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P16 but not retinoblastoma expression is related to clinical outcome in no-special-type triple-negative breast carcinomas

Giuseppe S Bogina[1], Gianluigi Lunardi[2], Lisa Marcolini[3], Matteo Brunelli[3], Laura Bortesi[1], Marcella Marconi[1], Francesca Coati[2], Matteo Valerio[2], Massimo Guerriero[4], Alberto Massocco[5], Maria C Pegoraro[6] and Giuseppe Zamboni[1]

[1]Department of Pathology, Sacro Cuore Hospital of Negrar, Verona, Italy; [2]Department of Oncology, Sacro Cuore Hospital of Negrar, Verona, Italy; [3]Department of Pathology, University of Verona, Verona, Italy; [4]Department of Statistic, University of Verona, Verona, Italy; [5]Department of Surgery, Sacro Cuore Hospital of Negrar, Verona, Italy and [6]Department of Oncology, ULSS 5 Ovest Vicentino, Vicenza, Italy

Triple-negative breast carcinomas represent a tumor group of pivotal clinical importance given the lack of target therapies. The prognostic significance of triple-negative breast carcinomas remains unclear because of their histological and molecular heterogeneity. Currently, neither prognostic nor predictive factors are available for these tumors. Retinoblastoma (Rb) pathway loss has been linked to clinical outcome in various cancer types, including breast cancer. We investigated the association between Rb and p16 protein expression and clinical outcome in no-special-type triple-negative breast carcinomas. Immunohistochemical staining for Rb, p16, p53 and CK5 was carried out on a section from archival specimens of 117 no-special-type triple-negative breast carcinomas. Immunopositive p16 (p16+) and immunonegative Rb (Rb−) staining were seen in 49.5% and in 24.8% of tumors, respectively. There was an inverse correlation between p16+ and Rb− ($P<0.001$). P16+ was correlated with G3 grade ($P<0.001$), high Ki-67 ($P=0.03$), p53 overexpression ($P<0.001$) and CK5 immunopositivity ($P=0.01$). Rb− was not associated with any clinicopathologic variable. Follow-up and therapy data were available in 95 patients. In 20 patients treated with surgery only, neither p16+ nor Rb− immunostaining were associated with disease-free survival and overall survival. In 75 patients treated with adjuvant chemotherapy, p16+ was associated with good response to therapy with significant increased disease-free survival ($P=0.001$) and showed a trend towards a statistical significance for increased overall survival ($P=0.056$); Rb− were not associated with disease-free survival and overall survival. In multivariate analysis, p16+ was independently associated with disease-free and overall survival, with a hazard ratio of 0.18 (95% CI: 0.06–0.51; $P=0.001$) and 0.21 (95% CI: 0.06–0.74; $P=0.015$), respectively. In patients with no-special-type triple-negative breast carcinomas, p16+ is related to good response to adjuvant chemotherapy and can be considered the best surrogate marker for Rb pathway loss.
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The term 'triple-negative breast carcinoma' is commonly used to define breast cancers that are immunohistochemically negative for estrogen (ER) and progesterone receptors (PR) and lacking overexpression and/or amplification of the *ERBB2* gene.[1–4] They account for 10–20% of all breast cancers patients[3–5] and, generally, have an aggressive clinical course. Moreover triple-negative breast cancers are not a single disease, but a heterogeneous entity having different morphologic and molecular features. In fact, they include special morphologic subtypes, sometimes associated with a better prognosis[1,4,6] and at least two molecular subtypes of triple-negative breast carcinomas, basal-like and non-basal breast carcinomas.[3] Although basal-like breast carcinomas are currently defined by gene expression profiling, triple-negative breast carcinomas with the expression of basal cytokeratins (CK 5/6, CK14 and CK17) and/or epidermal growth

Correspondence: Dr G Bogina, MD, Department of Pathology, Sacro Cuore Hospital, Via Don A Sempreboni 5, 37024 Verona, Negrar, Italy.
E-mail: giuseppe.bogina@sacrocuore.it
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factor receptor (EGFR) are often used as an immunohistochemistry surrogate for basal-like breast carcinomas.[7] Nevertheless, most clinicians use the triple-negative definition for reasons of convenience, as no rigorous staining and scoring protocols have been developed for these basal-like immunohistochemistry markers.[3] Chemotherapy is currently the mainstay of systemic treatment for triple-negative breast carcinomas because hormonal and HER2-directed therapies are not effective. Currently, neither prognostic nor predictive factors are available to guide treatment decisions. Consequently, in the adjuvant setting, overtreatment with potentially life-threatening side effects or undertreatment without effectiveness is possible.[3,5] Over the past few years, retinoblastoma (Rb), a tumor suppressor gene that regulates cell-cycle progression, has been associated with disease progression and therapeutic outcome in various cancer types.[8] Regarding breast carcinomas, Rb loss is more frequently observed in triple-negative breast carcinomas.[7,9,10] Molecular Rb pathways' loss were associated with improved response to chemotherapies and resistance to antiestrogen therapies.[11–14] Unfortunately, immunohistochemistry studies showing a decrease in or lack of Rb protein expression have provided conflicting results concerning prognosis and response to therapies.[10,14–20] Rb is inactivated by cyclin-dependent kinase (CDK)4-mediated phosphorylation, and the kinase activity of CDK4 is suppressed by p16INK4a (p16). Because inactivation of Rb results in the upregulation of p16 expression, high levels of p16 were used as an immunohistochemistry surrogate of Rb loss, mainly in squamous cell carcinomas harboring high-risk papilloma virus.[7] Based on morphologic similarity of basal-like breast carcinomas to papilloma virus-related squamous cell carcinomas of the head and neck, Subhawong *et al*[7] were the first to demonstrate directly that basal-like and other triple-negative breast carcinomas frequently demonstrate Rb − /p16 diffuse positive phenotype.

