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Molecular alterations in key-regulator genes among patients with T4 breast carcinoma

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Abstract

Background: Prognostic factors in patients who are diagnosed with T4 breast carcinomas are widely awaited. We here evaluated the clinical role of some molecular alterations involved in tumorigenesis in a well-characterized cohort of T4 breast cancer patients with a long follow-up period.

Methods: A consecutive series of 53 patients with T4 breast carcinoma was enrolled between 1992 and 2001 in Sardinia, and observed up for a median of 125 months. Archival paraffin-embedded tissue sections were used for immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH) analyses, in order to assess alterations in expression levels of survivin, p53, and pERK₁₋₂ proteins as well as in amplification of *CyclinD1* and *h-prune* genes. The Kaplan-Meier and Cox regression methods were used for survival assessment and statistical analysis.

Results: Overall, patients carrying increased expression of pERK₁₋₂ ($p = 0.027$) and survivin ($p = 0.008$) proteins as well as amplification of *h-prune* gene ($p = 0.045$) presented a statistically-significant poorer overall survival in comparison with cases found negative for such alterations. After multivariate analysis, the pathological response to primary chemotherapy and the survivin overexpression in primary carcinoma represented the main parameters with a role as independent prognostic factors in our series.

Conclusions: Although retrospective, our study identified some molecular parameters with a significant impact on prediction of the response to therapy or prognosis among T4 breast cancer patients. Further large prospective studies are needed in order to validate the use of such markers for the management of these patients.

Background

Since the staging systems of breast cancer were introduced during the course of the last century, the involvement of the skin has always been considered a morphologic characteristic leading to the classification of the tumour into the highest non-metastatic disease stage. In the current edition of the International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM staging system [1], primary breast cancers with extension to the skin are classified as T4. Patients with T4 carcinomas of any type, with or without lymph node involvement, and without distant metastases (T4 N0-2 M0), are classified as disease stage IIIB. According to this system, the breast carcinoma with skin involvement is included in stage III

and may be considered as locally-advanced breast cancer (LABC) [1-3].

In addition to the tumour size and the axillary lymph node involvement, other well-established prognostic factors currently used in breast cancer include histological subtype or grade, estrogen (ER) and progesterone (PR) receptor status, *HER2* amplification, and Ki67 proliferation index [4,5]. Novel tumour markers with potential clinical utility are thus awaited.

The molecular mechanisms underlying locally-advanced breast carcinomas are largely unknown. A distinct gene-expression profile has been described for T3/T4 tumours in comparison to the gene-expression pattern of T1/T2 tumours [6], suggesting that a distinct biological behaviour may characterize initial *vs.* locally-advanced breast carcinomas. The mitogen activated protein kinase (MAPK) pathway, a major signalling cascade involved in the control of cell growth and proliferation, has been indicated to play a role in the intracellular

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signalling process of breast carcinomas [7-9]. The ERK1-2 proteins, which represent the final components of such a signalling kinase cascade, have been found to be activated through phosphorylation (pERK₁₋₂) in human cancer and implicated in rapid malignant cell growth, mostly as a consequence of mutations in upstream components of the pathway [10,11]. Presence of pERK₁₋₂ could be thus considered as a marker for the increased activity of ERK1-2, which may induce cell proliferation, rapid cancer cell growth, and resistance to apoptosis [10]. Moreover, a genomic instability with an increased number of copies of the *CyclinD1* gene, which encodes a component of the p16^{CDKN2A}-RB pathway functionally interacting with the MAPK pathway [12,13], has been described to promote a deregulation of the cell cycle with subsequent induction of an uncontrolled cell proliferation and tumour growth [14]. Nevertheless, the p53 protein represent the final effector of the p14^{CDKN2A}-MDM2 pathway; in majority of human cancers, the *TP53* gene is functionally inactivated [15]. Lack or reduced expression levels of the p53 protein seems to be associated with a defective apoptotic response to genotoxic damage and, thus, to anticancer agents [16].

