfar points[27] out that CK 5 and CK 17 are discriminating. Rakha and Ellis affirm[16] that in their experience an acceptable degree of specificity and sensitivity is reached using at least two basal markers among CK 5/6, CK 14, CK 17 and EGFR. In the present study we followed Rakha and Ellis's indication, independently

Table 3. Morphological features of invasive basal-like, triple negative, Her2$^+$ and ER$^-$ cancers

Morphological features	Basal-like (n = 36)	Triple negative (n = 21)	Her2$^+$ (n = 73)	ER$^-$ (n = 40)
Geographic necrosis	26/36 (72%)	13/21 (62%)	6/73 (8%)	30/40 (75%)
Pushing border	23/36 (64%)	14/21 (67%)	4/73 (5%)	23/40 (57%)
Infiltrative border	13/36 (36%)	7/21 (33%)	69/73 (95%)	17/40 (43%)
Lymphoid stroma	19/36 (53%)	10/21 (48%)	8/73 (11%)	20/40 (50%)
Apocrine features	0/23 (0%)	0/21 (0%)	1/73 (1%)	2/40 (5%)
Metaplastic features	3/36 (8%)	1/21 (5%)	2/73 (3%)	1/40 (2%)
Squamoid features	5/36 (14%)	5/21 (24%)	0/73 (0%)	2/40 (5%)
Central fibrosis	6/36 (17%)	2/21 (10%)	10/73 (14%)	4/40 (10%)

Table 4. Primary antibodies

Antibody	Source	Clone	Dilution
CK 5/6	Dako	D5/16B4	1:70
CK 14	Bio-Optica	LL002	1:50
CK 8	DBA-Italia	C-51	
CK 18	Menarini	DC-10	1:500
ER	Bio-Optica	SP1	1:100
PR	Bio-Optica	SP2	1:50
Her2	Dako	Policlonal	1:600
Mib1	Bio-Optica	SP6	1:200
E-cadherin	Dako	NCH-38	1:50
EGFR	Zymed	31 G7	1:50
VEGF	Dako	VG1	1:50
p53	Bio-Optica	pAb 240	1:200
BRCA1	Hystoline	GLK-2	1:50
Vimentine	Dako	V9	1:300

of the positivity or negativity of any other parameter. There is also a tendency to define basal-like cancers as those tumors that are ER$^-$, PR$^-$ and Her-2$^-$, i.e., the so-called triple negative cancers.[28] However, 14–45% of basal-like cancers express at least one of these markers.[12,14,16,21,29-34] Therefore, it is clear that basal-like cancers are still to be unequivocally defined at the immunohistochemical level, and that expression of basal cytokeratins is not the unique requirement. From 3–20% of cancers are basal-like.[6,17,35,36] In our series, 36 out of 289 tumors (12.4%) were basal-like. The large majority of them were G3, with a prevalently solid architecture, a dense population of cells, a well-defined, pushing border of invasion and the absence of association with vascular invasion or lymph node involvement (as stressed by Fulford et al.[37]). They had sometimes a high mitotic and apoptotic rate, geographic necrosis, sometimes spindle or squamous metaplastic changes, glomeruloid microvascular proliferation and stromal lymphocytic response (as in the cases of Fulford et al.[37], Langerod et al.[38] and Diallo-Danebrock et al.[39]). Our ER$^-$ and triple negative cancers were histologically similar to basal-like cancers. Her-2$^+$ cancers had more frequently infiltrative borders. This finding is also very much in agreement with other authors' observations (reviewed in ref. 16).