The aim of this study was to determine the association between Rb and p16 protein expression and clinical outcome in a well-defined subset of no-special-type triple-negative breast carcinomas.

Materials and methods

The targets of our study were all no-special-type triple-negative breast carcinomas, diagnosed at the Institute of Pathology of Sacro Cuore Hospital, Negrar, Verona, between January 1998 and December 2010.

Case selection process is shown in Figure 1.

From a dedicated database including all breast cancer, we selected ER/PR $<1\%$ cases, without HER2 assessment (group 1: 1998–2005) and ER/PR $<1\%$ and HER2 − (HER2 = 0/1 + immunohistochemistry and 2 + immunohistochemistry, silver *in situ* hybridization non-amplified) (group 2: 2006–2010). Group 1 tissue samples were assessed for HER2 by immunohistochemistry and by silver *in situ* hybridization in 2 + immunohistochemistry cases. We obtained 172 triple-negative breast carcinomas diagnosed according to our standard algorithm: 137 were triple-negative breast carcinomas of no special type, whereas 35 were of special type.

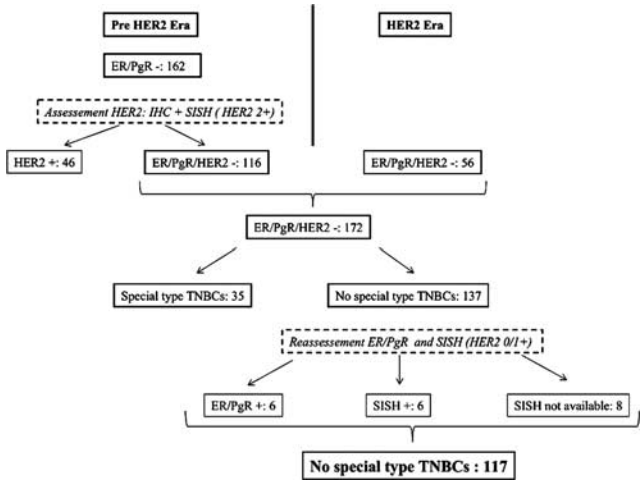

Figure 1 Cases selection algorithm.

Subsequently, no-special-type triple-negative breast carcinomas samples were reassessed for ER/PR status by immunohistochemistry and by HER2-silver *in situ* hybridization in cases HER2 0/1 + immunohistochemistry. After this reassessment, 6 cases were ER and/or PR $\geq 1\%$, 6 cases were HER2-silver *in situ* hybridization amplified and for 8 cases silver *in situ* hybridization was not available. At the end of the analysis, our sample consisted of 117 no-special-type triple-negative breast carcinomas.

Tissue samples were fixed in 10% buffered formalin for 20–24 h before processing and embedding. Tumors were classified histologically according to the World Health Organization Histological Classification.[21] Tumor grading was assessed according to Elston and Ellis system.[22]

Data from each patient regarding medical history, results of staging procedures, therapy and follow-up were retrieved. Patients having bilateral breast cancer or others malignant tumors were excluded from the outset.

To evaluate the possible clinical impact of Rb and p16 expression, we considered disease-free survival, defined as elapsed months since diagnosis of primary tumors to first appearance of any type of relapse (locoregional recurrence or distant metastasis) or censored at the date of last follow-up, and overall survival, defined as elapsed months since diagnosis of primary tumors to death or censored at the date of last follow-up.

Patients having distant metastases at the time of diagnosis were excluded from statistical analysis concerning disease-free survival and overall survival.

In compliance with the Declaration of Helsinki, this investigation was approved by our reference Ethic Committee.

Immunohistochemistry and Silver *In Situ* Hybridisation

From each tumor, the most informative block was selected for immunohistochemistry and silver *in situ* hybridization. Each block contained normal breast tissue as internal control. For each case, we performed ER, PR, Ki-67, HER2, p16, Rb, p53 and CK5 immunohistochemistry staining and HER2-silver *in situ* hybridization using serial sections from the same paraffin-embedded block. The protocol used for each single antibody is reported in Table 1. The silver *in situ* hybridisation method, used for ERBB2 gene amplification, was Brightfield Double ISH (VMS-Roche Diagnostics), using INFORM-HER Dual ISH DNA Probe Cocktail, and ultraVIEW AP Red ISH DIG Kit + ultra VIEW SISH DNP Kit detection system.

According to ASCO-CAP guidelines, we defined as positive tumors with ER/PR $\geq$1%. HER2 immunohistochemistry expression was scored according to FDA-approved guidelines as 0 (no staining or weak/moderate, incomplete/complete staining in $\leq$10% of cells), 1+ (weak and incomplete staining in $>$10% of cells), 2+ (weak/moderate complete staining in $>$10% of cells) and 3+ (strong, complete staining in $>$10% of cells). For silver *in situ* hybridization analysis, the *ERBB2*/chromosome 17 ratio was calculated and scored as follows: ratio $<$2 *ERBB2* gene not amplified; ratio $\geq$2: *ERBB2* gene amplified.