Finally, two additional mechanisms seem to play a central role in breast cancer progression and resistance to treatment. The increased expression of survivin, a member of the inhibitor-of-apoptosis (IAP) protein family, has been demonstrated to be associated with resistance to apoptosis [17-19]. It has been reported that survivin and other IAP proteins cooperate to activate kinase cascades which control cell motility, thus stimulating tumour cell invasion and promoting metastasis [19]. Survivin is indeed overexpressed in most cancer cells and tissues of different histological origin, being correlated to overall survival and acting as a poor prognostic factor in some cancer patients [20-22]. In breast carcinomas, the up-regulation of survivin has been hypothesized to act as a factor exerting resistance against tamoxifen-induced apoptosis [23,24]. The second additional mechanism involved in breast cancer pathogenesis includes an increased activity of the human homologue of the *Drosophila* prune (h-prune), which belongs to a superfamily of phosphoesterases [25]. It has been demonstrated that h-prune is able to promote cell motility through either induction of its phosphodiesterase activity (very recently, a multi-domain adaptor protein, ASAP1, has been reported to stimulate the h-prune phosphodiesterase activity [26]) or interaction with specific protein partners (mainly, nm23-H1) [27-29]. The h-prune protein has been found expressed at higher levels in breast, colorectal, and gastric carcinomas, participating to the promotion of both tumour invasiveness and metastasis formation [25,27]. In breast cancer, overexpression of h-prune has been demonstrated to be

involved in cancer progression, identifying subsets of patients with higher tumour aggressiveness (although it seems to have no role as independent prognostic factor in clinical outcome of patients with invasive breast carcinoma) [30]. As previously demonstrated [25,30], gene amplification may play an important role in inducing overexpression of h-prune among breast cancer patients.

In the present study, we examined the expression of survivin, p53, and pERK₁₋₂ proteins as well as the amplification of *CyclinD1* and *h-prune* genes in a well-characterized cohorts of patients with T4 breast carcinoma and a long follow-up, in order to determine their association with clinical and pathological parameters as well as with patients' outcome.

Methods

Cases and tissue samples

Paraffin-embedded samples of 53 consecutive patients with T4 breast cancer were included into the study. Cases were enrolled between 1992 and 2001, and observed up to September 2008 for a median of 125 months (range, 82-194). Patients were assessed by physical examination and mammography, confirmed via core-needle biopsy. All patients completed a treatment plan including primary chemotherapy, surgery, radiation therapy, adjuvant chemotherapy, and hormone therapy, when indicated (see below). The median age was 51

Table 1 Patient and tumour characteristics at baseline

Characteristics	Patients	
	N	%
Age		
<50	23	43
>50	30	57
Tumor stage		
T4abc	38	72
T4d	15	28
Axillary lymph nodes		
N0	0	0
N+	53	100
Hormone receptor status		
ER+/ER-	28/25	53/47
PR+/PR-	17/36	32/68
Proliferative index		
Ki67+	17	32
Ki67-	27	51
unknown	9	17
Tumour Grading		
G2/G3	38/15	72/28
HER2 status		
HER2+	10	19
HER2-	43	81

years (range, 32-67). Baseline characteristics are summarized in Table 1.

Fifteen patients (28%) had initially inflammatory breast carcinoma (T4d) and 38 (72%) had initially non-inflammatory cancer (T4abc); all patients had clinical involvement of axillary lymph nodes (as N+). According to the American Joint Committee on Cancer (AJCC) TNM staging system [1], all 53 cases included into this study were classified with the highest stage of non-metastatic disease (Stage IIIB). Estrogen (ER) and progesterone (PR) status was assessed by standard immunohistochemistry; nuclear staining in $\geq 10\%$ was considered positive (according to the indication that a significant difference in 5-year recurrence-free survival between ER-positive and ER-negative patients has been reported for a cut-off of 10% [31]). HER2 status was assessed by fluorescence *in situ* hybridization (FISH) analysis.

The study was approved by the Institutional Review Board at the University of Cagliari. A written informed consent was obtained for using tissue specimens in molecular analyses.

Treatment plan

All patients were treated with primary chemotherapy using anthracycline-containing regimens, such as FEC (5-Fluorouracil; Epirubicin; Cyclophosphamide) or PEV (Cisplatin; Epirubicin; Vinorelbine). After completing the neoadjuvant chemotherapy, patients underwent surgery consisting of modified radical mastectomy (MRM) or breast-conserving surgery (BCT). Postoperative adjuvant chemotherapy consisted of six cycles of CMF (cyclophosphamide, methotrexate, fluorouracil). Locoregional radiotherapy was performed during the fourth course of CMF. After completing adjuvant chemotherapy, patients with hormone receptor-positive tumours received tamoxifen for 5 years.

Clinical evaluations were performed every 3 months for 2 years and every 6 months thereafter. Instrumental examinations (e.g., mammography, liver ultrasound, chest X-ray, bone scan, and echocardiogram) were performed every 6 months for the first 2 years, and every 12 months thereafter.

