Expression and Prognostic Value of Activating Transcription Factor 2 (ATF2) and its Phosphorylated Form in Mammary Carcinomas

STEFAN KNIPPEN[1], THOMAS LÖNING[2], VOLKMAR MÜLLER[1], CHRISTINE SCHRÖDER[1], FRITZ JÄNICKE[1] and KARIN MILDE-LANGOSCH[1]

[1]*Department of Gynecology, University Medical Center Hamburg-Eppendorf, Hamburg;*
[2]*Albertinen Pathology, Hamburg, Germany*

Abstract. *Background: The transcription factor ATF2 is overexpressed in various tumors, but its role in breast cancer is still not understood. Materials and Methods: In a study of mammary carcinomas, the expression of ATF2 and its phosphorylated active forms was analyzed by Western blot analysis (WB; pThr69/pThr71-ATF2, n=134) and immunohistochemistry (IHC; p-ATF2-Thr6, n=110). Results were correlated with histological and clinical data, survival data, expression of ERK1/2 and two matrix metalloproteinases. Results: Patients with high ATF2 expression as detected by WB had a significantly shorter overall survival (p=0.038). This tendency was corroborated by IHC. In contrast, high p-ATF2 expression as found by WB correlated significantly with a well-differentiated phenotype, but not with prognosis. Immunohistochemically detected p-ATF2 overexpression was even associated with prolonged survival (p=0.047). Conclusion: Although high ATF2 expression is associated with a poor prognosis, our data do not point to an oncogenic role of active p-ATF2 in mammary carcinomas.*

Activating transcription factor 2, ATF2 (CREB2), forms homodimers or Jun-ATF2 heterodimers which bind to 12-*O*-tetradecanoylphorbol-13-acetate (TPA) responsive elements (TREs) or cyclic adenosine monophosphate (cAMP) response elements (CREs) in the promoter regions of target genes (1). ATF2 is expressed ubiquitously with increased concentrations in some human tumors. Its transactivating function and stability are enhanced by phosphorylation (24). This post-translational modification occurs by extracellular signal-regulated kinases (ERK) 1/2 and c-Jun *N*-terminal kinase (JNK/p38; phosphorylation on thr69 and thr71) in response to various stimuli (5, 6) or by the kinase ATM (ataxia telangiectasia mutated) following DNA damage (phosphorylation on ser490 and ser 498; (7)).

Although ATF2 overexpression in various tumors has been observed (8), the biological role of this transcription factor in human cancer is poorly understood. In MCF7 breast cancer cells, ATF2 phosphorylation by p38 is activated and DNA binding of ATF2 to the cyclin D1 promoter is enhanced after addition of estradiol which contributes to cell proliferation (9). In an immunohistochemical study on melanomas, strong nuclear ATF2 staining was a significant predictor of poor outcome, and more often found in metastatic sites compared with primary tumors (10). Moreover, ATF2 confers radiation resistance to melanoma cells (11). In mouse spindle cell carcinomas of the skin, ATF2 down-regulation results in morphological changes from the typical mesenchymal to an epithelial morphology. Moreover, these transfectants exhibited a slower growth rate and reduced tumorigenicity and invasiveness in SCID mice (12). In nude mice bearing ovarian cancer xenografts, paclitaxel treatment resulted in a reduced ATF2 expression in tumor cells (13).

In contrast to these results which indicate an oncogenic function of ATF2 in skin and ovarian tumors, loss of heterozygosity (LOH) analysis suggests a tumor suppressor function of ATF2 in lung and breast cancer where chromosome 2q, the locus of the *ATF2* gene, is frequently deleted (14). Additional clues came from the study of $ATF2^{+/-}$ mice which spontaneously developed mammary carcinomas (15). Moreover, ATF2 down-regulation in MCF12A cells which were derived from normal human mammary epithelial cells resulted in increased tumorigenicity, and in breast cancer cells, reduced *ATF2* mRNA levels compared to microdissected normal cells were detected (15). In the light of the sparse information about the role of ATF2 in breast cancer *in vivo*, we decided to study ATF2 protein expression and phosphorylation in a well-