Ki-67 was scored evaluating the percentage of positive-stained nuclei (irrespective of staining intensity): based on the median value (40%), tumors were divided in low and high Ki-67. P16, Rb, p53 and CK5 were scored using a semiquantitative method that takes into account both the percentage and intensity of staining. The percentage of positive tumor cells was established by the assignment of a proportion score: 0 = none; 1 = 1–10%; 2 = 11–30%; 3 = 31–50%; 4 = 51–75%; and 5 = 76–100%. Thereafter, an intensity score, which represented the average intensity of positive tumor cells, was assigned: 0 = none; 1 = weak; 2 = intermediate; and 3 = strong. The proportion and intensity scores were then multiplied to obtain a total score, which ranged from 0 to 15. The positivity threshold of each biomarker was chosen according to significant values obtained from the literature: Rb $>$0;[7,9,10,16,23] p16 $\geq$12;[7,9,23–25] p53 $\geq$8;[7,25,26] and CK5 $>$0.[27,28]

In particular, a p16 score $>$12 correlates with diffuse immunoreactivity in the tumor, as opposed to the more common and less significant patchy immunoreactivity that many cancers show for p16.

All slides were scored by a single dedicated breast pathologist (GB) who did not have knowledge about patients' outcome.

Statistics

For statistical analysis, data were imported and merged in STATA/IC for windows version 12.

χ^2 or Fisher's exact test were used to compare statistically the categorical variables.

Cumulative incidence of disease-free survival and overall survival in the groups was described by the Kaplan–Meier method and was compared with the use of the log-rank test. Patients alive and not relapsing or alive, regardless of relapsing, were censored at the date of their last follow-up visit for disease-free survival and overall survival, respectively. A two-sided *P*-value $<$0.05 was considered statistically significant. Univariate and multivariate Cox proportional hazard regression model was used to evaluate the independent prognostic relevance of the following factors for disease-free survival and

Table 1 Protocol used for each single antibody

Antibody	*Company*	*Clone*	*Source*	*Dilution*	*Immunostainer*	*Antigen retrieval*	*Incubation*	*Detection*
Confirm ER Rmab	VMS	SP1	Rmab	PD	BenchMarch XT VMS	CC1 pH 8.2 × 30′	37 °C × 20 min	UltraView Universal Dab Detection
Confirm PR Rmab	VMS	1E2	Rmab	PD	BenchMarch XT VMS	CC1 pH 8.2 × 30′	37 °C × 16 min	UltraView Universal Dab Detection
Confirm anti-Ki-67	VMS	K-2	Mmab	PD	BenchMarch XT VMS	CC1 pH 8.2 × 30′	37 °C × 16 min	UltraView Universal Dab Detection
Pathway HER-2/*neu*	VMS	4B5	Rmab	PD	BenchMarch XT VMS	CC1 pH 8.2 × 30′	37 °C × 16 min	UltraView Universal Dab Detection
Confirm anti-p53	VMS	D07	Mmab	PD	BenchMarch XT VMS	CC1 pH8,2 × 30′	37 °C × 40 min	UltraView Universal Dab Detection
P16	SCBT	JC8	Mmab	1:100	BenchMarch XT VMS	CC1 pH 8.2 × 30′	37 °C × 20 min	UltraView Universal Dab Detection
Cytokeratin 5	Leica	XM26	Mmab	1:100	Bond MaX Leica	ER2 pH 6.0 × 30′	RT × 15 min	Bond Polymer Refine Detection
RB gene protein	Leica	13A10	Mmab	1:50	Bond MaX Leica	ER2 pH 9.0 × 30′	RT × 15 min	Bond Polymer Refine Detection

Abbreviations: Leica, Leica Biosystems, Newcastle, UK; Mmab, mouse monoclonal antibody; RB, retinoblastoma; Rmab, rabbit monoclonal antibody; SCBT, Santa Cruz Biotechnology, Santa Cruz, CA, USA; VMS, Ventana Medical Systems, Tucson AZ, USA.

overall survival: age (≤45 years *vs* >45 years); tumor size (T1 *vs* T2–T4); node status (negative *vs* positive ≤3 *vs* positive >3); histological grade (grade 2 *vs* grade 3); vascular invasion (absent *vs* present); Ki-67 immunostaining percentage (≤40% *vs* >40%); p16 (<12 *vs* ≥12); and Rb (0 *vs* ≥1) immunostaining score. In multivariate analysis, all variables were initially included into the model and then removed by backward stepwise selection if their *P*-value was >0.05. To verify the proportional hazard assumption, estat phtest (test based on Shoenfeld residuals) and linktest (test for model goodness of fit) STATA commands were used.

Results

From reassessment of ER, PR and HER2, as described above, we obtained 152 triple-negative breast carcinomas: 117 of no special type and 35 of special type (14 apocrine, 10 metaplastic, 5 lobular, 3 adenoid cystic and 3 medullary carcinoma). We focused our analysis on 117 no-special-type triple-negative breast carcinomas. Their clinicopathologic features and therapy data are summarized in Table 2.

