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FOXP3 expression in cancer cells and anthracyclines efficacy in patients with primary breast cancer treated with adjuvant chemotherapy in the phase III UNICANCER-PACS 01 trial

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Background: Predictive markers of response to chemotherapy are lacking in breast cancer patients. Forkhead Box Protein 3 (FOXP3) is an anti-oncogene whose absence in cancer cells could confer resistance to DNA damaging agent.

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So we made the hypothesis that FOXP3 expression predicts the response to anthracyclines in breast cancer patients and that adjuvant chemotherapy adding taxanes to anthracyclines confers an overall survival (OS) benefit over anthracyclines alone, in patients with FOXP3-negative tumors.

Patients and methods: Expression of FOXP3 in cancer cells was evaluated by immunohistochemistry in tumor samples from 1097 patients who participated in the PACS01 randomized trial that evaluated in adjuvant setting the adjunction of docetaxel (Taxotere) to anthracyclines in patients with localized breast cancer. Kaplan–Meier analysis and Cox regression model were used to assess OS according to the presence or absence of FOXP3 expression in tumor cells.

Results: Four hundred and five tumors were found to express FOXP3 (37%). FOXP3 expression in breast cancer cells was associated with better OS ($P = 0.003$). Uni- and multivariate survival analyses according to treatment arm revealed that FOXP3 expression in breast cancer cells is independently associated with improved OS in patients treated with anthracycline-based adjuvant chemotherapy, but not in patients treated with sequential anthracycline–taxane. Moreover, *in vitro* experiments showed that FOXP3 induction in breast cancer cell lines using histone deacetylase inhibitor enhances anthracyclines efficacy.

Conclusion: FOXP3 expression in tumor cells may be an accurate predictive biomarker of anthracycline efficacy in breast cancer.

Key words: adjuvant, anthracycline, breast cancer, FOXP3

introduction

Forkhead Box Protein 3 (FOXP3) is a transcription factor critically involved in the development and functions of immunosuppressive regulatory T cells (Treg) [1]. Until recently, FOXP3 expression was thought to be restricted to Treg and some populations of activated T cells. However, recent studies have provided clear evidence that various types of human cancer cells expressed FOXP3 transcript, as well as the mature protein [2–4]. Importantly, biological functions of FOXP3 in tumor cells and their significance presently remain unclear because of conflicting clinical results. Recent data suggest that FOXP3 expression in tumor cells could be a poor prognostic factor in breast cancer [5]. In contrast, FOXP3 was recently demonstrated to be a tumor suppressor gene, acting as a transcriptional repressor of breast cancer oncogenes such as Skp2 (S-phase kinase protein 2) and HER2 (human epidermal growth factor receptor 2) [6, 7]. In line with these findings, we recently described that the presence of FOXP3+ tumor cells represents a novel independent prognostic factor of improved survival in a small retrospective series of breast carcinoma patients treated with neoadjuvant chemotherapy [8]. Interestingly, exploratory analysis according to chemotherapeutic regimen suggested that the favorable value of FOXP3 expression in breast cancer cells was only significant in patients treated with anthracyclines but not in patients treated with taxanes. These data support the contention that FOXP3 expression could modulate breast cancer cell sensitivity to anthracycline chemotherapy. Furthermore, recent biological studies have linked the expression of FOXP3 in tumor cells with sensitivity to DNA damaging agent and topoisomerase inhibitors [9]. Thus, we made the hypothesis that FOXP3 expression in breast cancer cells may be a predictive factor of response to anthracyclines. In the present study, we try to validate this hypothesis in a large prospective adjuvant cohort of node-positive breast cancer patients treated in the PACS01 trial [10], and we have analyzed the predictive value of FOXP3 expression in cancer cells for the efficacy of adjuvant chemotherapy according to chemotherapy regimen (anthracycline-alone versus sequential anthracycline–taxane regimen).

Interestingly, this study reveals that FOXP3 expression in breast cancer cells is independently associated with improved overall survival (OS) in patients treated by six cycles of anthracycline-based adjuvant chemotherapy but not in patients treated with sequential anthracycline–taxane regimen. Moreover, pharmacological induction of FOXP3 expression in breast cancer cell lines by the histone deacetylase (HDAC) inhibitor valproic acid could affect the response to anthracyclines.

patients and methods

patients

This study is a biomarker parallel study to the PACS01 trial. The PACS01 trial included 1999 patients with node-positive breast cancer between 1998 and 2001. This randomized trial compared six cycles of FEC100 (epirubicin 100 mg/m^2, cyclophosphamide 500 mg/m^2, and 5-fluorouracil 500 mg/m^2) with three cycles of FEC100 followed by three cycles of docetaxel (Taxotere, Sanofi-Aventis, Paris, France) (100 mg/m^2). Results have been reported previously [10] and showed that adding three cycles of docetaxel reduced the risk of relapse by 17%. After adjuvant chemotherapy, patients with hormone receptor-positive disease received endocrine therapy. Radiation therapy was recommended for all patients treated with conservative surgery and was carried out at investigator's discretion after mastectomy. No patients with HER2-overexpressing disease received trastuzumab in the adjuvant setting.