Correspondence to: Karin Milde-Langosch, Department of Gynecology, University Medical Center Hamburg-Eppendorf, Martinistr. 52, D-20246 Hamburg, Germany. Tel: +49 4042803 8380, e-mail: milde@uke.uni-hamburg.de

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characterized cohort of breast cancer patients. Since the same samples or a sub-cohort had been investigated for expression of two matrix metalloproteinases and ERK1/2 MAP kinases in previous experiments, we correlated ATF2 expression with these factors in order to gain insights into the role of the ATF2 transcription factor in human mammary carcinomas.

Materials and Methods

Tissue samples. The study was performed in accordance with the principles of the declaration of Helsinki after approval by the local Ethics Committee. One hundred and thirty-four primary breast cancer samples from the Department of Gynecology, University Medical Center Hamburg-Eppendorf (median patient age 57 years, range 25-90 years) were analyzed by Western blot analysis (WB), and 110 of them by immunohistochemistry (IHC). Patients underwent surgery including lumpectomy and dissection of axillary lymph nodes or radical mastectomy. Tumors were rapidly chilled after removal and frozen in liquid nitrogen. An adjacent stained tissue section was used to confirm that the tumor cell content of the sample was at least 50% (mostly >75%). After surgery, the patients received adjuvant endocrine therapy and/or anthracycline-containing chemotherapy as well as radiotherapy according to National Guidelines.

According to their histology, 112 carcinomas were of ductal type, 15 were lobular and 5 were of special types (4 mucinous, 1 tubular). Nodal involvement was reported in 39 cases, 88 were nodal negative (unknown 7). By immunohistochemical oestrogen receptor (ER) assay, 36 cases were ER negative and 104 tumors ER positive (unknown 1). The pathological staging of the primary tumors was carried out as recommended by the UICC (16): 26 tumors were classified as stage 1, 82 as stage 2 and 16 as stage 3 or 4 (unknown 5). According to histological examination, 7 specimens were of low malignancy grade (G1), 71 tumors were classified as G2 and 55 cases were high-grade carcinomas (G3). Follow-up data were available in 129 cases. Recurrences were reported in 40 patients, and 29 died of the disease. The median follow-up time of those patients still alive was 88 months (range 15 - 169 months). All tumor samples had been analyzed for expression and phosphorylation of the MAP kinases ERK1 and ERK2 (17). Seventy-one samples from our cohort were characterized with respect to the expression of the invasion-associated matrix metalloproteinases MMP-1 and MMP-9 (18, 19).

Western blot analysis. The mammary carcinoma cell line MCF7, which was used as a control on WB was cultivated as described elsewhere (20). Protein extraction and WB were performed as described elsewhere (18). Equal amounts of protein (20 μg/well or 10 μg/well) of each sample were loaded, and equal loading was verified by immunoblotting with actin antibodies (goat anti-actin, I-19; 1:10,000; Santa Cruz Biotechnology, Heidelberg, Germany). Electrophoresis was performed in 8-10% polyacrylamide separating gels with a 3% stacking gel and proteins were transferred to polyvinylidene difluoride membranes (Immobilon P; Millipore, Eschborn, Germany). After overnight blocking, membranes were incubated for 1 h at room temperature (ATF2) or overnight at 4˚C (p-ATF2) with the primary antibodies rabbit anti-ATF2 (N-96; Santa Cruz), diluted 1:2,000, or mouse anti-p-ATF2 (Thr69/71; Cell Signaling Technology, Beverly, MA, USA), diluted 1:800. As secondary antibody, peroxidase-conjugated anti-rabbit-IgG or anti-mouse-IgG (1:8000; Santa Cruz) was used and visualised by chemiluminescence reagents (Super Signal West Pico kit; Pierce, Rockfort, IL, USA) with Hyperfilm ECL films (Amersham, Braunschweig, Germany). As a control for comparable exposure of membranes and as a standard for densitometry, 20 μg MCF7 proteins were always loaded in one well. Band intensities were quantified by densitometry (GS-700 Imaging densitometer; BioRad, München, Germany) and calculated as a percentage intensity of the control sample. The use of different protein amounts (20 μg/well or 10 μg/well) was considered during all calculations.