Response Assessment

The clinical measurement of the response to neoadjuvant therapy was defined according to the International Union Against Cancer (UICC) criteria [32]. Pathological complete response (pCR) was defined as the histological absence of residual invasive disease in both the breast and the axilla. Presence of histological invasive residual disease in breast tissue or detection of cancer-positive lymph nodes in the axilla were defined as <pCR. Major pathological response (MpR) in breast tissue was defined

as no more than 2 cm of residual disease (pT0 plus pT1) [27].

Immunohistochemistry (IHC)

Immunohistochemical staining was done on formalin-fixed, paraffin-embedded sections, as previously described [30]. Four- to five-micrometer sections were immunostained with each specific monoclonal antibody (anti-survivin, anti-p53, and anti-phosphorylated ERK₁₋₂). Slides were viewed using a BX61 Olympus Microscope supplied with DP50 camera and Viewfinder Lite 1.0 Version (Pixera Corporation) image analysis system. Labelling intensity and cellular staining was independently evaluated by two observers. Intensity and distribution of IHC staining was used to classify samples as positive (tissue sections presenting strong to moderate staining in more than 10% of cells) or negative (including tissue sections showing weak to absent staining) for expression of candidate genes.

Fluorescence in situ Hybridization (FISH)

For *h-prune* and *CyclinD1* gene amplification analysis, double-colour FISH analysis was performed using the PAC 279-H19 clone, spanning the *h-prune* gene region at chromosome 1q21, and the BAC RP11-300I6 clone specific for the *CyclinD1* gene at chromosome 11q13, according to previously reported protocols [25,30]. Nuclei were counterstained with 4',6-diamidino-2-phenyl-indole (DAPI). Three distinct experiments were performed for each case. Digital images were captured using an Olympus BX-61 epifluorescence microscope, equipped with the appropriate filters, a COHU video, and the Cytovision software.

Hybridization signals on at least 100 intact, well-preserved, and non-overlapping nuclei were evaluated by at least two investigators. A gain of gene copy was defined as presence of multiple (three or more) signals in at least 10% of nuclei

Statistical analysis

Chi-square and Fisher's exact tests were used to evaluate possible associations between covariates (ER; PR; Ki67 proliferative index; HER2; expression for survivin, p53, and phosphorylated ERK₁₋₂; amplification of *h-prune* and *CyclinD1*) and clinical outcome in terms of treatment responses and median survivals. Univariate correlations between prognostic variables and survival outcomes were carried out using the Kaplan-Meier method. Variables were also evaluated for independent correlations on survival by Cox regression analysis. Statistical comparisons were performed using the SPSS statistical software package, version 15.0 (SPSS Inc., Chicago, IL, USA). All tests were two-tailed and

P values of less than 0.05 were considered to be statistically significant.

Results

Patients' collection

To evaluate the pathogenetic and prognostic roles of five candidate molecular markers (expression levels of survivin, p53, and pERK1-2 proteins; amplification of *CyclinD1* and *h-prune* genes), we have examined fifty-three patients with diagnosis of T4 breast carcinoma (T4-N0-2-M0, according to the TNM classification by Sobin *et al.* [1]).

All patients were evaluated for response to primary chemotherapy. No disease progression was observed during the treatment. The clinical response rate was 100% (95% CI, 65,2-89,5): a complete clinical response was observed in 8 patients (15%). According to Sataloff's classification [33], pathological complete response in primary tumour (pCR) was observed in 8 patients (15%); major pathological response (MpR), corresponding to pT0-pT1 classification after primary chemotherapy, was observed in 18 (34%) breast tissues. The pathological lymph node assessment revealed absence of involvement (pN0) in 12 (23%) patients after primary chemotherapy.

All 53 patients became suitable for surgery. Modified radical mastectomy was performed in 36 patients (68%) and breast conserving treatment (BCT) was feasible in 17 patients (32%). Records of the clinical follow-up covered a median period of 125 months (range 70-182); 10-year disease-free survival (DFS) and overall survival (OS) within the entire series were 32% and 43%, respectively.

Immunohistochemistry and FISH analysis

Assessment of the expression levels of survivin, p53, and pERK1-2 proteins by immunohistochemistry (IHC) as well as characterization of the chromosomal copy number of *cyclinD1* and *h-prune* genes by fluorescence *in situ* hybridization (FISH) analysis was carried out on paraffin-embedded tissue sections from primary breast carcinomas of our series.