Tumor blocks were initially collected for 1190 patients, for biomarker studies. Among these tumor blocks, 1097 were found to have sufficient tumor tissue to perform immunohistochemistry (Figure 1). Clinical characteristics and magnitude of the docetaxel efficacy were not different among this biomarker study group and the rest of the population included in PACS01 study (data not shown). These 1097 tumors were centrally immunostained with FOXP3-specific antibody. Of these 1097 tumors, 405 (36.9%) were found to be FOXP3 positive (FOXP3+). Using χ^2 test, we observed no difference for clinical characteristic between patients tested for FOXP3 expression and patients not tested except for T stage (more T2 and T3 in patients not tested for FOXP3) (data not shown).

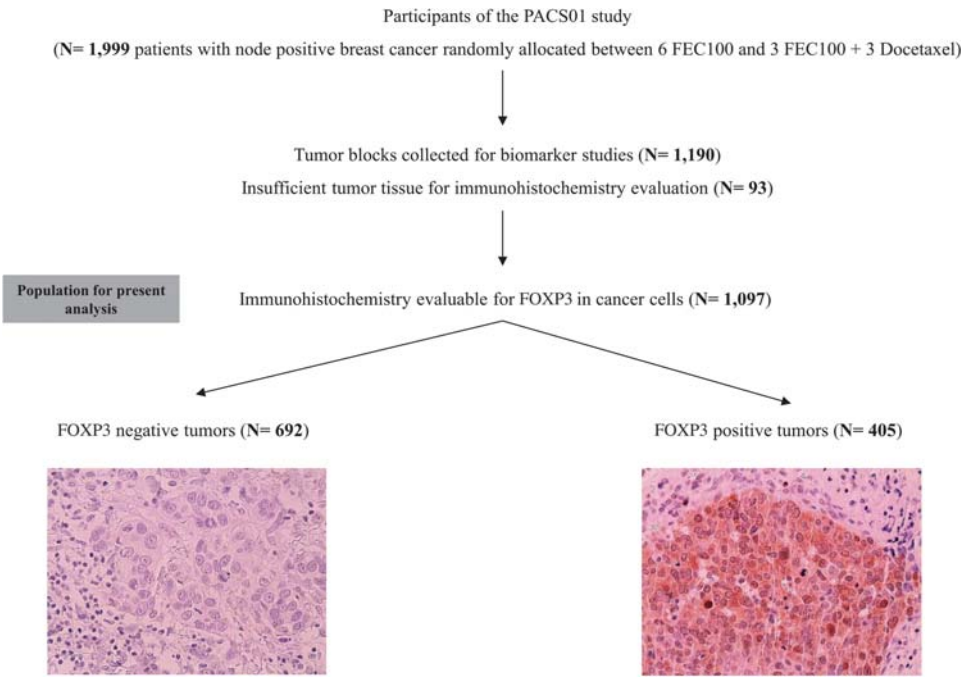

Figure 1. CONSORT diagram. Origin of patients analyzed for breast cancer cell expression of FOXP3, and examples for negative (left: no visible cytoplasmic or nuclear staining) and positive (right: cytoplasmic and/or nuclear staining) tumor samples.

FOXP3 staining

FOXP3 expression in cancer cells was analyzed immunohistochemically on formalin-fixed paraffin-embedded tumor sections. Labeling was carried out using Ventana Benchmark XT automat (Ventana, Tucson, AZ). After deparaffinazition, antigen retrieval was carried out by heating slides for 60 minutes in pH 7.8 EDTA solution (CC1; Ventana). Antihuman FOXP3 monoclonal antibody (mAb) clone ab20034 (Abcam, Cambridge, UK) at 1/100 dilution was incubated during 32 min at 37°C, and the ultraVIEW system (Ventana) was used for the revelation. The slides were after counterstained with hematoxylin and mounted in Entellan (Merck, Darmstadt, Germany). Consecutive slides from 20 appropriate cases with known mAb clone ab20034 reactivity were stained using antihuman FOXP3 mAb clone PCH101 (eBioscience, San Diego, CA) at 1/50 dilution. For this staining, antigen retrieval was carried out by heating slides for 25 min at 95°C in pH 6, 10 mM trisodium citrate. The incubation was carried out at room temperature for 60 min. Positive and negative staining controls were carried out with human paraffin tonsil sections using FOXP3 monoclonal antibody and an isotype-matched negative control antibody. Expression of FOXP3 was evaluated independently by two of the authors (SL and GM), both blinded for clinicopathologic data. Discrepancies between the two observers were reviewed jointly to reach consensus. Staining of at least 30% of the tumor cells was considered positive for FOXP3 expression.