Immunohistochemistry. For immunohistochemical staining of ATF2 and p-ATF2, tissue microarrays (TMAs) with core tissue biopsies (diameter 0.6 mm) punched from individual donor paraffin blocks and transferred into recipient blocks were used. The resulting TMA blocks contained samples from 110 tumors (four cores per patient) which had been already analyzed by WB as well as 27 metastases (lymph nodes: 16 cases; others: 11 cases) of mammary tumors. Serial sections of 8 μm were cut from the TMA blocks using an adhesive-coated tape system (Tape-Transfer System; Instrumedics, Hackensack, New Jersey, USA). Slides were deparaffinised in xylene and rehydrated, followed by antigen retrieval by autoclaving in citrate buffer, pH 6.0.

ATF2 immunostaining was performed using a rabbit anti-ATF2 antibody (N-96; Santa Cruz), diluted 1:450 and a mouse antibody specific for ATF2 proteins phosphorylated at Thr69 (F1; Santa Cruz), diluted 1:50, and the Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). The evaluation of the results was performed independently by two observers. Immunostaining was scored as proposed by Remmele and Stegner (21), resulting in an immunoreactive score (IRS) of 0-12. Four parallel cores per tumor were analyzed, and in case of variations, the mean IHC score was calculated. Immunohistochemical detection of ER and PR had been carried out before as described elsewhere (18).

Statistical analysis. The SPSS program, version 15.0 (SPSS; München, Germany) was used for inter-relationships between the analyzed ATF2 and p-ATF2 proteins and histological or clinical factors as well as previously analysed proteins by Spearman-Rho and chi-square tests. Using the same program, Kaplan-Meier analysis and log-rank statistics of overall survival and recurrence-free survival were performed. Overall survival was defined as the time interval between surgery and death from breast cancer. For multivariate analysis, Cox regression analysis was performed. Probability values less than 0.05 were regarded as statistically significant.

Results

Western blot results for ATF2 and p-ATF2 and correlations with established prognostic parameters. In order to analyze the expression and role of the transcription factor ATF2 in mammary carcinomas, WB experiments were performed for ATF2 and its active, phosphorylated form p-ATF2 (Thr69/71; Figure 1). ATF2 was detectable as a single band around 70 kDa. The reported splice variant of 28 kDa which is expressed in myometrial cells during pregnancy (22) was not detected in our cases. Moreover, no bands were found between 30 and 45 kDa which might represent the partly

