Original Articles

Jun and Fos family protein expression in human breast cancer: Correlation of protein expression and clinicopathological parameters

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Summary

Objectives: The activator protein-1 (AP-1) is a dimeric transcription factor formed by members of the Jun and Fos protein family. AP-1 plays a role in a variety of physiological functions including cell proliferation and differentiation, although both c-Jun and c-Fos have also been implicated in oncogenic transformation and tumor progression. To further elucidate the role of AP-1 in breast cancer, we have investigated the expression of the AP-1 proteins c-Jun, JunB, JunD, phosphorylated c-Jun, c-Fos, Fra1, Fra2 and the tumor supressor protein p53.

Methods: Protein expression was evaluated on a breast cancer tissue microarray with 58 lymph node positive or negative breast cancer specimens, 29 corresponding lymph node metastases, and 11 tissue samples from surrounding tumor-free tissue, each cored as triplicate. Jun and Fos protein family expression was evaluated by immunohistochemistry and was correlated with clinicopathological parameters.

Results: High expression levels were observed for c-Jun, JunD, c-Fos and Fra2, whereas JunB and Fra1 exhibited lower staining. c-Jun protein expression was correlated to Fra1 staining ($p = 0.007$, Kendall's Tau) and Fra1 was further associated with c-Fos ($p < 0.001$), JunD ($p = 0.001$) and Fra2 ($p = 0.011$) expression. JunD expression correlated with c-Fos ($p < 0.001$), JunB ($p = 0.035$) and c-Jun ($p = 0.05$). Activated c-Jun correlated with c-Fos expression ($p = 0.041$).

JunB was negatively correlated to tumor stage, ($p = 0.093$, corr coeff. = -0.293, Spearman's correlation) but was significantly increased in nodal negative tumors ($p = 0.004$, Mann Whitney test). In addition, increased Fra1 expression showed a trend towards an increased overall survival ($p = 0.077$, RR = 0.534, Cox regression).

Conclusion: Our results suggest an important role for JunB and Fra1 in the biological behavior of malignant breast tumors.

Key words: AP-1; Jun; Fos; Breast cancer.

Introduction

Although AP-1 (activator protein 1) was one of the first proteins identified to be involved in transcriptional processes, its role in human tumors is still poorly understood. AP-1 is a dimer consisting of members of the basic region leucine zipper (bZIP) DNA binding proteins. The dimer is either formed by a homodimer of members of the Jun-(c-Jun, JunB, JunD) protein family or by a heterodimer consisting of one Jun-family member and one Fos-(c-Fos, FosB, Fra1, Fra2) or a more distantly related ATF (activating transcription factor) or Maf (musculoaponeurotic fibrosarcoma) protein family member [1-4]. The AP-1 dimers bind to specific DNA response elements and enable transcription of diverse target genes, such as EGFR, CyclinD1, p53, p16 ^{INK4a}, VEGFD, uPA, uPAr, proliferin, MMPs, CD44, cathepsin, and MTS. Since v-Jun and v-Fos were first identified as viral oncoproteins, their role in tumorigenesis is widely accepted, although c-Jun and c-Fos posses decreased oncogenic potential compared to their viral homologues [5-9].

AP-1 activity is induced by a variety of internal and external stimuli, including growth factors, hormones, bacterial and viral infections, UV-radiation, and activation of other oncogenes. AP-1 can fulfill a variety of cellular processes and is thought to play an important role in proliferation, differentiation, wound healing, UV response, and apoptosis. Significant changes in its expression and activation are implicated in tumorigenesis and the aggressive biologic behavior of a tumor [3, 4, 9, 10]. Interestingly, several studies suggest that either overexpression or lack of expression of certain AP-1 proteins leads to oncogenic transformation, promotion of tumor growth, tumor cell invasion, angiogenesis and drug resistance [11-13]. AP-1 proteins that lack potent transactivation domains exhibit weak (Fra1 and Fra2) or no transforming activity (JunB and JunD) and need to dimerize with AP-1 proteins containing an active transactivation domain, to induce oncogenic transformation [14].