The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane et al. [11] were followed throughout this study.

statistical analysis

The association between presence of FOXP3+ tumor cells and clinicopathologic/biological variables was examined using the χ^2 test or Fisher's exact probability test if required. OS was defined as the time from date of diagnosis until death (all causes). Survivors were censored at the last follow-up. The Kaplan–Meier method was used for calculation of survival probabilities and the log-rank test for comparison of survival curves. Median follow-up with its 95% confidence interval (CI) was calculated using the reverse Kaplan–Meier method. Cox's proportional hazards regression was used for univariate and multivariate analysis of OS. Univariate Cox proportional hazards models of all potential baseline predictors (FOXP3 expression, HER2, treatment arm, tumor grade, hormonal receptor expression, axillary lymph node involvement and tumoral T stage) were built to compute the hazard ratios (HRs) with their 95% CIs. As our initial aim was to examine the prognostic role of FOXP3 expression according to the adjuvant regimen (i.e. predictive value), interaction between FOXP3 expression in tumor cells and treatment was tested in univariate Cox analysis allowing to compare each combination of FOXP3 expression and treatment to the reference FOXP3 negative and adjuvant treatment with FEC alone. As exploratory purpose, OS was also described and compared according to FOXP3 in each treatment group allocated by randomization.

All predictors with P values<0.10 in univariate Cox analyses were used in multivariate analysis, carried out by the backward elimination procedure. FOXP3 and treatment interaction terms were included in the models. According to Harrell rules, we have limited the included variables for constructing multivariate models to one variable for 10 events both in whole population and in subgroup analyses [12]. We computed the Akaike Information Criterion for goodness of fit of multivariate models and Harrell's C-statistic for discrimination of each variable and for final multivariate Cox models (a Harrell's C index = 0.5 indicates no predictive discrimination and a Harrell's C index = 1.0 indicates perfect separation of patients). The multivariate models were internally validated using bootstrapping (2000 replications). All analyses were carried out using Stata V11 software (StataCorp LP, College Station, TX). P values were two-tailed and considered significant when <0.05.

in vitro experiments

Material and methods used for *in vitro* experiments are described in the supplemental appendix (available at *Annals of Oncology* online).

results

patient characteristics

Table 1 reports the patient characteristics for the whole population and for patients with FOXP3-negative (FOXP3−) and FOXP3+ tumor cells. The median follow-up was 8.06 years (95% CI 8.0–8.16). At data cut-off, 218 patients had died. Six FEC100 was received by 547 patients (49.9%) and 550 patients (50.1%) received three FEC100 + three docetaxel as adjuvant chemotherapy. Only 178 patients (16.3%) had HER2-overexpressing breast carcinoma. In this cohort, 692 tumors (63%) did not express FOXP3 and 405 (37%) expressed FOXP3, the staining being both cytoplasmic and nuclear (Figure 1). Cases with known reactivity of FOXP3 mAb clone ab20034 showed a similar staining (but with a slight higher intensity) with mAb clone PCH101 (data not shown). Patients with FOXP3+ tumor cells were found to have significantly lower tumor grade ($P< 0.001$) and significantly lower axillary lymph node involvement ($P = 0.026$).

Table 1. FOXP3 expression in cancer cells according to clinicopathologic parameters

		FOXP3− tumors $N = 692$	FOXP3+ tumors $N = 405$	Total $N = 1097$	P value χ^2
HER2					
	Not overexpressed	570 (82.5%)	346 (85.9%)	916 (83.7%)	0.146
	Overexpressed	121 (17.5%)	57 (14.1%)	178 (16.3%)	
	Total	691 (100%)	403 (100%)	1094 (100%)	
Treatment Arm					
	FEC	357 (51.6%)	190 (46.9%)	547 (49.9%)	0.135
	FEC + Docetaxel	335 (48.4%)	215 (53.1%)	550 (50.1%)	
	Total	692 (100%)	405 (100%)	1097 (100%)	
SBR					
	SBR I/II	331 (50.5%)	248 (64.6%)	579 (55.7%)	<0.0001
	SBR III	324 (49.5%)	136 (35.4%)	460 (44.3%)	
	Total	655 (100%)	384 (100%)	1039 (100%)	
No. of involved nodes					
	N1: 1 to 3	411 (59.4%)	272 (67.2%)	683 (62.3%)	0.026
	N2: 4 to 9	222 (32.1%)	110 (27.2%)	332 (30.3%)	
	N3: ≥10	59 (8.5%)	23 (5.7%)	82 (7.5%)	
	Total	692 (100%)	405 (100%)	1097 (100%)	
T stage					
	T1	319 (50.7%)	202 (54.7%)	521 (52.2%)	0.458
	T2	284 (45.2%)	154 (41.7%)	438 (43.9%)	
	T3	26 (4.1%)	13 (3.5%)	39 (3.9%)	
	Total	629 (100%)	369 (100%)	998 (100%)	
Hormone receptor status					
	Negative	153 (22.2%)	73 (18.1%)	226 (20.7%)	0.106
	Positive	537 (77.8%)	331 (81.9%)	868 (79.3%)	
	Total	690 (100%)	404 (100%)	1094 (100%)	
Locoregional relapse					
	No	650 (93.9%)	383 (94.6%)	1,033 (94.2%)	0.664
	Yes	42 (6.1%)	22 (5.4%)	64 (5.8%)	
	Total	692 (100%)	405 (100%)	1,097 (100%)	
Controlateral relapse					
	No	668 (96.5%)	386 (95.3%)	1,054 (96.1%)	0.314
	Yes	24 (3.5%)	19 (4.7%)	43 (3.9%)	
	Total	692 (100%)	405 (100%)	1,097 (100%)	
Distant metastasis					
	No	498 (72%)	326 (80.5%)	824 (75.1%)	0.002
	Yes	194 (28%)	79 (19.5%)	273 (24.9%)	
	Total	692 (100%)	405 (100%)	1,097 (100%)	
Death					
	No	536 (77.5%)	343 (84.7%)	879 (80.1%)	0.004
	Yes	156 (22.5%)	62 (15.3%)	218 (19.9%)	
	Total	692 (100%)	405 (100%)	1,097 (100%)	