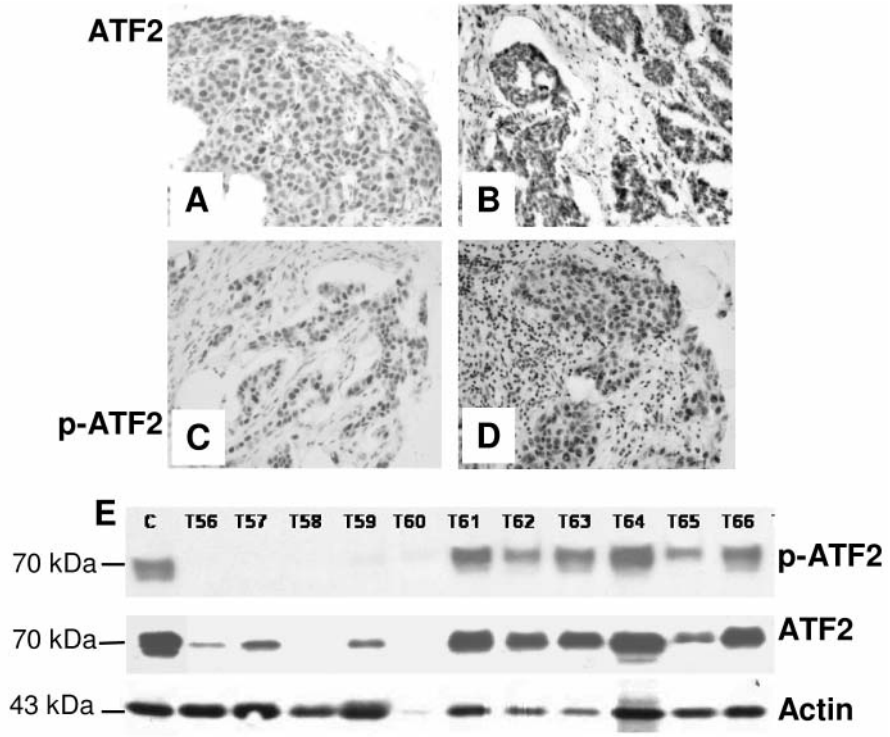

Figure 1. *ATF2 and p-ATF2 expression in breast cancer tissues as detected by immunohistochemistry (A-D) and Western blot analysis (E). A, B: Examples of strong nuclear ATF2 expression and negative staining in two mammary carcinomas (×200). C, D: Examples of strong nuclear p-ATF2 staining (C) and weak, mainly cytoplasmic p-ATF2 expression (D) in two breast cancer samples. E: WB analysis of p-ATF2, ATF2 and actin in representative carcinomas. C, control.*

Table I. *Correlations between ATF2 and p-ATF2 results obtained by Western blot (WB) and immunohistochemistry (IHC).*

	ATF2		p-ATF2 (Thr69/71)		ATF2 (Thr69)	
	Western blot		Western blot		IHC	
	Correlation	*p*-value	Correlation	*p*-value	Correlation	*p*-value
p-ATF2 (pThr69/71) WB	0.392	**<0.001**				
ATF2 IHC	0.215	0.099	0.103	0.459		
p-ATF2 (pThr69) IHC	–0.267	**0.041**	–0.050	0.724	0.191	**0.046**

Correlation coefficients and *p*-values after Spearman Rho analysis are shown.

homologous and cross-reactive ATF7 and CREB-P proteins (23, 24). Relative to the control, ATF2 expression in the tumors ranged from 0 to 132% (mean 24.3%, median 17.9%). For p-ATF2, the relative expression ranged from 0 to 263% (mean 45.3%, median 20.1%). In protein extracts from 7 normal tissue samples, weak ATF2 expression was observed (relative expression 0.1-7.7%, mean 3.1%), whereas p-ATF2 expression ranged from 8.6 to 27.2% (mean 15.5%). By Spearman-Rho test using the continuous densitometric results, there was a significant correlation of ATF2 with its phosphorylated form, p-ATF2 (Thr 69/71; Table I). According to the relative expression values corrected for equal actin loading, the tumors were divided into three groups of equal size for each protein, representing negative/weak, moderate and strong expression. Using these groups, statistical correlations with classical histological or clinical prognostic indicators were calculated by Chi-square tests (Table II). For ATF2, no significant association with age,

Table II. *P-values for associations of ATF2 and p-ATF2 results with classic prognostic parameters, ERK1/2, MMP-1 and MMP-9.*