For IHC analysis, a lack of expression for the p53 protein was observed in 13/53 (25%) patients; conversely, a positive immunostaining was detected in 5/53 (9%) cases for pERK1-2, and 21/53 (40%) cases for survivin (Table 2). The FISH analysis was performed using specific probes corresponding to the *h-prune* and *cyclinD1* genomic regions at chromosomes 1q21 and 11q13 (respectively) as well as control clones spanning the peri-centromeric regions at chromosomes 1 and 11, respectively. Multiple FISH signals in >10% analyzed nuclei were found in 8/53 (15%) cases, for *h-prune*, and 12/53 (23%) cases, for *cyclinD1* (Table 2). A normal copy number (diploid signals) was detected for centromeric control probes, confirming the specificity of the

amplification at 1q21 and 11q13 loci and excluding any procedure artifact. Absence of karyotypic anomalies in cells from normal tissues surrounding the tumours strongly indicated that amplification of the *h-prune* and *cyclinD1* genomic regions was highly specific for breast cancer cells. Representative examples of IHC staining and FISH results are shown in Figure 1.

Correlation with clinico-pathological parameters

Using Pearson's Chi-Squared test, molecular alterations were evaluated for association with histological tumour characteristics: ER and PR status, *HER2* amplification, Ki67 proliferation index. No statistically significant correlation between any of the molecular alteration and pathological parameters was observed (including triple negative tumours: ER-, PR-, *HER2*-), with the exception of the detection of positive pERK1-2 immunostaining in the group of patients negative for Ki67 expression only [the pERK1-2 expression was not detected in tumours expressing Ki67 (0/16), whereas 4/26 (15%) Ki67+ tumours presented a positive pERK1-2 immunostaining; $p = 0.041$] (Table 2).

To investigate the role in predicting the response to primary chemotherapy, all tumour characteristics (histological parameters and molecular alterations) were compared to clinical and pathological outcome in our series. As shown in Table 3, the Ki67 proliferation index and *HER2* amplification were significantly associated with a better clinical outcome [5/7 (71%) complete clinical responses *vs.* 11/35 (31%) partial clinical responses, for Ki67 proliferation index; 8/8 (100%) complete clinical responses *vs.* 29/45 (64%) partial clinical responses, for *HER2* amplification]. Conversely, the expression of pERK1-2 was significantly associated with a worse clinical outcome [0/8 complete clinical response *vs.* 5/45 (11%) partial clinical responses] (Table 3). Considering the histological classification, a higher prevalence of positive Ki67 proliferation index was found in cases achieving the pathological response [5/7 (71%) pCR *vs.* 11/35 (31%) non-pCR]; a positive pERK1-2 expression was instead observed in patients who did not achieve the pathological response [0/8 pCR *vs.* 5/45 (11%) non-pCR] (Table 3).

Each molecular alteration was then evaluated for its impact on overall survival. Using the Kaplan-Meier method, survival curves indicated that patients carrying pERK1-2 positive staining ($p = 0.027$), *h-prune* amplification ($p = 0.045$), and survivin overexpression ($p = 0.008$) presented a statistically-significant poorer overall survival in comparison with those resulted negative for such alterations (Figure 2A). No significant association with overall survival was observed for p53 down-expression and *cyclinD1* amplification (Figure 2A). As summarized in Figure 2B, median overall survivals were consistently

Table 2 Comparison between IHC or FISH results and histopathological parameters

Characteristics	FISH analysis				IHC analysis					
	CyclinD1		h-prune		p53		pERK ₁₋₂		survivin	
	positive cases	%	positive cases	%	positive cases	%	positive cases	%	positive cases	%
Total patients (N = 53)	12	23	8	15	13	25	5	9	21	40
Estrogen receptor (ER)										
negative (N = 20)	2	10	1	5	4	20	2	10	9	45
positive (N = 24)	7	29	5	11	5	21	2	8	7	29
Progesterone receptor (PR)										
negative (N = 28)	5	18	2	7	5	18	2	7	13	46
positive (N = 16)	4	25	4	25	4	25	2	12	3	19
Ki67										
negative (N = 26)	4	15	3	12	4	15	4	15	11	42
positive (N = 16)	4	25	2	12	5	31	0	0	5	31
HER2										
0-1 (N = 16)	5	31	3	19	2	12	2	12	9	56
2-3 (N = 37)	7	19	5	14	11	30	3	8	12	32

higher in breast cancer patients with absence of *h-prune* amplification (median OS: 96 months in comparison to 59 months of patients with *h-prune* polysomy) and negative immunostaining for pERK₁₋₂ (median OS: 95 months in comparison to 43 months of patients with pERK₁₋₂+ tumours) and survivin (median OS: 97 months in comparison to 45 months of patients with survivin overexpression).