Table 2 Clinicopathologic features of 117 TNBCs of no special type

	Patients: 117
Median age (years) (range)	59 (26–97)
Tumor size (mm)	
≤20	60
>20	57
Tumor grading	
G1	0
G2	12
G3	105
Vascular invasions	
Yes	29
No	83
Unknown	5
No. of positive nodes	
0	67
1–3	23
≥4	18
Unknown	9
Ki-67 (%)	
≤40	70
>40	47
Chemotherapy	
Yes	83
No	27
Unknown	7
Chemotherapeutic regimens	
CMF	15
CMF + A	11
CMF + A + T	11
FEC	17
FEC + T	16
A + C	9
Other	4

Abbreviations: A, anthracycline; C, cyclophosphamide; CMF, cyclophosphamide; FEC, 5-fluorouracil, epirubicin, cyclophosphamide; T, taxane; TNBCs, triple-negative breast carcinomas, methotrexate, 5-fluorouracil.

Association of P16 and Rb Expression with Pathologic Features

P16 immunopositivity (p16 +) was seen in 58 of 117 triple-negative breast carcinomas (49.5%), whereas Rb immunonegativity (Rb −) was seen in 29 of 117 triple-negative breast carcinomas (24.8%) (Table 3). There was a clear inverse correlation between p16 + and Rb − ($P<0.001$): tumors with p16 strong immunostaining were generally devoid of Rb immunostaining in the tumor compartment, although stroma and lymphocytes were positive (Figures 2a–c), whereas tumors with p16 absent/low immunostaining were Rb immunopositive (Figures 2d–f). P16 + was correlated with G3 grade ($P<0.001$), high Ki-67 ($P=0.03$), p53 overexpression ($P<0.001$) and CK5 immunopositivity ($P=0.01$). Other clinicopathologic variables, such as age, tumor size, vascular invasions and lymph node status, were not associated with p16 + . Rb − was not associated with any clinicopathologic variables.

Association of P16 and Rb Expression with Disease Outcomes

Disease-free survival and overall survival analysis was possible in 95 out of 117 patients: in 14, follow-up and/or therapy was unknown, whereas 8 were excluded because they had distant metastases at the time of diagnosis. Twenty out of 95 patients were treated with surgery only, whereas 75 patients were treated with surgery and adjuvant chemotherapy. Disease progression occurred in 27 patients: 6 experienced locoregional recurrence, 11 distant metastasis and 10 both locoregional recurrence and distant metastasis. The median relapsing time was 23 months. Twenty patients were dead at the time of analysis. The median time to death was 37.5 months. Patients alive and disease free at the time of analysis had a median follow-up of 80 months. In patients treated with surgery only, neither p16 + nor Rb − immunostaining were associated with disease-free survival and/or overall survival ($P=0.11$ and 0.40 for p16 and $P=0.32$ and 0.67 for Rb, respectively) (Figure 3). In patients treated with surgery and adjuvant chemotherapy, p16 + was associated with good response to therapy: Kaplan–Meier analysis showed a statistically significant increased disease-free survival ($P=0.001$) and a trend towards a statistical significance for increased overall survival ($P=0.056$) (Figures 4a and b). Conversely, Rb − was not associated with disease-free survival and

Table 3 P16 and Rb expression in 117 TNBCs of no special type in relation to clinicopathologic variables

	P16− *(59 patients)*	*P16+* *(58 patients)*	P-*value*	*Rb−* *(37 patients)*	*Rb+* *(80 patients)*	P-*value*
Age (years)						
≤45	9	12	0.47	9	12	0.29
>45	50	46		28	68	
Tumor size (mm)						
≤20	34	26	0.19	17	43	0.55
>20	25	32		20	37	
Vascular invasions						
Yes	16	13	0.52	6	22	0.16
No	39	44		31	53	
Unknown	4	1		0	5	
No. of positive nodes						
0	30	37	0.36	22	45	0.64
1–3	14	9		8	15	
≥4	10	8		4	14	
Unknown	5	4		3	6	
Tumor grade						
G2	12	0	<0.001	3	9	0.75
G3	47	58		34	71	
Ki-67 (%)						
≤40	42	28	0.014	18	52	
>40	17	30		19	28	
pRb						
Negative	6	31	<0.001			
Positive	53	27				
P53						
Negative	38	16	<0.001	15	39	0.43
Positive	21	42		22	41	
CK5						
Negative	28	14	0.012	6	24	0.17
Positive	31	44		31	56	

Abbreviation: TNBCs, triple-negative breast carcinomas.
p16 positive: IHC score ≥12; Rb and CK5 positive: IHC score ≥1; p53 positive: IHC score ≥8.

overall survival ($P=0.66$ and 0.89, respectively) (Figures 4c and d).

Results of multivariate Cox proportional hazards regression analysis, adjusted for age, tumor size, node status, histological grading, vascular invasion, Ki-67, Rb and p16 immunostaining are reported in Table 4. In this model, p16+ was independently associated with disease-free survival and overall survival, with a hazard ratio of 0.18 (95% CI: 0.06–0.51; $P=0.001$) and 0.21 (95% CI: 0.06–0.74; $P=0.015$), respectively.