	Western blot analysis				Immunohistochemistry			
	n	ATF2	n	p(Thr69/71)-ATF2	n	ATF2	n	p(Thr69)-ATF2
Clinical/histological parameter								
Histological grade	133	0.363	128	***0.041***	110	0.070	109	0.597
Stage	129	0.576	125	0.407	105	0.307	104	***0.039***
Nodal involvement	127	0.513	123	0.170	103	**0.045**	103	0.435
ER	133	0.690	128	**0.008**	109	0.134	108	0.447
PR	133	0.881	128	0.045*	109	0.097	108	0.747
Age (cut-off 50 years)	134	0.358	129	0.406	109	0.233	109	0.317
Histological type	132	0.571	127	**0.028**	107	0.398	107	0.934
MAP kinases ERK1/ERK2								
ERK1 (WB)	128	**0.014**	133	0.037*	108	0.491	107	0.250
ERK2 (WB)	133	**0.013**	128	0.418	108	0.620	107	0.259
p-ERK1 (WB)	133	0.867	128	**0.001**	108	0.899	107	0.773
p-ERK2 (WB)	133	0.447	128	**0.003**	108	0.835	107	0.127
p-ERK1/2 (IHC)	118	***0.008***	112	0.116	106	0.829	105	**0.002**
Invasion-associated proteins								
MMP-9	69	**0.004**	66	*0.041**	61	0.691	60	0.210
Activated MMP-1	71	0.700	64	0.177	63	0.668	62	0.659
Proenzyme MMP-1	71	0.056	64	**0.020**	63	0.869	62	0.774

P-values obtained by Chi-square tests are given. For statistical analysis, the following groups were compared: age: ≤50 years *vs*. >50 years; histological grade: G1/G2 *vs*. G3; histological type: ductal *vs*. other; staging: pT1/pT2 *vs*. pT3/pT4; nodal involvement *vs*. nodal-negative tumors; ER-positive *vs*. ER-negative tumors; PR-positive *vs*. PR-negative tumors. For ERK-1, ERK-2 and their phosphorylated forms, 3 groups of equal size with low, moderate and high expression values were compared [16]. Bold: significant associations; italics: inverse correlations; * non-linear association.

histological grade and type, clinical stage, nodal involvement, nor ER and PR status was found. For p-ATF2, a significant positive association with ER status was detected (p=0.008), as well as significant associations with low grading (p=0.041) and a non-ductal histology (p=0.028; Table II).

IHC results for ATF2 and p-ATF2 and correlations with established prognostic parameters. In all tumors, positive staining was mostly nuclear as expected (Figure 1). Positive immunoreactivity in normal tissue adjacent to the tumors was found in some fibroblasts (Figure 1), and in normal glandular or ductal epithelia (not shown). By Spearman analysis, a weak positive correlation of ATF2 and pATF2 IHC results was found (p=0.046; Table I). Comparison of WB and IHC results showed a weak inverse association of ATF2 WB and p-ATF2 IHC results as well as a non-significant trend pointing to a positive correlation of ATF2 WB and IHC results (Table I). For comparison with clinical and histological data by Chi-square tests, 3 groups were used (IRS 0-2, 3-8, 9-12) representing cases with negative/weak (n=11), moderate (n=72) and high contents (n=27) of ATF2 and negative/weak (n=10), moderate (n=55) and high contents (n=45) of p-ATF2. For ATF2, a significant association of higher immunoreactivity with nodal involvement (p=0.045) and trends towards an association with high grading (p=0.070) and a negative PR status (p=0.097) were observed. In contrast, high p-ATF2 expression was significantly associated with low clinical stage (p=0.039), but with no other analyzed histological or clinical parameter (Table II). In the metastases, the mean IRS score for ATF2 (9.9; range 4-12) was higher than that in primary tumors (7.0; range 0-12). In contrast, p-ATF2 expression in metastatic lesions (IRS 6.8; range 0-12) was below that in primary tumors (IRS 8.2; range 2-12). Neither difference was statistically significant.

Associations with ERK1/ERK2. The trans-activating potential of ATF2 is mainly regulated by phosphorylation, *i.e.* by mitogen-activated protein (MAP) kinases. Our cohort of breast cancer patients had been analysed for expression of the MAP kinases ERK1 and ERK2 and their phosphorylated active forms by immunoblots and for p-ERK1/2 expression by IHC (17). By comparison of the WB results, we found significant correlations of ATF2 with ERK1 (p<0.014) and ERK2 (p<0.013) expression. In addition, high p-ATF2 levels were significantly associated with high p-ERK1 (p<0.001) and p-ERK2 expression (p<0.003). There was an inverse association of ATF2 WB data and p-ERK1/2 IHC results and a close association of p-ATF2 IHC and p-ERK1/2 IHC results (p=0.002; Table II).