Using the Cox model adjusted according to age at diagnosis for a multivariate analysis, pathological response to primary chemotherapy and survivin overexpression remained the only parameters with a significant impact on prognosis in our series of breast cancer patients; no other association with overall survival was observed for the remaining variables (Table 4).

Discussion

In this study, we evaluated the impact of some specific molecular alterations (activation of ERK1-2 proteins, amplification of *CyclinD1* and *h-prune* genes, silencing of *TP53* gene, overexpression of survivin protein) as predictive and prognostic factors among patients with T4 breast carcinoma. The analyzed molecular alterations have been largely demonstrated to play an important role in: *a*) deregulating the cell cycle with subsequent induction of abnormal cell proliferation and tumour growth (ERK1-2 phosphorylation and *CyclinD1* amplification); *b*) impairing the apoptotic machinery with subsequent induction of resistance to anticancer agents (p53 downexpression and survivin overexpression); and *c*) promoting metastasis formation (*h-prune* amplification). Our findings indicated that subsets of T4 breast cancer patients with pERK₁₋₂ staining, survivin expression, or *h-prune* amplification in primary tumour tissues

presented a worse overall survival. After multivariate analysis, the pathological response to primary chemotherapy and the survivin overexpression in primary carcinoma represented the main parameters with a role as independent prognostic factors predicting the clinical outcome in such a series of breast cancer patients.

Although an increased expression of survivin in tumour tissues has been already demonstrated to correlate with a poor clinical outcome in a variety of malignancies [20-22], our results clearly indicated an analogous significant impact on prognosis of such a molecular alteration among T4 breast cancer patients. From the pathogenetic point of view, survivin has been found to provide protection against apoptotic stimuli by inhibiting activation of caspase-9 toward the initiation of the intrinsic mitochondrial pathway of apoptosis [18]. Recently, it has been demonstrated that survivin as well as other members of the IAP protein family are strongly involved in metastasis formation; search for survivin-IAP antagonists may indeed provide new antimetastatic therapies for cancer patients [19]. Nevertheless, survivin seems to be upregulated through the activation of the MAPK-ERK pathway [34]; in other words, the overexpression of survivin may be associated with the increased levels of ERK1-2 phosphorylation (in our series, all 5 cases expressing pERK₁₋₂ proteins also presented survivin overexpression). Interestingly, our findings indicated that presence of pERK₁₋₂ expression in primary T4 carcinomas may be indeed correlated with clinical outcome (see Figure 2), suggesting that the cascade of molecular events activating ERK1-2 and upregulating survivin has indeed an important prognostic role in such patients. One could speculate that the lack of a significant association with prognosis for