We did not find any correlation of basal-like cancers with OS, DFS and R; however, in the group of cases with invasion of lymph nodes, basal-like tumors tended to be more represented than luminal cancers although the difference was not significant. This is in disagreement with the results of other authors;[13] possible reasons for this disagreement could be as follows: low number of basal-like cancers in our series; use of different antibodies and staining techniques; lack of reliable quality control when we dealt with negativity instead of positivity; lack of consensus regarding the definition of ER and Her-2 positivity;[40] not enough representative tissue arrays given the heterogeneity of breast cancers; difficulty in establishing a threshold of positivity for ER, PR and Her-2; the fact that basal-like tumors often express CK 8 and 18 as luminal tumors do.[16,18]

In lymph node-negative cases (67%), T lost its discriminating power and only G, ER$^-$ and Mib-1 expression in more than 15% of neoplastic cells were significantly related to shorter OS. This reinforces the concept that proliferation and ER signaling are the best biological prognosticators, particularly in lymph node-negative cases.[19,41,42] Triple negative cases of the present series had a poor prognosis as a group and the negative discriminating value of ER negativity and PR negativity was reinforced by Her-2 negativity, probably because the benefit of targeted therapy was lacking in Her-2 negative cases.[16] Her-2 positivity was not associated with shorter OS and DFS probably because the negative effect of its amplification is balanced by the use of targeted therapy.

In conclusion, our results are in agreement with the conclusion of Gusterson[18] that NPI is the most reliable method of predicting survival of operable breast cancers and that the genes related to proliferation[41] and to ER signalling[42] are the best biological prognosticators even in single cases. In lymph node-negative cases, the most predictive parameters indicating a worse prognosis and therefore a correct therapeutic approach are ER negativity and Mib-1 expression in more than 15% of neoplastic cells. Among the subtypes, only the triple negative type is an independent

indicator of a worse prognosis, because ER negativity is associated with Her-2 negativity. Hence, as Gusterson affirms,[18] it is premature to conclude (reviewed in ref. 43) that histological subtypes, as identified by gene expression, are the best prognosticators.

Materials and Methods

Patients. Patients submitted to surgery for invasive breast cancer at the Department of Human Pathology and Oncology of the University of Siena, between January 1993 and December 1998 were included in the present observational study. We excluded patients with distant metastases at the time of diagnosis, patients who received a neo-adjuvant chemotherapy and patients who did not follow a standardized program of clinical and instrumental follow-up at our outpatient clinic, as well as patients who had no sufficient paraffin-embedded tissue available for tissue microarrays (TMA).

The study population included 289 female patients with a mean age of 61 ± 12 y (range 32–82); 218 (75.4%) women were postmenopausal.

Surgical treatment consisted of modified radical mastectomy in 144 cases and partial mastectomy in 145 cases; all patients received a level II axillary dissection, with a mean number of 16 + 5 lymph nodes removed (range 8–37). Postoperative radiation therapy to the residual breast was administered in all cases of partial mastectomy.

Adjuvant postoperative therapy was administered to 215 patients, with 102 women receiving chemotherapy and 143 receiving hormone therapy, 113 as an exclusive treatment and 30 following a primary adjuvant chemotherapy. Patients were evaluated every 6 mo for the first five y, and then annually; mean follow-up was 113 mo (range 18–120).

The data on patients OS, DFS and R were recorded.

Histopathology. Histopathological findings were reviewed. For each case, histopathology defined the cancer histotype, pathological stage assessed according to the criteria established by the International Union Against Cancer, grade according to the modified Scarff-Bloom-Richardson criteria, presence of