Discussion

RB1 gene, located on chromosome 13 (13q14), encodes a nuclear phosphoprotein that regulates cell-cycle progression through the G1- to-S-phase transition. In quiescent cells, Rb is hypophosphorylated and, assembling transcriptional repressor complexes on the promoters of E2F-regulated genes, blocks cell-cycle progression. In cells entering cell cycle, extracellular signals induce the expression of D-type cyclins, which bind to and activate cyclin-dependent kinases (CDK4 and CDK6); these complexes in turn lead to the phosphorylation of Rb and its dissociation from E2F family members that then transcriptionally activate many genes required for cell-cycle progression.[29] P16, the principal member of the INK4 family of CDK inhibitors, is codified by the *CDKN2A* gene localized on chromosome 9p21. The binding of p16 to CDK4/6 inhibits CDK4 and CDK6, retaining Rb in its hypophosphorylated status, preventing cell-cycle progression from G1 to S phase. Elevated expression of p16 is a strong mechanism of inhibition of proliferation and cellular senescence induction, resulting in tumor development block. On the other hand, p16 overexpression is observed in a number of cancers with inactivated Rb: a cell with a compromised Rb pathway will initiate a regulatory-induced overexpression of p16 because of negative feedback regulation.

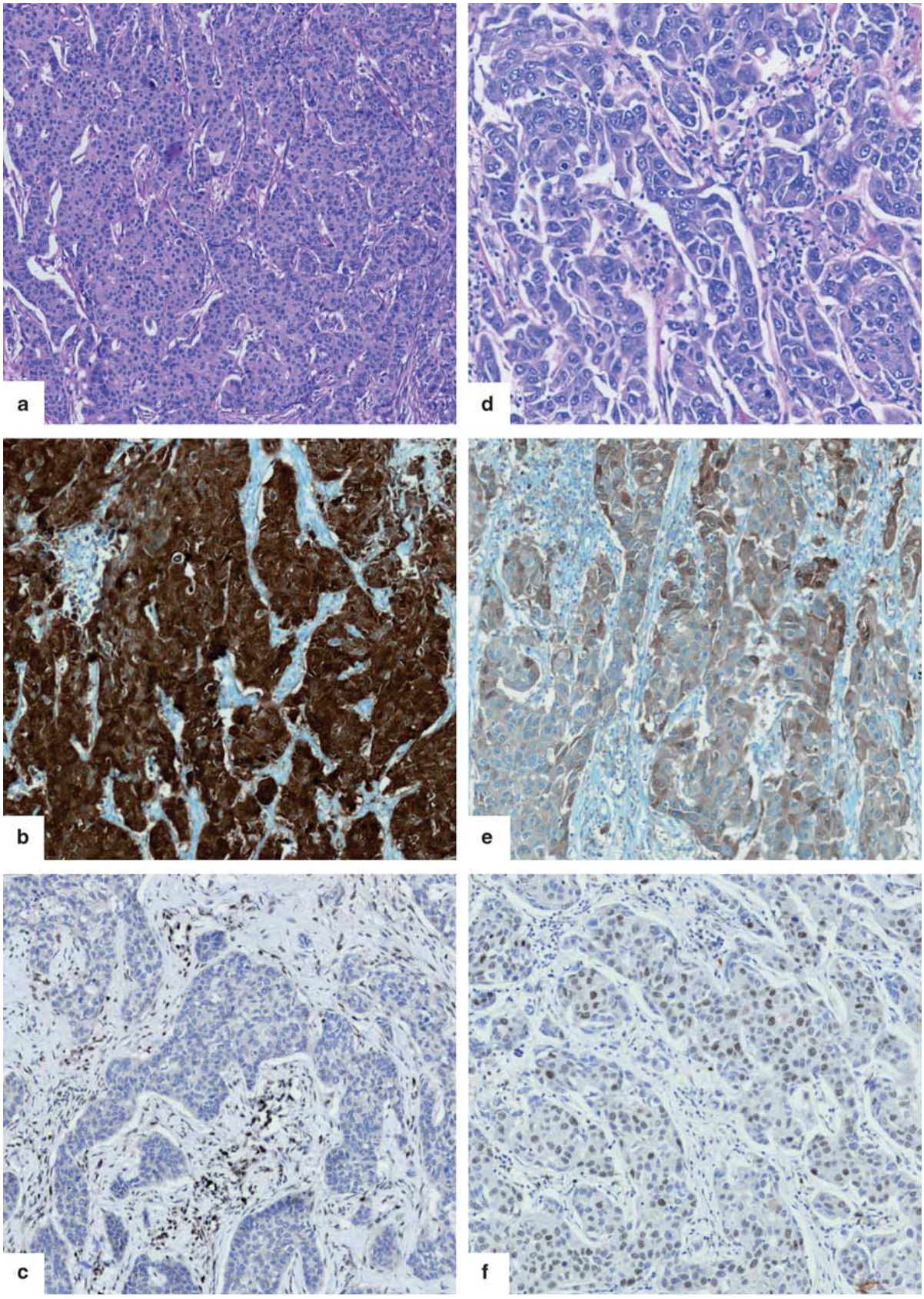

Figure 2 Two different examples of high-grade, no-special-type triple-negative breast carcinomas. Case 1 (**a–c**): Hematoxylin–eosin (× 20) (**a**) with strong immunoreactivity for p16 (**b**) and negativity for Rb (stromal and lymphoid control cells positive) (**c**). Case 2 (**d–f**): Hematoxylin–eosin (× 20) (**d**) with p16 low (**e**) and Rb + (**f**) immunostaining.