FOXP3, Forkhead Box Protein 3; HER2, human epidermal growth factor receptor 2; SBR, Scarff, Bloom et Richardson score.

association between FOXP3, treatment arm, classical clinicopathologic factors and OS

In the whole cohort of patients, OS was significantly shorter for patients with FOXP3− tumor (log-rank tests $P = 0.0023$) (Figure 2A). Clinical and pathological factors significantly associated with shorter OS by univariate Cox regression were HER2 overexpression, treatment arm with six FEC100, high tumor grade, negative hormonal receptor, high number of involved lymph nodes, T stage, and absence of FOXP3 expression in tumor cells (HR = 1.59, 95% CI 1.18–2.13; $P = 0.003$) (Table 2). Subgroup univariate survival analysis according to FOXP3 tumor expression and adjuvant treatment arm demonstrates that FOXP3 expression was associated with shorter survival only in the FEC-alone-treated group (Figure 2B). OS was significantly shorter in patients treated with six FEC100 for a FOXP3− tumor (log-rank $P = 0.0005$, Figure 2B). Interaction between FOXP3 and treatment arm was not significant; however, the statistical power of the interaction test was 13%. Eight-year OS rates were, respectively, 83%, (95% CI 75% to 89%) and 75% (95% CI 69% to 80%), in patients with FOXP3+ and FOXP3− tumors treated with the FEC regimen alone and,