Table 5. Correlations between different parameters and incidence of relapses

		Relapse n (%)	p
pT	T1a	1/13 (7.1)	
	T1b	8/47 (17)	
	T1c	23/137 (16.8)	
	T2	26/54 (31)	
	T4b	6/7 (85.7)	**<0.001**
pN	N0	28/194 (14.4)	
	N1a	13/52 (25)	
	N2a	12/28 (49.2)	
	N3a	11/15 (73.3)	**<0.001**
Tumor grade	1	8/49 (16.3)	
	2	31/160 (19.4)	
	3	25/80 (31.3)	**<0.05**
ER	Negative	14/40 (35)	
	Positive	50/249 (20.1)	**<0.05**
PgR	Negative	14/61 (23)	
	Positive	50/222 (22.5)	0.944
Her2	Negative	52/216 (24.1)	
	Positive	12/73 (16.4)	0.174
Triple negative	Yes	9/21 (42.9)	
	No	55/268 (20.5)	**<0.05**
Immunophenotype	Basal	7/36 (19.4)	
	Luminal A	52/222 (23.3)	
	Luminal B	5/31 (16.1)	0.695
LVI	Absent	32/198 (16.2)	
	Present	29/72 (40.3)	**<0.001**
Mib-1	≤15%	36/196 (18.4)	
	>15%	21/74 (28.4)	0.07
CD44	Absent	38/167 (22.8)	
	Present	19/86 (22.1)	0.905
Caderina E	Absent	5/20 (25)	
	Present	41/186 (22)	0.763
Egfr	Absent	55/241 (22.8)	
	Present	6/21 (28.6)	0.550
Vegfr	Absent	15/58 (25.9)	
	Present	45/201 (22.4)	
p53	Absent	48/203 (23.6)	
	Present	3/23 (13)	0.249
BRCA1	Absent	49/205 (23.9)	
	Present	1/8 (12.5)	0.455
Vimentina	Absent	51/213 (23.9)	
	Present	0/7	0.140

Table 6. Prognostic parameters for DFS and OS at univariate analysis on the whole population (289 patients)

		5 y DFS %	10 y DFS %	p	5 y OS %	10 y OS %	p
pT	T1a	100	92.9		100	92.9	
	T1b	91.2	83.2		97.8	93.3	
	T1c	88.8	82.8		98.5	86.4	
	T2	82.9	67.7		98.7	77.3	
	T4b	57.1	19	**<0.001**	83.3	62.5	**<0.05**
pN	N0	91.6	85.2		99.5	90.4	
	N1a	89.8	72.7		97.9	85.1	
	N2a	71	56.1		92.6	61.7	
	N3a	53.3	26.7	**<0.001**	93.3	53.3	**<0.001**
Grade	1	93.8	83.3		97.9	91.5	
	2	87.3	80.1		99.4	87	
	3	83.3	67.1	**<0.05**	97.4	75.4	**<0.05**
ER	Absent	82.5	65		95	72.5	
	Present	88	79.2	**<0.05**	99.2	86.8	**<0.05**
Triple negative	Yes	80.9	57.1		95.2	66.7	
	No	87.8	78.8	**<0.05**	100	86.3	**<0.05**
LVI	Absent	91.3	83.3		99.5	88.4	
	Present	75.6	57.8	**<0.001**	97	71.7	**<0.01**
Mib-1	≤15%	90.2	81.1		98.9	89.3	
	>15%	84.8	70.7	0.06	97.2	77.4	**<0.05**

Table 7. Prognostic parameters for DFS and OS at univariate analysis in lymph node negative patients (194 patients)

		5 y DFS (%)	10 y DFS (%)	p	5 y OS (%)	10 y OS (%)	p
Tumor grade	1	97.4	94.7		100	100	
	2	93.4	88.6		99	93.3	
	3	82.9	69.5	**<0.005**	97.8	75.6	**<0.0005**
ER	Absent	87.5	70.8		95.8	79.2	
	Present	92.2	87.3	**<0.05**	99.4	92	**<0.05**
LVI	Absent	92.7	87.2		99.3	99.1	
	Present	84.6	73	**<0.05**	96.15	80.7	0.11 n.s.
Mib-1	≤15%	96.3	90.2		99.2	95.4	
	>15%	83.6	76.5	**<0.05**	97.6	80.9	**<0.005**

peritumoral LVI (**Table 1**). Tumors were also evaluated for biological characteristics (**Table 2**) and the presence of geographic necrosis, border appearence, lymphocytic stromal response, apocrine features, metaplastic features, basaloid cell change and large central acellular zone (**Table 3**).

The evaluation was limited to the invasive portion of the tumor.