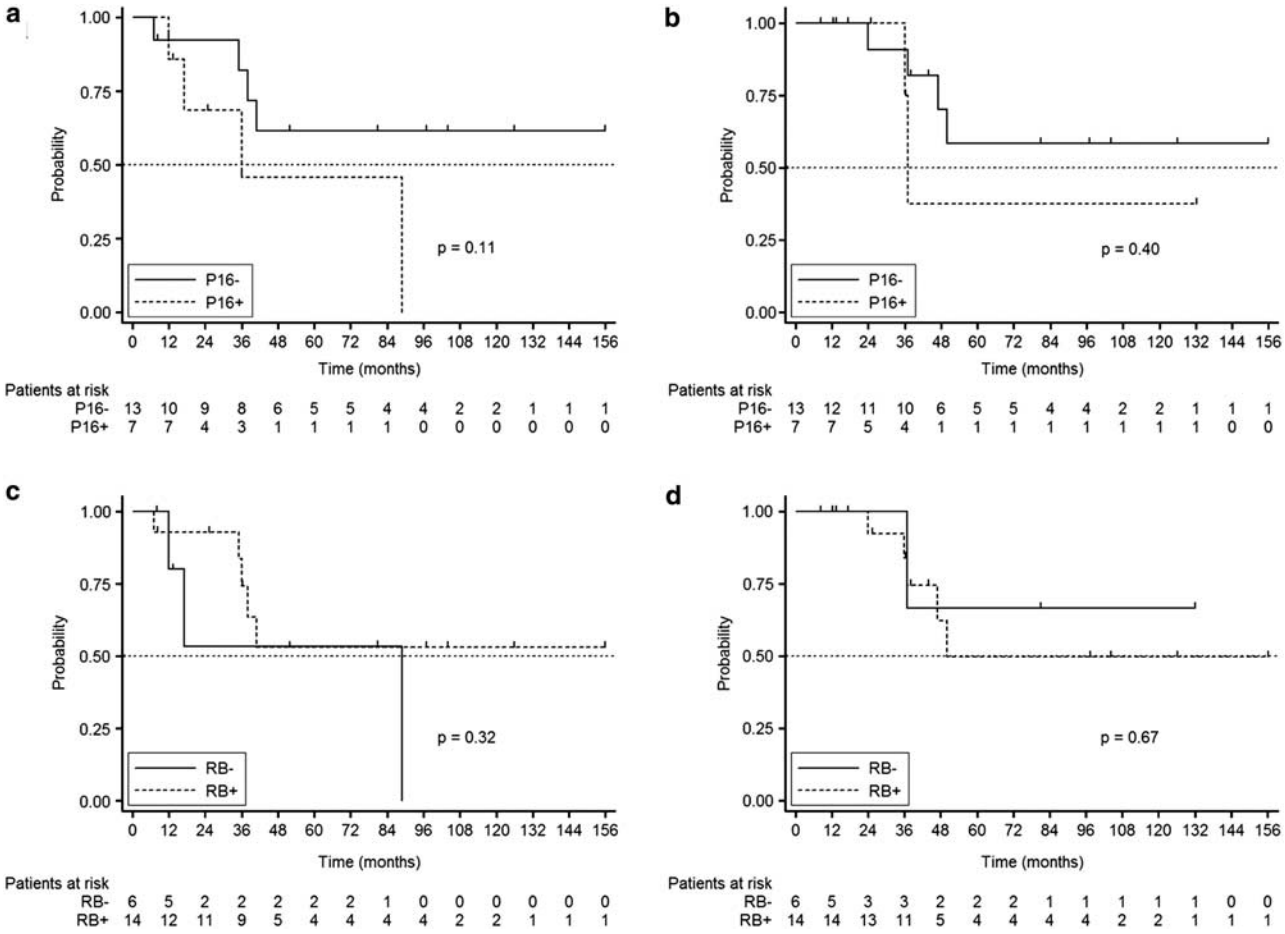

Figure 3 Disease outcome related to p16 and Rb protein expression in patients treated with surgery only. (**a** and **b**) P16+ was not associated with disease-free survival (**a**) and overall survival (**b**). (**c** and **d**) Rb− was not associated with disease-free survival (**c**) and overall survival (**d**).

Elevated expression of p16, in conjunction with a high proliferative index, is believed to be indicative of Rb functional loss.[23,30] In recent molecular studies, Rb loss signature has been linked to poor prognosis in breast cancer patients receiving adjuvant endocrine therapy and to good prognosis in patients receiving chemotherapy.[11–14] Rb immunohistochemistry studies have provided conflicting results concerning prognosis and response to therapies, and also because of the use of different immunohistochemistry cutoff.[10,14–20] The results reported by Derenzini *et al*[16] indicated that only the absence but not hyperphosphorylation of Rb protein predicts clinical outcome. However, it should be noted that immunohistochemistry expression of Rb has provided contradictory interpretations concerning correlation with *RB1* gene status. Herschkowitz *et al*[9] showed that Rb loss of heterozygosity, occurring at a frequency of 72.2% in basal-like breast carcinomas, did not correlate with Rb absent immunostaining, but it significantly correlates with p16 strong immunostaining.