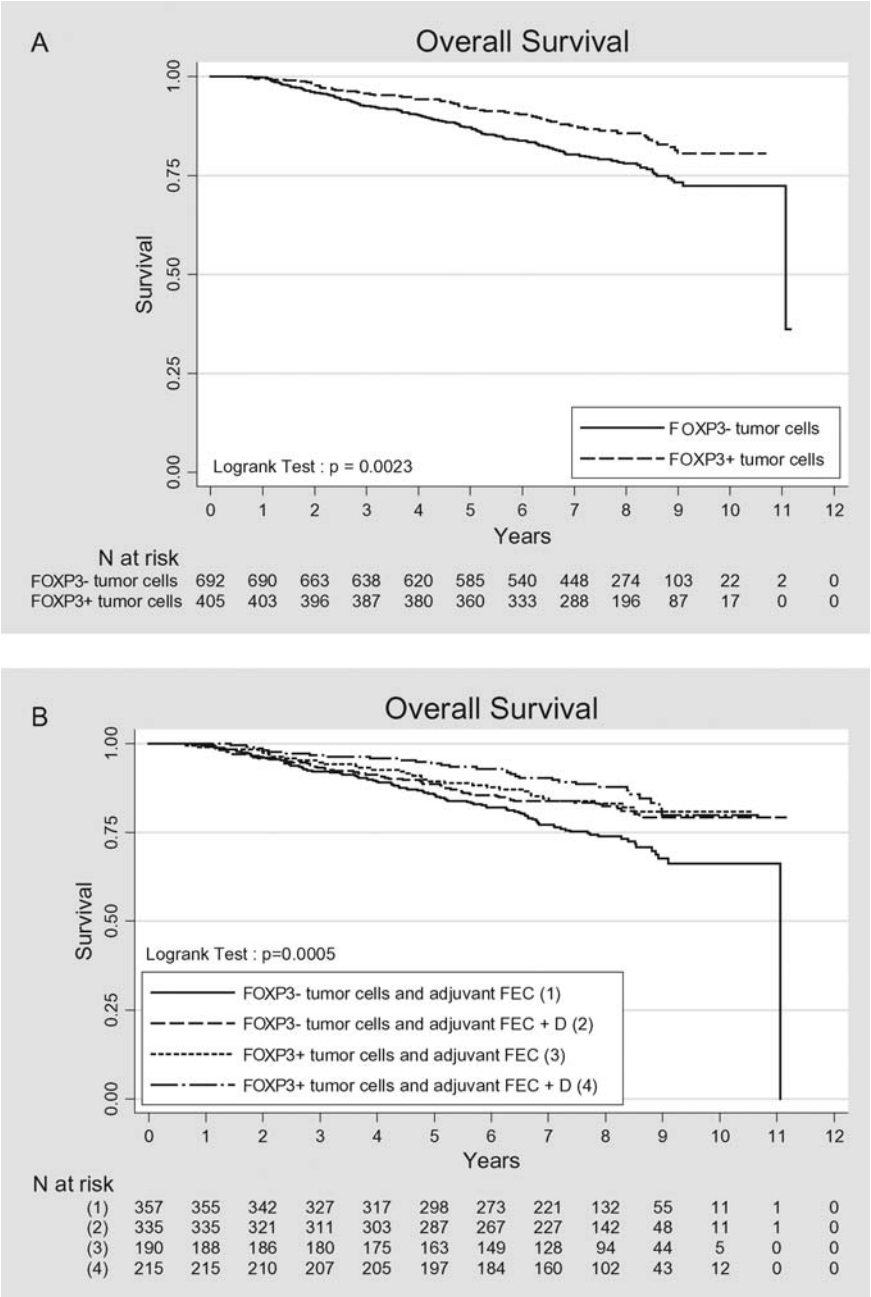

Figure 2. Kaplan–Meier curves for overall survival (OS) stratified (A) according to presence (FOXP3+) or absence (FOXP3−) of FOXP3 expression in breast cancer cells and (B) according to presence or absence of FOXP3 expression in breast cancer cells and treatment arm (FEC or FEC + D). *P* values were calculated using the log-rank test. D, docetaxel; FOXP3, Forkhead Box Protein 3.

Table 2. Univariate analysis (Cox regression) for factors associated with overall survival ($n = 1097$)

		Death/*n* (218/1097)	HR	95% CI	*P* value	Harrell's C statistic
FOXP3 in tumor cells						
	Positive	62/405	1		0.003	0.5535
	Negative	156/692	1.59	1.18–2.13		
	Total	218/1097				
HER2						
	Not overexpressed	170/916	1		0.006	0.5408
	Overexpressed	47/178	1.57	1.14–2.17		
	Total	217/1094				
Treatment arm						
	FEC + docetaxel	90/550	1		0.004	0.5489
	FEC	128/547	1.49	1.14–1.96		
	Total	218/1097				
SBR						
	SBR I/II	79/579	1		<0.0001	0.6138
	SBR III	125/460	2.23	1.68–2.96		
Total	204/1039					
Hormone receptor status						
	Positive	162/868	1		0.016	0.5466
	Negative	55/226	1.45	1.07–1.96		
	Total	217/1094				
No of involved nodes						
	N1: 1 to 3	87/683	1		<0.0001	0.6308
	N2: 4 to 9	95/332	2.43	1.81–3.25		
	N3: ≥10	36/82	4.07	2.76–6		
	Total	218/1097				
T stage						
	T1	63/521	1		<0.0001	0.6062
	T2	113/438	2.37	1.74–3.22		
	T3	13/39	2.9	1.59–5.27		
	Total	189/998				

CI, confidence interval; FOXP3, Forkhead Box Protein 3; HER2, human epidermal growth factor receptor 2; HR, hazard ratio; SBR, Scarff, Bloom et Richardson score.

respectively, 89% (95% CI 83% to 93%) and 86% (95% CI 80% to 90%) in patients with FOXP3+ and FOXP3− tumors treated with the sequential FEC–docetaxel regimen. As our initial aim was to examine the prognostic role of FOXP3 expression according to the adjuvant regimen, multivariate analysis was carried out using a combined variable according to FOXP3 expression in each treatment group. By multivariate analysis, T stage (T2 stage: HR = 1.85, 95% CI 1.34–2.54; T3 stage: HR = 1.69, 95% CI 0.82–3.45), high number of involved nodes (N2: HR = 2.08, 95% CI 1.51–2.86; N3: HR = 2.89, 95% CI [1.83–4.57]) and high tumor grade (SBRIII: HR = 1.81, 95% CI 1.32–2.46) remained independently associated with shorter survival (Table 3). Absence of FOXP3 expression was also independently associated with a poor survival, but only in FEC-alone arm, while other groups share similar prognosis (HRs = 0.62, 0.62 and 0.57, respectively, for FOXP3− tumor and FEC + docetaxel treatment, FOXP3+ tumor and FEC treatment, and FOXP3+ tumor and FEC + docetaxel treatment) (Table 3). Of note, interaction between treatment and FOXP3 was not significant ($P = 0.4$) (HR = 2.2, 95% CI 0.8–2.2). Harrell's C statistic was 0.703, indicating good discrimination by the multivariate model. Bootstrapping also confirms the stability of the multivariate Cox model, and that the four variables previously determined remain independently associated with survival.

induction of FOXP3 expression in breast cancer cell lines enhances the cytotoxic effect of anthracyclines