Immunohistochemical analysis on TMA. For each case, a representative area of the tumor was selected based on HE-stained sections from paraffin blocks. A hollow needle was used to remove 1 mm tissue cores (needles with varying diameters of 0.6 up to 2 mm were available) from these areas. An HE-stained slide arranged on the donor block surface was used for orientation. These tissue cores were then transferred to a recipient paraffin wax block into a ready-made hole, guided by a defined x-y position in a precisely spaced array pattern. 4 μmicron-thick sections were cut from this block using a microtome, mounted on a microscope slide and stained by immunohistochemistry. The characteristics of the antibodies used are listed in **Table 4**. Following deparaffinization in xylene, slides were rehydrated through a graded series of alcohol and placed in running water. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase and methanol. Samples were steamed for antigen retrieval with 10 mM citrate buffer (pH 6.0) for 35 min. Following protein block, slides were incubated with antibody and washed with normal swine serum in Tris buffer saline (TBS). 3,3'-Diaminobenzidine tetrahydrochloride (DAB) was used for the visualization of the antibody/enzyme complex. Slides were counterstained with Harris's hematoxylin and examined by light microscopy. Assessment of staining was based on a semiquantitative approach and tumor immunoreactivity was

scored as follows: 0 = negative, 1 = weak positive, and 2 = moderate/strong positive in combination with the percentage of cells showing positive staining. ER and PR were scored positive if at least 5% of neoplastic cells showed nuclear staining. Her-2 was scored positive if a 2+ or 3+ result was found. Details of Mib-1 expression by means of immunohistochemistry and choice of cut-off levels are reported elsewhere.[44] In brief, patients were stratified according to different percentages of Mib-1 expression staining and cut-off point analysis of DFS and OS was used to select the cut-off value for Mib-1 expression positivity (data not shown). The best cut-off point for semiquantitative Mib-1 expression that maximized the separation of the survival curves was 15% of neoplastic cells, which was therefore selected as the cut-off value. For evaluation of immunohistochemical staining, positive controls and normal breast tissues were used. For Her-2, p53 and EGFR a known positive external control was utilized. Different cores of the same tumor were scored individually, then the mean of the readings was calculated, once the uninformative cores were eliminated. Stainings were scored as positive or negative independently of their intensity. The observers (Megha T and Malagnino V) scored each staining pattern without previous knowledge of the outcomes on two separate occasions and a good intra- and interobserver correlation between the results was found.

Statistical analysis. Correlation between clinicopathological variables was investigated by means of univariate analysis. The chi-square test was used to assess the statistical significance of the association between categorical variables.

DFS and overall survival OS were calculated from the date of surgery; follow-up was closed at 10 y. We evaluated the prognostic significance of the different variables with respect to DFS and OS by means of the log-rank test and compared such prognostic significance to that of the other clinical and pathological factors considered by means of Cox regression analysis.

The Statistical Package for the Social Sciences software (version 11.0) (SPSS™, Chicago, IL USA) was used.