Only a few studies have investigated the correlation between p16 and clinical outcome in breast cancers. In these studies, strong immunoexpression of p16 was associated with improved response to neoadjuvant chemotherapy in invasive breast cancer.[24]

Despite Rb/p16 pathway perturbation being a more common event in triple-negative breast carcinomas,[7,9,10] only a few studies investigated Rb/p16 correlation with clinical outcome in this subset of tumors.[10,24] In triple-negative breast carcinomas, there are not established markers predicting chemotherapy response, although it is very heterogeneous.[14] This heterogeneity is also because of the presence of some special histotypes of triple-negative breast carcinomas, which are associated with different prognosis.[1,4,6]

The aim of our study was to evaluate the association between p16 and Rb immunostaining and clinical outcome in a well-defined subset of no-special-type triple-negative breast carcinomas.

In agreement with other authors,[7,9,14] we confirmed an inverse relationship between p16 and Rb protein expression: tumors with p16 absent/low immunostaining were Rb immunopositive, whereas those with p16 strong immunostaining were

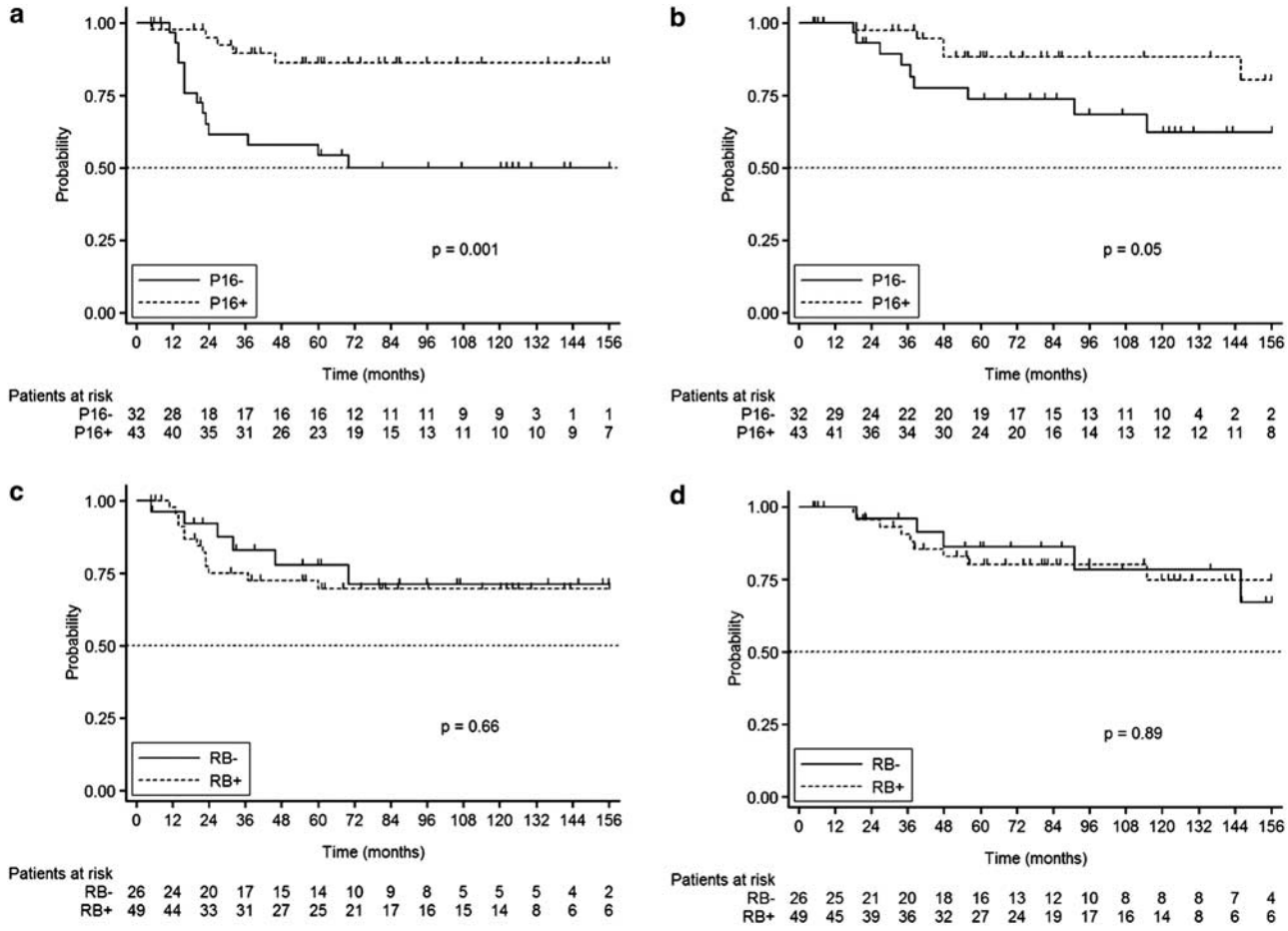

Figure 4 Disease outcome related to p16 and Rb protein expression in patients treated with surgery and chemotherapy. (**a**) P16+ cases showed a statistical significance for increased disease-free survival. (**b**) P16+ cases showed a trend towards a statistical significance for increased overall survival. (**c** and **d**) Rb− was not associated with disease-free survival (**c**) and overall survival (**d**).

generally devoid of Rb immunostaining. P16+ was correlated with high-grade tumour, high Ki-67, p53 overexpression and CK5 immunopositivity. Conversely, Rb− showed no association with any variable analyzed.