Since clinical data suggested that FOXP3 expression in tumor cells might be positively associated with increased sensitivity to anthracyclines, we have decided to investigate whether FOXP3 is directly responsible for the tumor cell sensitivity to anthracyclines *in vitro*. In this regard, previous studies demonstrated that HDAC inhibitors such as valproic acid were able to increase *FOXP3* gene expression [13]. We have thus tested the sensitivity of various breast cancer cell lines to anthracyclines after treatment with valproic acid. We have first verified using six breast cancer cell lines that treatment with low dose of valproic acid enhances *FOXP3* expression in four out of the six tumor cell lines (Figure 3A, left). This pharmacological induction of FOXP3 is associated with downregulation of Skp2 protein (Figure 3A, right). We then observed that, while valproic acid has no intrinsic cytotoxic

Table 3. Multivariate analysis (Cox regression) for factors associated with overall survival ($n = 1097$)

		Death/*n* (181/953)	HR	95% CI	*P* value	Bootstrapping[a]	
						95% CI	*P* value
FOXP3 and treatment arm	FOXP3− and FEC	80/308	1		0.011[b]		0.0162[b]
	FOXP3− and FEC + D	50/292	0.62	0.43–0.88	0.008	0.42–0.9	0.012
	FOXP3+ and FEC	27/171	0.62	0.4–0.97	0.035	0.39–0.99	0.045
	FOXP3+ and FEC + D	24/182	0.57	0.36–0.9	0.016	0.35–0.91	0.02
T stage							
	T1	62/499	1		0.001		0.001
	T2	110/420	1.85	1.34–2.54		1.33–2.56	
	T3	9/34	1.69	0.82–3.45		0.78–3.63	
Number of involved nodes							
	N1: 1 to 3	76/601	1		<0.0001		<0.0001
	N2: 4 to 9	78/284	2.08	1.51–2.86		1.49–2.89	
	N3: 10	27/68	2.89	1.83–4.57		1.77–4.72	
SBR							
	SBR I/II	69/526	1		<0.0001		<0.0001
	SBR III	112/427	1.81	1.32–2.46		1.31–2.49	
AIC							2307.243
Harrell's C statistic							0.7037

[a]Two thousand replications.

[b]Test of the variable.

AIC, Akaike Information Criterion; CI, confidence interval; D, docetaxel; FOXP3, Forkhead Box Protein 3; HR, hazard ratio.

effect on tumor cells, it enhances the cytotoxic effect of epirubicin, but only in cell lines in which FOXP3 expression was increased (Figure 3B, left). In contrast, valproic acid has no effect on docetaxel cytotoxic activity (Figure 3B, right). To validate that valproic acid effect mainly reside in its effect on FOXP3 expression, we applied our experimental setting on MDA-MB-231 cells knocked down for FOXP3 production by small interfering RNA (siRNA). Our results show that, when FOXP3 upregulation is prevented, the valproic acid treatment does not affect the susceptibility to low dosage of epirubicin (Figure 3C). Same results were obtained at higher dosage of epirubicin (data not shown).

Altogether, these results demonstrate that cancer cell line susceptibility to epirubicin, but not docetaxel, is increased upon FOXP3 induction.

discussion

The present data give rise to the hypothesis that absence of FOXP3 expression in breast cancer cells could identify a subset of patients with anthracycline-resistant tumor, who would potentially have a high benefit from adding docetaxel in adjuvant chemotherapy. Moreover, we demonstrate *in vitro* that pharmacological exposition of breast cancer cell lines to valproic acid could enhance anthracycline cytotoxic effects by restoring FOXP3 expression.

Not all breast tumors equally respond to a given chemotherapy regimen, and this may lead to unnecessary exposure to drug side-effects and delay efficient treatment, especially in adjuvant setting in which no early evaluation is possible. Unfortunately, commonly used clinical and biological factors are poor predictors of response or resistance to chemotherapy. Therefore, it is of considerable clinical importance to identify biological markers that can select those patients who are most likely to respond to a specific chemotherapy regimen. FOXP3 is a transcription factor involved in regulating immune system development, notably in the generation of Treg [14, 15]. Initially, FOXP3 expression was thought to be restricted to hematopoietic tissues. However, although data are scant, FOXP3 expression by other tissues has also been observed, notably in human tumor cells [2]. Zuo et al. recently reported that FOXP3 acts as a transcriptional repressor for breast cancer oncogenes Skp2 and HER2, thereby suppressing breast tumor cell growth [6, 7]. Moreover, induction of FOXP3 expression in breast cancer cell lines could induce apoptosis and abrogate tumor growth in murine models [6, 7, 16]. In line with our initial hypothesis, recent studies have suggested that altered regulation of the cell cycle may be involved in resistance or sensibility to chemotherapy, notably to DNA damaging agents such as anthracyclines. For example, in breast cancer, loss of p27, a CDK inhibitor that plays a central role in cell cycle regulation, is associated with poor prognosis [17–19] and resistance to anthracycline treatment [20]. It appears now that loss of p27 in breast cancer results from increased protein degradation, mainly mediated by an SCF-type ubiquitin ligase complex that contains Skp2 [21, 22], which is downregulated by FOXP3 [6]. In our study, we confirmed *in vitro* that Skp2 expression is inversely correlated with expression of FOXP3 (Figure 3A).