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References

1. Petersen OW, Nielsen HL, Gudjonsson T, Villadsen R, Ronnov-Jessen L, Bissell M. The plasticity of human breast carcinoma cells is more than ephitelial to mesenchymal conversion. Breast Cancer Res 2001; 3:213-7.
2. Birnbaum D, Bertucci F, Ginestier C, Tagett R, Jacquemier J, Charafe-Jauffret E. Basal and luminal breast cancers: basic or luminous?. Int J Oncol 2004; 25:248-9.
3. Sorlie T, Tibshirani R, Parker J, Hastie T, Marron JS, Nobel A, et al. Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 2003; 100:8418-23.
4. Yehiely F, Moyano JV, Evans JR, Nielsen TO, Cryns VL. Deconstructing the molecular portrait of basal-like breast cancer. Trends Mol Med 2006; 12:537-44.
5. van de Rijn M, Perou CM, Tibshirani R, Haas P, Kallioniemi O, Kononen J, et al. Expression of cytokeratins 17 and 5 identifies a group of breast carcinomas with poor clinical outcome. Am J Pathol 2002; 161:1991-6.
6. Abd El-Rehim DM, Pinder SE, Paish CE, Bell J, Blamey RW, Robertson JF, et al. Expression of luminal and basal cytokeratins in human breast carcinoma. J Pathol 2004; 203:660-1.
7. Lakhani SR, Reis-Filho JS, Fulford L, Penault-Llorca F, van der Vijver M, Parry M, et al. Breast Cancer Linkage Consortium. Prediction of BRCA1 status in patients with breast cancer using estrogen receptor and basal phenotype. Clin Cancer Res 2005; 11:5175-80.
8. Laakso M, Loman N, Borg A, Isola J. Cytokeratin 5/14-positive breast cancer: true basal phenotype confined to BRCA1 tumors. Mod Pathol 2005; 18:1321-8.
9. Nagle RB, Böcker W, Davis JR, Heid HW, Kaufmann M, Lucas DO, et al. Characterization of breast carcinomas by two monoclonal antibodies distinguishing myoepithelial from luminal epithelial cells. J Histochem Cytochem 1986; 34:869-81.
10. Malzahn K, Mitze M, Thoenes M, Moll R. Biological and prognostic significance of stratified epithelial cytokeratins in infiltrating ductal breast carcinomas. Virchows Arch 1998; 433:119-29.
11. Gusterson BA, Ross DT, Heath VJ, Stein T. Basal cytokeratins and their relationship to the cellular origin and functional classification of breast cancer. Breast Cancer Res 2005; 7:143-8.
12. Nielsen TO, Hsu FD, Jensen K, Cheang M, Karaca G, Hu Z, et al. Immunohistochemical and clinical characterization of the basal-like subtype of invasive breast carcinoma. Clin Cancer Res 2004; 10:5367-74.
13. Gaedcke J, Traub F, Milde S, Wilkens L, Stan A, Ostertag H, et al. Predominance of the basal type and HER-2/neu type in brain metastasis from breast cancer. Mod Pathol 2007; 20:864-70.
14. Rakha EA, Tan DS, Foulkes WD, Ellis IO, Tutt A, Nielsen TO, et al. Are triple-negative tumours and basal-like breast cancer synonymous? Breast Cancer Res 2007; 9:404.
15. Livasy CA, Karaca G, Nanda R, Tretiakova MS, Olopade OI, Moore DT, et al. Phenotypic evaluation of the basal-like subtype of invasive breast carcinoma. Mod Pathol 2006; 19:264-71.
16. Rakha EA, Ellis IO. Triple-negative/basal-like breast cancer: review. Pathology 2009; 41:40-7.
17. Rakha EA, El-Rehim DA, Paish C, Green AR, Lee AH, Robertson JF, et al. Basal phenotype identifies a poor prognostic subgroup of breast cancer of clinical importance. Eur J Cancer 2006; 42:3149-56.
18. Gusterson B. Do 'basal-like' breast cancers really exist? Nat Rev Cancer 2009; 9:128-34.
19. Wirapati P, Sotiriou C, Kunkel S, Farmer P, Pradervand S, Haibe-Kains B, et al. Meta-analysis of gene expression profiles in breast cancer: toward a unified understanding of breast cancer subtyping and prognosis signatures. Breast Cancer Res 2008; 10:65.
20. Blamey RW, Pinder SE, Ball GR, Ellis IO, Elston CW, Mitchell MJ, et al. Reading the prognosis of the individual with breast cancer. Eur J Cancer 2007; 43:1545-7.
21. Tischkowitz M, Brunet JS, Begin LR, Huntsman DG, Cheang MC, Akslen LA, et al. Use of immunohistochemical markers can refine prognosis in triple negative breast cancer. BMC lancer 2007; 7:134.
22. Cheang MC, Voduc D, Bajdik C, Leung S, McKinneys, Chia SK, et al. Basal-like breast cancer defined by five biomarkers has superior prognostic value than triple-negative phenotype. Clin Cancer Res 2008; 14:1368-76.
23. Reis-Filho JS, Tutt AN. Triple negative tumours: a critical review. Histopathology 2008; 52:108-18.
24. Reis-Filho JS, Lakhani SR. Breast cancer special types: why bother? J Pathol 2008; 216:394-8.
25. Sotiriou C, Piccart MJ. Taking gene-expression profiling to the clinic: when will molecular signatures become relevant to patient care? Nat Rev Cancer 2007; 7:545-53.
26. Cheang MC, van de Rijn M, Nielsen TO. Gene expression profiling of breast cancer. Annu Rev Pathol 2008; 3:67-97.
27. Moinfar F. Is 'basal-like' carcinoma of the breast a distinct clinicopathological entity? A critical review with cautionary notes. Pathobiology 2008; 75:119-31.
28. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, Collichio F. The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 2007; 13:2329-34.
29. Rouzier R, Perou CM, Symmans WF, Sneige N. Triple negative breast carcinoma and the basal phenotype: from expression profiling to clinical practice. Adv Anat Pathol 2007; 14:419-30.
30. Calza S, Hall P, Auer G, Björhle J, Klaar S, Kronenwett U. Intrinsic molecular signature of breast cancer in a population-based cohort of 412 patients. Breast Cancer Res 2006; 8:34.
31. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, et al. Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 2006; 355:560-9.
32. Bidard FC, Conforti R, Boulet T, Michiels S, Delaloge S, André F. Does triple-negative phenotype accurately identify basal-like tumuor? An immunohistochemical analysis based on 143 'triple-negative' breast cancers. Ann Oncol 2007; 18:1285-6.
33. Bertucci F, Birnbaum D. Reasons for breast cancer heterogeneity. J Biol 2008; 7:6.
34. Irvin WJ Jr, Carey LA. What is triple-negative breast cancer? Eur J Cancer 2008; 44:2799-805.
35. Perou CM, Sørlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature 2000; 406:747-52.
36. Tang P, Wang J, Bourm P. Molecular classification of breast carcinoma with similar terminology and different definitions: are they the same? Hum Pathol 2008; 39:506-13.
37. Fulford LG, Reis-Filho JS, Ryder K, Jones C, Gillett CE, Hanby A, Easton D. Basal-like grade III invasive ductal carcinoma of the breast: patterns of metastasis and long-term survival. Breast Cancer Res 2007; 9:4.