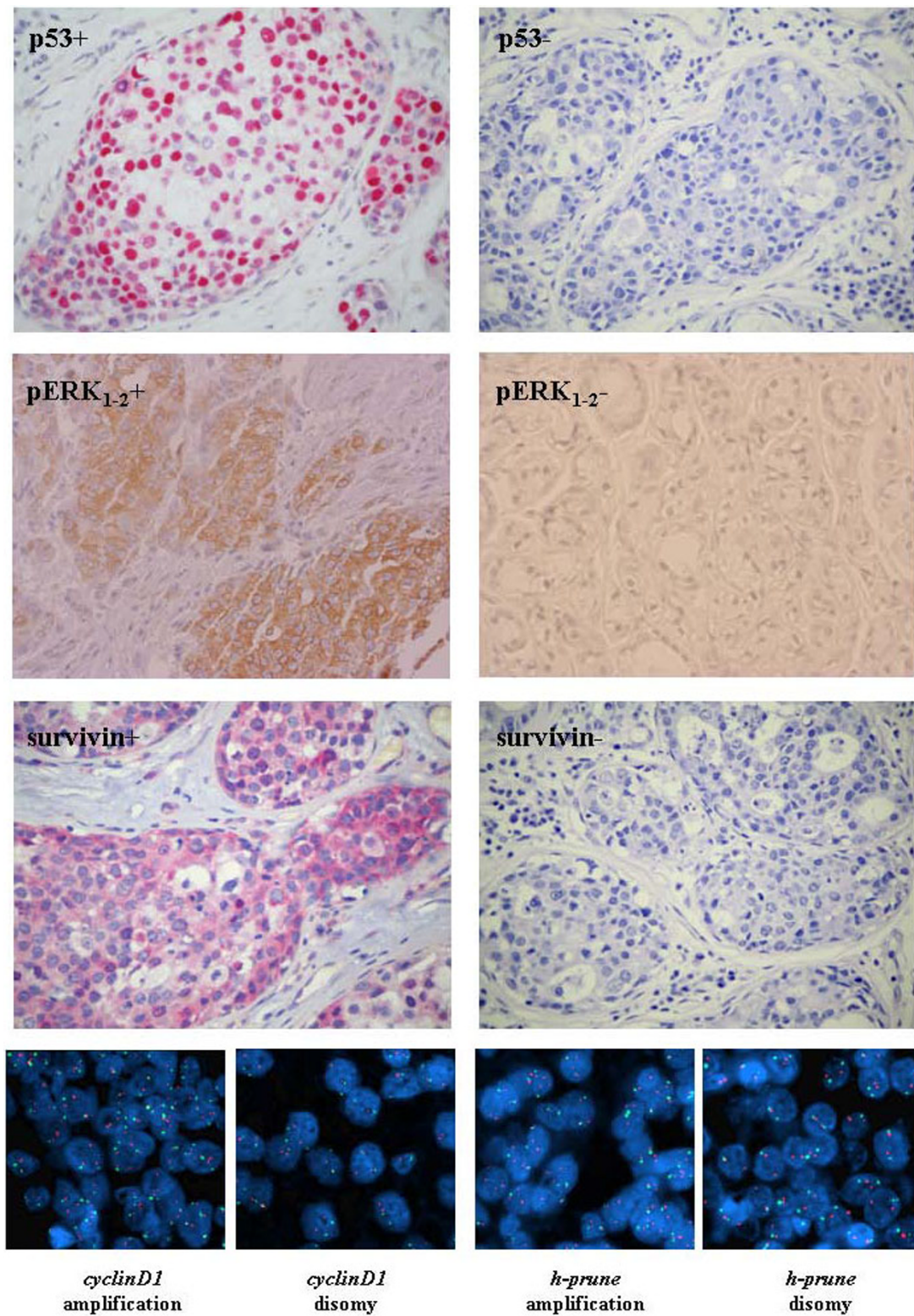


Figure 1 Immunohistochemistry and FISH analysis. (Up-middle) Typical examples of T4 breast carcinoma tissue sections positive (left) or negative (right) for p53, pERK₁₋₂, and survivin protein expression. (Bottom) Typical examples of double-colour FISH results. Nuclei extracted from paraffin-embedded tissues after hybridization with probes specific for *cyclinD1* or *h-prune* loci (red signals) and control chromosome centromeres (green signals).

Table 3 Comparison between histopathological or molecular parameters and response to therapy

A													
Outcome	ER			PR			Ki67			HER2			
	positives	%	P	positives	%	P	positives	%	P	positives	%	P	
Clinical response	0.132			0.640			0.047			0.022			
Complete response	3/7	43		3/7	43		5/7	71		8/8	100		
Partial response	16/29	55		11/29	38		9/27	33		27/37	73		
< Partial response	5/8	62		2/8	25		2/8	25		2/8	25		
Pathological response	0.469			0.235			0.042			0.095			
pCR	4/7	57		3/7	43		5/7	71		7/8	87		
<pCR	20/37	54		13/37	35		11/35	31		30/45	67		
B													
Outcome	cyclinD1			h-prune			pERK ₁₋₂			p53			survivin
	positives	%	P	positives	%	P	positives	%	P	positives	%	P	
Clinical response	0.457			0.824			0.032			0.391			0.089
Complete response	1/8	12		1/8	12		0/8	0		1/8	12		1/8 12
Partial response	8/37	22		5/37	14		3/37	8		10/37	27		15/37 41
< Partial response	3/8	37		2/8	25		2/8	25		2/8	25		5/8 62
Pathological response	0.660			0.822			0.007			0.972			0.234
pCR	2/8	25		1/8	12		0/8	0		2/8	25		3/8 37
<pCR	10/45	22		7/45	16		5/45	11		11/45	24		18/45 40

pCR, pathological complete response

pERK₁₋₂ staining in multivariate analysis may be due to the fact that we identified only a limited fraction (5/53; 9%) of carriers and, thus, the subgroup analysis relied on a small number of subjects.