Subhawong *et al*,[7] in a subset of 33 triple-negative breast carcinomas, did not show significant differences in the Rb−/p16+ phenotype between basal-like (CK5 and/or EGFR immunohistochemistry positivity) and unclassifiable (CK5 and EGFR immunohistochemistry negativity) triple-negative breast carcinomas, nor significant correlation between Rb−/p16+ phenotype and p53 overexpression. As shown in our study, the author demonstrated the correlation between Rb−/p16+ phenotype and higher Ki-67 index both in basal-like and unclassifiable triple-negative breast carcinomas.

We demonstrated that patients with p16+ tumors had a better response to adjuvant chemotherapy than patients with p16−: Kaplan–Meier analysis showed a statistically significant increased disease-free survival ($P=0.001$) and a trend towards a statistical significance for increased overall survival ($P=0.056$) (Figures 4a and b). In multivariate Cox proportional hazards regression analysis, p16+ was independently associated with disease-free survival and overall survival, with a hazard ratio of 0.18 (95% CI: 0.06–0.51; $P=0.001$) and 0.21 (95% CI: 0.06–0.74; $P=0.015$), respectively (Table 4). Instead, in patients treated with surgery only, prognosis was not affected by p16 immunostaining level (Figures 3a and b). Conversely, Rb immunostaining level was not associated with clinical outcome, neither in patients treated with surgery only nor in patients treated with adjuvant chemotherapy (Figures 3c, d and 4c, d).

There are many studies showing significant association between Rb pathway loss or its immunohistochemistry surrogates and clinical outcome in breast cancers, but these studies are irrespective of hormone receptors status, or divided into positive and negative hormone receptors, but irrespective of HER2 status.[13,14,16,18,20,25,31] However, only few studies focused this issue in triple-negative breast carcinomas.

Arima *et al*[24] showed, in a subset of 60 triple-negative breast carcinomas patients treated with neoadjuvant chemotherapy, that p16 strong immunostaining

Table 4 Multivariate analysis of predictors of the disease-free survival and overall survival time in no special type TNBCs patients, treated with chemotherapy

Factor	*HR*	*95% CI*	P-*value*
Disease-free survival			
P16 protein expression			
p16 −	1.00		
p16 +	0.18	0.06–0.51	0.001
Nodes metastases			
Negative	1.00		
Positive	3.75	1.49–9.41	0.005
Overall survival			
P16 protein expression			
p16 −	1.00		
p16 +	0.21	0.06–0.74	0.015
Tumor size (mm)			
≤20	1.00		
>20	4.41	1.29–15.01	0.018
Vascular invasions			
Absent	1.00		
Present	3.04	0.95–9.70	0.060

Abbreviations: CI, confidence interval; HR, hazard ratio; TNBCs, triple-negative breast carcinomas.

was significantly related to chemotherapy response, as reflected by complete pathological response. However, in the same patients, p16 strong immunostaining was not associated with disease-free survival or overall survival in log-rank analysis. Interestingly, the authors demonstrated biological differences between p16 + and p16 − basal-like breast carcinoma cells, with depletion of p16 increasing the percentage of CD44 + /CD24 − stem-like cancer cells and reducing chemosensitivity. The reduction of cell proliferation may be a reason for the chemoresistance of p16-depleted cells.

Treré *et al*[10] demonstrated, in a subset of 24 triple-negative breast carcinomas, that patients lacking Rb immunohistochemistry expression had a favorable clinical outcome if treated with adjuvant therapy.

In both these studies,[10,24] patient selection was different compared with ours: they considered triple-negative breast carcinomas as the tumors with hormone receptors <10% and they did not exclude special histotypes (about 20% of our cases). In addition, having analyzed HER2 status with either immunohistochemistry and silver *in situ* hybridization methods, we excluded cases HER2 0/1 + immunohistochemistry but silver *in situ* hybridization amplified (4.4% of our cases).

We chose ≥1% as the threshold for hormone receptors positivity because the ASCO-CAP panel recommended considering endocrine therapy in patients whose breast tumor shows at least 1% ER-positive cells. Also, we think it is very important to select between different histotype of triple-negative breast carcinomas, because of distinct prognostic implications that may be derived; for example, adenoid–cystic and medullary carcinomas and the low-grade apocrine and metaplastic carcinomas are associated with a better prognosis.[1,4,6] As stated by Montagna *et al*,[6] the identification of special-type triple-negative breast carcinomas has a significant clinical utility and should be considered in therapeutic algorithm. HER2 immunohistochemistry-negative/FISH-positive cases have been reported in a percentage varying from 0 to 3.2%,[32] the reason why we considered necessary to determine *ERBB2* gene amplification in tumors 0 or 1 + immunohistochemistry to avoid inclusion of false triple-negative breast carcinomas cases.

In conclusion, at the best of our knowledge, this is the first study demonstrating that, in a well-defined subset of no special-type triple-negative breast carcinomas, patients with p16 strong immunostained tumors had a good response to adjuvant chemotherapy and p16 immunoexpression can be considered the best surrogate marker for Rb pathway loss.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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