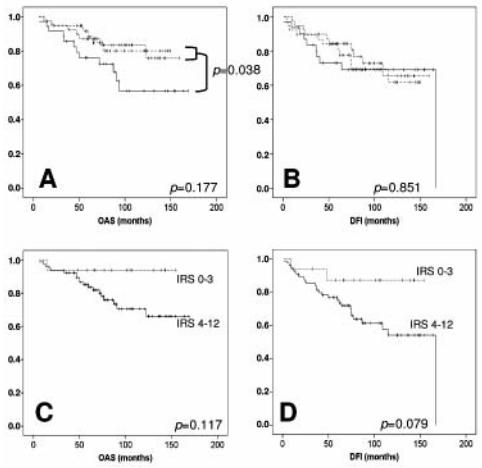

Figure 2. *Prognostic impact of ATF2 expression in breast cancer patients. Differences in overall survival (OAS; A, C) and disease-free interval (DFI; B, D) are shown. A, B: Western blot results (solid line: strong expression; dashed line: moderate expression; dotted line: weak expression). C, D: Immunohistochemistry results.*

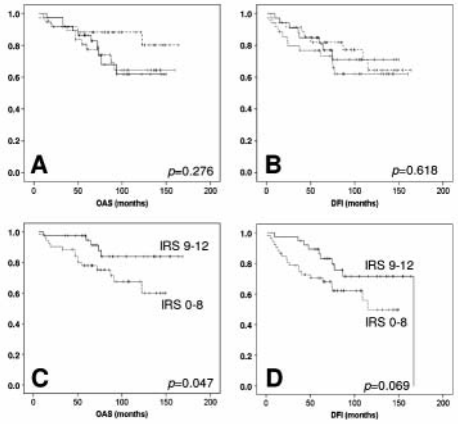

Figure 3. *Prognostic impact of p-ATF2 expression in breast cancer patients. Differences in overall survival (OAS; A, C) and disease-free interval (DFI; B, D) are shown. A, B: Western blot results (solid line: strong expression; dashed line: moderate expression; dotted line: weak expression). C, D: Immunohistochemistry results.*

Associations with matrix metalloproteinases MMP-1 and MMP-9. In the 71 patients mentioned above, the expression of MMP-9 (total protein) and MMP-1 (pro-enzyme and activated form) which are implicated in breast cancer invasion had been analysed before. MMP-9 expression in this sub-cohort was significantly associated with high ATF2 protein levels as shown in WB (p=0.004, Table II). In addition, there were associations of ATF2 and p-ATF2 expression as detected by WB with MMP-1 proenzyme levels (p=0.056 and p=0.020; Table II).

Correlation of ATF2 and p-ATF2 with overall and recurrence-free survival. By Kaplan-Meier analysis, patients with strong ATF2 expression as detected by IHC (score 4-12; n=11) and WB (highest tertial) had a shorter overall survival compared with patients with tumors with lower ATF2 expression (Figure 2). When cases with weak and moderate ATF2 expression in WB were combined, this difference reached statistical significance (p=0.038; Figure 2A). In addition, low ATF2 IHC results were associated with a longer disease-free interval (Figure 2D). For the phosphorylated ATF2 (Thr69/71) proteins, WB analysis did not reveal any prognostic significance (Figure 3A, B). Regarding the IHC results, high p-ATF2 (Thr69) expression was associated with increased overall (p=0.047) and recurrence-free survival (p=0.069) which is in contrast to the results obtained with total ATF2 (Figure 3C, D). In multivariate Cox regression analysis including stage, nodal status, grading, ER status and ATF2 (or p-ATF2) expression as detected by WB or IHC, no independent impact of the transcription factors on overall or disease-free survival was detected (not shown).