In accordance with the anti-oncogenic properties of FOXP3, we recently described, in an exploratory retrospective immunohistochemical study of 103 patients treated with neoadjuvant chemotherapy for localized breast cancer, that FOXP3 expression in breast cancer cells was associated with better OS [8]. By multivariate analysis, presence of FOXP3+

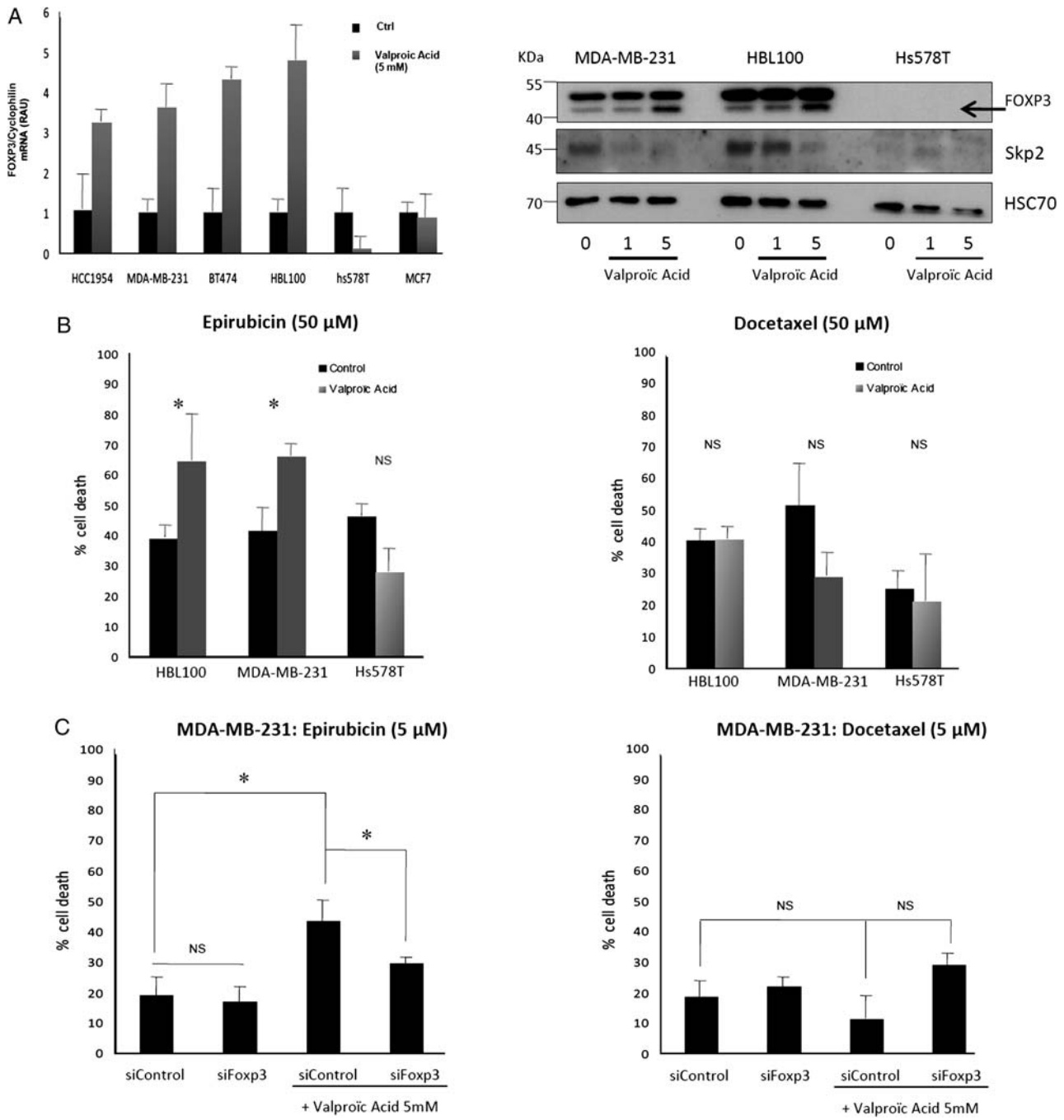

Figure 3. Cytotoxic activity of epirubicin is enhanced by valproic acid in a FOXP3-dependent manner: (A) Left: FOXP3 expression was determined using RT-PCR in six breast cancer cell lines exposed or not to valproic acid (5 mM) ($n = 5$). Right: expression of FOXP3 and Skp2 proteins were determined by western blotting in breast cancer cell lines exposed or not to valproic acid. (B) Cytotoxic activity of epirubicin (left) and docetaxel (right) was determined in two breast cancer cell lines in which FOXP3 expression is enhanced by valproic acid (HBL-100 and MDA-MB-231), and in one breast cancer cell line in which FOXP3 expression is not enhanced by valproic acid (Hs578T), using crystal violet dye. Results are presented as percentage of cell death relative to untreated control cultures ($n = 5$ experiments). (C) Cytotoxic activity of epirubicin (left) and docetaxel (right) was determined using crystal violet dye in MDA-MB-231 cells transfected or not with FOXP3 SiRNA. Cells were previously treated or not with valproic acid. Results are presented as percentage of cell death relative to untreated control cultures ($n = 3$ experiments). *: $P < 0.05$. Error bars represent mean ± SD. FOXP3, Forkhead Box Protein 3; NS, non-significant; SD, standard deviation.