The well-established prognostic factors currently used into the management of breast cancer patients include the disease stage as well as the degree of differentiation (tumour grade), the proliferation index, and the hormone receptor status (ER, PR, and, recently, HER2) in primary tumours [35,36]. In our series of patients with T4 breast carcinoma, no statistically-significant correlation between any of the analyzed molecular alterations and such pathological parameters was inferred. The only exception was represented by the correlation between the pERK₁₋₂ staining and the Ki67 proliferation index. None of the tumours expressing a high Ki67 proliferation index showed an increased level of pERK₁₋₂ protein; conversely, all cases with activated ERK₁₋₂ protein presented a low Ki67 proliferation index. Activation of ERK1-2 proteins has been demonstrated to promote cell cycle progression, participating to induction of cell growth and enhancement of cell survival [10]. Our findings led us to speculate that: a) induction of cell proliferation via pERK₁₋₂ and Ki67 molecules may represent two unrelated phenomena; and b) among patients with low Ki67 expression levels (who may have an unfavourable prognosis [37], though the role of Ki67 proliferation index as prognostic and predictive marker is yet to be conclusively defined [38]), the presence of pERK₁₋₂ overexpression seems to identify a subgroup with an even

worse prognosis. Taking into consideration the response rates, patients whose tumours had high Ki67 expression levels or *HER2* amplification presented the highest rates of response to primary chemotherapy (for Ki67, a significant association was found with both clinical and pathological responses; for *HER2*, a significant association was surprisingly observed with clinical response only) (see Table 3). These latter findings are consistent with data previously reported [39-41]. Among the molecular parameters, only pERK₁₋₂ expression seemed to be significantly correlated with response to primary chemotherapy (significant lower rates were observed for both clinical and pathological responses; see Table 3), reflecting the fact that the activation of ERK1-2 proteins may increase the resistance to apoptosis, reducing the sensitivity to chemotherapy [10].

Several mechanisms have been recently described to participate in progression of breast cancer through activation of the h-prune complex. It is now clear the existence of a network of interacting proteins which indeed regulate the phosphodiesterase activity of h-prune, contributing to promote (ASAP1) or inhibit (nm23-H1) either cancer cell motility and tumour adhesiveness *in vitro* either tumour invasiveness and metastasis formation *in vivo* [25-29]. The increased expression of h-prune protein has been demonstrated to deeply modify this equilibrium of opposite stimuli, playing an important role in promotion of cancer progression [25]. Among others, the main mechanism leading to h-prune overexpression is represented by the amplification of