38. Langerod A, Zhao H, Borgan O, Nesland JM, Bukholm IR, Ikdahl T, et al. TP53 mutations status and gene expression profiles are powerful prognostic markers of breast cancer. Breast Cancer Res 2007; 9:30.
39. Diallo-Danebrock R, Ting E, Gluz O, Herr A, Mohrmann S, Geddert H, et al. Prognostic and predictive impact of protein expression profiling in high risk breast cancer patients treated with high-dose chemotherapy. Verh Dtsch Ges Pathol 2007; 91:187-96.
40. Wolff AC, Hammond ME, Schwartz JN, Hagerty KL, Allred DC, Cote RJ, et al. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. Arch Pathol Lab Med 2007; 131:18-43.
41. Haibe-Kains B, Desmedt C, Piette F, Buyse M, Cardoso F, Van't Veer L, et al. Comparison of prognostic gene expression signatures for breast cancer. BMC Genomics 2008; 9:394.
42. Marchiò C, Natrajan R, Shiu KK, Lambros MB, Rodriguez-Pinilla SM, Tan DS. The genomic profile of HER2-amplified breast cancers: the influence of ER status. J Pathol 2008; 216:399-407.
43. Peppercorn J, Perou CM, Carey LA. Molecular subtypes in breast cancer evaluation and management: divide and conquer. Cancer Invest 2008; 26:1-10.
44. Marrelli D, Pinto E, Neri A, Megha T, Gioffrè W, Cioppa T, et al. Mib-1 proliferation index is an indipendent predictor of Lymph node metastasis in invasive breast cancer: a prospective study on 675 patients. Oncol Rep 2006; 15:425-9.