tumor cells constituted an independent prognostic factor for better OS. Interestingly, the favorable prognostic value of FOXP3 in breast cancer cells was limited to patients treated with anthracycline-based chemotherapy and associated with pathological complete response but was not significant in patients treated with taxanes [8], suggesting a prognostic value associated with the use of anthracyclines. In this study, we confirmed in a larger prospective randomized trial that the

prognostic value of FOXP3 expression in breast cancer cells could be dependent on the adjuvant regimen. Indeed, we show here that the OS of patients treated with anthracyclines plus taxanes was not different between patients with FOXP3+ or FOXP3− tumor. Moreover, survival of these patients was not significantly different compared with patients with FOXP3+ tumor and treated with anthracyclines alone. Only patients with FOXP3− tumor and treated with anthracyclines alone harbored worse OS compared with the three previous groups.

Our results on the favorable prognostic role of FOXP3 in breast tumor seem in contradiction with those initially published by Merlo et al. [5], but this could be explained by differences in the studied populations. Indeed, while all patients of PACS01 underwent adjuvant chemotherapy, only a limited number of patients reported by Merlo et al. received adjuvant chemotherapy. As we demonstrated the implication of FOXP3 expression in the anthracycline response, the prognostic value of FOXP3 could be different in the absence of chemotherapy treatment.

Importantly, we also provide a therapeutic key to reverse anthracycline resistance. Indeed, using valproid acid, a HDAC inhibitor that induces FOXP3 expression, we could demonstrate that the pharmacological induction of FOXP3 is associated with downregulation of Skp2 protein and enhances epirubicin toxicity. This effect seemed to be FOXP3 dependent, as it was abrogated in our FOXP3 siRNA experiments. By contrast, cytotoxic effect of docetaxel was not affected by the expression of FOXP3 in our six breast cancer cell lines.

We choose valproic acid because it was demonstrated to potentiate epirubicin-induced cell death in preclinical mouse models of breast cancer [23]. Several reports have suggested that HDAC inhibitors can synergize with cytotoxic agents [24–27], especially with anticancer drugs directly targeting DNA such as topoisomerase II inhibitors [24, 25]. Indeed, valproic acid-induced chromatin decondensation facilitated binding of topoisomerase II inhibitors to the DNA substrate and consequently increased DNA strand breaks by recruitment of topoisomerase IIβ [28]. Recently, phase I/II trials suggested that combined valproic acid and epirubicin as well as FEC100 regimen has an acceptable toxicity profile and antitumor efficacy, even in breast cancer patients who experienced treatment failure with anthracyclines, as well as in patients with tumors though to be resistant to anthracyclines [29, 30]. Here, we provide a mechanistical explanation and proposed that valproic acid could be used to reverse anthracycline resistance in FOXP3− tumors.

In conclusion, the results of the present study suggest that FOXP3 expression in cancer cells may be an accurate predictive biomarker of response to anthracycline-based chemotherapy in breast cancer and emphasize the anti-oncogenic properties of this protein, which may constitute a novel target for future intervention in breast cancer. Moreover, these results strengthen the importance of the analysis of FOXP3 expression in breast cancer patients, as such information can be used as an additional tool for the stratification of patients in clinical trials evaluating the benefit of taxanes or anthracyclines. It could also help clinicians for the selection of patients who would potentially derive the highest benefit from adding docetaxel in adjuvant chemotherapy and of patients who might benefit from additional therapies, like HDAC inhibitors, such as valproic acid, in future clinical trials.

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disclosure

The authors have declared no conflicts of interest.

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Relevant impact of central pathology review on nodal classification in individual breast cancer patients

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Background: In the MIRROR study, pN0(i+) and pN1mi were associated with reduced 5-year disease-free survival (DFS) compared with pN0. Nodal status (N-status) was assessed after central pathology review and restaging according to the sixth AJCC classification. We addressed the impact of pathology review.

Patients and methods: Early favorable primary breast cancer patients, classified pN0, pN0(i+), or pN1(mi) by local pathologists after sentinel node procedure, were included. We assessed the impact of pathology review on N-status ($n = 2842$) and 5-year DFS for those without adjuvant therapy ($n = 1712$).

Results: In all, 22% of the 1082 original pN0 patients was upstaged. Of the 623 original pN0(i+) patients, 1% was downstaged, 26% was upstaged. Of 1137 patients staged pN1mi, 15% was downstaged, 11% upstaged. Originally, 5-year DFS was 85% for pN0, 74% for pN0(i+), and 73% for pN1mi; HR 1.70 [95% confidence interval (CI) 1.27–2.27] and HR 1.57 (95% CI 1.16–2.13), respectively, compared with pN0. By review staging, 5-year DFS was 86% for pN0, 77% for pN0(i+), 77% for pN1mi, and 74% for pN1+.

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