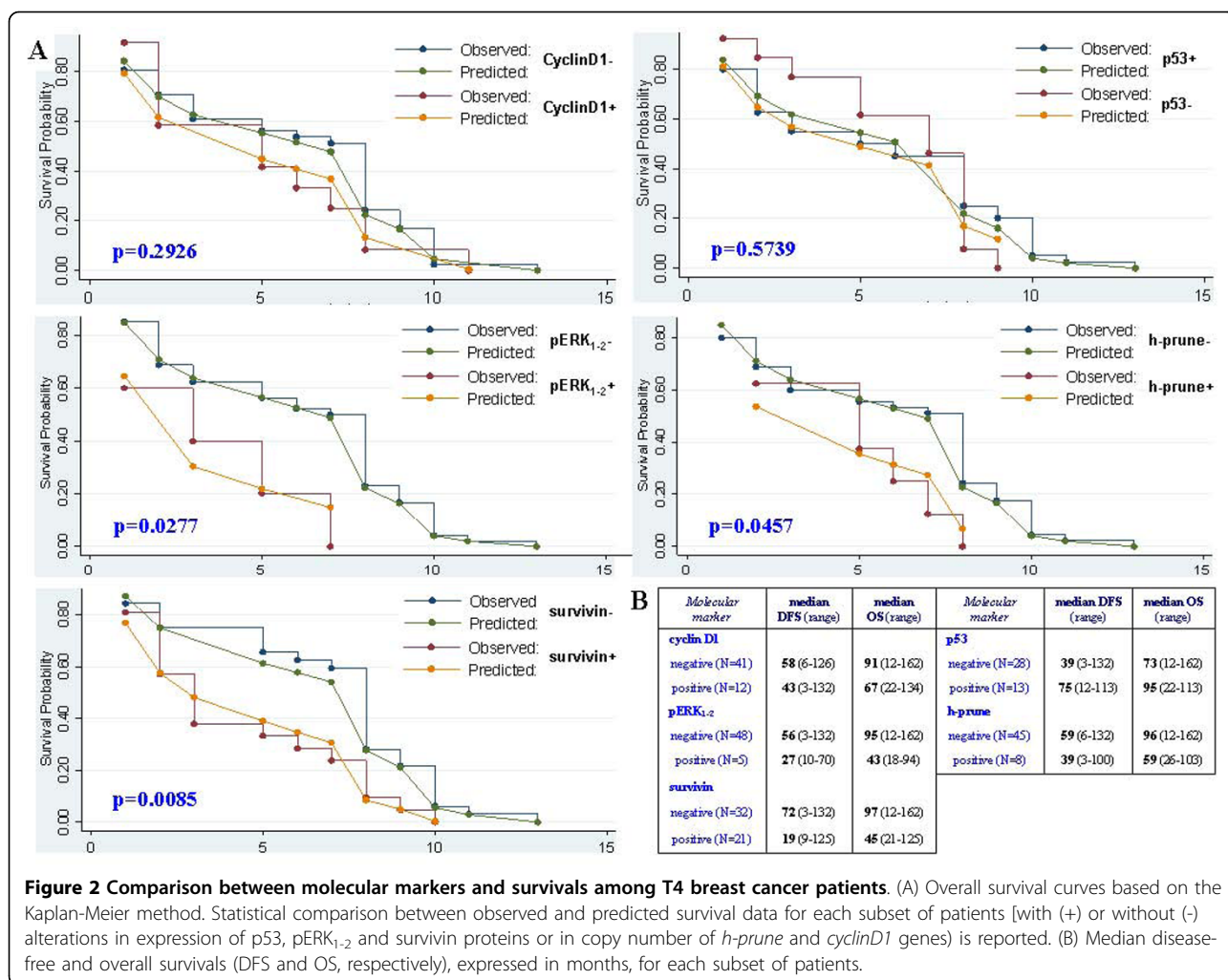


Figure 2 Comparison between molecular markers and survivals among T4 breast cancer patients. (A) Overall survival curves based on the Kaplan-Meier method. Statistical comparison between observed and predicted survival data for each subset of patients [with (+) or without (-) alterations in expression of p53, pERK₁₋₂ and survivin proteins or in copy number of *h-prune* and *cyclinD1* genes] is reported. (B) Median disease-free and overall survivals (DFS and OS, respectively), expressed in months, for each subset of patients.

gene copy number [25,42]. Considering tumours with at least three gene copies, a small fraction (8/53; 15%) of T4 breast carcinomas from our series presented *h-prune* amplification at chromosome 1q21.3 (see Table 2); such a frequency is quite identical to that described in our

previous report (173/1,016; 17%) [30]. All breast cancer patients included into the present study showed axillary nodal involvement; among them, occurrence of *h-prune* amplification was able to identify a subset with a worse overall survival (see Figure 2). As for pERK₁₋₂ staining, the low number of events could explain the absence of a significant association of the *h-prune* amplification with prognosis in the multivariate analysis.

Table 4 Multivariate analysis of different parameters for overall survival

Characteristic	Hazard Ratio	95% CI	P
<i>cyclinD1</i>	0.75	0.26-2.12	0.591
<i>h-prune</i>	2.37	0.85-7.03	0.078
pERK ₁₋₂	1.08	0.37-3.18	0.875
p53	0.74	0.34-1.58	0.443
survivin	3.40	1.20-4.76	0.012
estrogen receptor (ER)	1.88	0.57-6.91	0.281
progesterone receptor (PR)	1.33	0.36-4.84	0.639
proliferation index (Ki67)	1.81	0.48-6.85	0.377
HER2	3.24	0.96-14.5	0.059
pathological response	8.83	1.10-50.2	0.040

Stratified by age. CI, confidence interval. In bold, significant values.

Conclusions

Although our study was retrospective, some important indications about either the prediction of the response to therapy or the role on prognosis in T4 breast cancer patients have been inferred. There is no doubt that the pathological response after primary chemotherapy remains one of the major predictor of survival; however, the molecular marker represented by survivin overexpression may be also considered as a useful prognostic factor in these patients. To validate the incorporation of survivin or the other promising molecular parameters

(h-prune and pERK₁₋₂) as markers for management of T4 patients, further large prospective studies are awaited. Nevertheless, translational studies investigating additional molecular biomarkers should contribute to more accurately identify subsets of patients who would be expected to be more or less likely to respond to specific therapeutic interventions.

List of abbreviations

AJCC: American Joint Committee on Cancer; ER; Estrogen Receptor; FISH: fluorescence *in situ* hybridization; IHC: immunohistochemistry; MAPK: mitogen activated protein kinase; PR: Progesterone Receptor; UICC: International Union Against Cancer.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BM conceived of the study. MS performed molecular analysis. MB performed statistical analysis. FA participated to collection of cases. MD participated to collection of cases. VP participated to collection of cases. MP performed molecular analysis. PS participated to interpretation of results. AC participated to data management. GrP performed some molecular analyses. MI participated to design of the study. GiP participated to interpretation of data and drafted the manuscript. All authors read and approved the final manuscript.

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