Discussion

As a member of the AP-1 family of transcription factors, ATF2 has been implicated in the regulation of various target genes which are involved in the proliferation, invasion and survival of cancer cells. Yet, experimental models gave conflicting results as for the role of ATF2 in breast cancer cells: In MCF10A breast epithelial cells, H-ras or TGFβ induced ATF2 activation *via* p38-MAPK which resulted in enhanced cell invasion and migration (8, 25). In contrast, ATF2 deficiency led to a susceptibility to mammary tumors in mice (15). Thus, the question if ATF2 displays oncogenic or tumor-suppressor function in breast cancer cells is still unanswered.

By determination of ATF2 and p-ATF2 protein expression in well-characterized breast cancer samples, we added interesting new information to this issue. Interestingly, the ATF2 results derived from immunoblots and IHC did not significantly correlate with each other, nor did the p-ATF2 expression levels detected with both methods. This might be due to tissue heterogeneity or ATF2 expression in stromal cells, which cannot be separated from ATF2 in the epithelial tumor cells in WB experiments, whereas in IHC, immunoreactivity was scored in cancer cells only. For phospho-ATF2, this result might also be due to different antibodies which were used for technical reasons: In WB, only activated ATF2 proteins phosphorylated at Thr69 and Thr71 (3, 4) were detected, whereas in IHC, a Thr69-specific antibody was used. Another reason for discrepancies could be cross-reactivity of the anti-pATF2 antibodies with other members of the subfamily, namely p-ATF7 and p-CRE-BPa. For our WB results, we can rule out such cross-reactivities due to the different molecular

weights of these proteins, whereas such discrimination is not possible by IHC. With both methods, high ATF2 expression was associated with reduced overall survival, which points to an oncogenic rather than a tumor-suppressing influence of ATF2 in breast cancer. In contrast, high expression of p-ATF2 as found by WB and IHC was not associated with a poor prognosis. The results for pThr69-ATF2 obtained by IHC even point to an association of high p-ATF2 levels with prolonged disease-free and overall survival (Figure 3) which is in contrast to the results obtained for total ATF2. Statistically, we found significant associations of high p-ATF2 protein levels with a well-differentiated, ER-positive phenotype (WB) and early stages (IHC) which are favourable prognostic indicators. ATF2 phosphorylation at Thr69 and Thr71 is mainly carried out by stress-activated protein kinases and the MAP kinases ERK1/2, p38 and JNK (4, 5). In our samples, high amounts of p-ATF2 were significantly associated with p-ERK1/2 overexpression. This correlation was independently found by WB and IHC and suggests that ATF2 is phosphorylated by p-ERK1 and p-ERK2 *in vivo*. In a prior study, we found that high levels of p-ERK1/2 were associated with a better prognosis (17). This is in concordance with the our new finding that, in contrast to total ATF2, phosphorylated ATF2 is not a negative prognostic indicator. The signalling pathway which leads to activation of ERK1/2 and, consequently, ATF2, might be inactivated in high-risk mammary carcinomas. The observation that p-ATF2 correlates with p-ERK1 and p-ERK2, but not with total ERK1/2, suggests that the close association of these proteins is not due to transcriptional activation of ERK expression by ATF2. Instead, this result and the strong correlation of total ATF2 and ERK1/ERK2 point to common regulatory mechanisms. ATF2 expression correlated significantly with MMP-9, a matrix metalloproteinase with collagenase IV activity that is associated with a poor prognosis in carcinomas (26). Yet, this association was not found for p-ATF2, which rules out a direct transcriptional activation of MMP-9 expression by p-ATF2. Two prior experimental studies found that MMP9 expression was not activated by p-ATF2 (25) and support our interpretation that the correlation of MMP-9 and ATF2 in our cohort might be the result of an indirect mechanism. In conclusion, our results point to opposing roles of ATF2 and its phosphorylated forms, p-Thr69/p-Thr71-ATF2 or p-Thr69-ATF2, in mammary carcinomas. Although high ATF2 expression is associated with shorter survival, the phosphorylated ATF2 protein is mainly overexpressed in prognostically favourable tumors. The regulatory mechanisms leading to these interesting results should be further analyzed.

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