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In general, AP-1 proteins are considered to promote tumor growth, but several studies suggest some components of the AP-1 family to behave as tumor supressors under certain conditions or to play a rather ambivalent role in tumorigenesis, as suggested for JunB, JunD, Fra1, FosB and even c-Fos. For example, JunB can antagonize or substitute the proliferative effect of c-Jun depending on multifactoral circumstances [9, 15-19]. These data imply that different components of the AP-1 transcription factor complex appear to have distinct effects on cell proliferation or transformation, and that the result of AP-1 activity seems to be strongly dependent on the composition of dimer, the level of expression, cell type and differentiation. However, AP-1 can also exert inverse effects and might even be able to promote malignant transformation of the cell.

In order to further elucidate the possible effects of AP-1 family members in malignant breast tissue, we have performed an immunohistochemical analysis of the expression of c-Jun, JunB, JunD, c-Fos, Fra1 and Fra2 and the tumor supressor protein p53, which is suggested to be repressed through interaction with c-Jun [20]. To investigate the activation status of c-Jun, an antibody specifically detecting the phosphorylated serine residues 63 and 73 of c-Jun was applied.

Material and Methods

Tissue collection and tumor specimens

Archival tissue samples of 58 primary breast cancer specimens and 29 corresponding lymph node metastases were obtained from patients undergoing surgery for breast cancer at the General Hospital of Vienna from 1990 to 1994. At least a total of 55 primary breast cancers and 29 lymph node metastases were immunohistochemically evaluated. Seventeen patients were premenopausal, four peri- and 25 postmenopausal and for nine patients the menopausal status was not available. Estrogen- and progesterone-receptor status was available in 48 and 47 cases and was positive in 26 and 21 cases, respectively. Forty-one of the primary breast tumors were lymph node positive and 14 were lymph node negative. Of the 41 nodal positive tumors, affected lymph nodes of 29 patients were available and cored on the tissue microarray. Forty-eight percent of the patients developed distant metastases. There was not data available for 15% of the patients. None of the patients received any form of neoadjuvant endocrine or cytotoxic therapy that could have confounded the results prior to tissue biopsy. Overall survival was available for all of the patients. Histologically, 43 specimens were invasive ductal carcinomas, ten were invasive lobular, and for two specimens no data was available. Histological grading classified one of the tumors G1, 23 tumors G2, three tumors G2.5, 27 tumors G3, and one tumor G4. Ten of the carcinomas were assessed as Stage I, 27 Stage II, seven Stage III, and six as Stage IV.

Construction of the breast cancer tissue microarray

The tissue array contained tissue samples from 59 patients, including 58 primary breast carcinomas, 29 corresponding lymph node metastases, 11 tumor-free mammary tissues and four tumor-free lymph nodes. In addition, two relapses, two distant metastases derived from the skin, one post therapeutic primary tumor and one lymph node metastasis, of which no primary tumor was available, were arrayed but excluded from analysis because of the small sample size. To minimize loss of samples and to reduce cellular heterogeneity of a tumor or the staining pattern, each tissue sample was arrayed as a triplicate. In summary, 324 tissue cores were arrayed on a glass slide for further analysis. Areas of the block containing tumor cells were identified via a corresponding H&E stained slide. Representative regions were marked for subsequent extraction by an experienced pathologist. Core biopsies of 0.6 mm in diameter were taken from each donor block and arrayed into a recipient block in a 0.8 mm spaced grid using the tissue puncher/arrayer MTA1 (Beecher Instruments, Silver Spring, MD). After tissue microarray construction was completed, 3 µm sections were cut and transferred on coated glass slides. The presence of tumor tissue was validated on H&E stained sections of the tissue microarray.

Immunohistochemistry

Consecutive tissue microarray slides were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval sections were placed in citrate puffer (10 mmol, pH 6.0) and boiled in a microwave oven for 3 x 5 minutes. The endogenous peroxidase was blocked with 3% hydrogen peroxide in PBS. To avoid non-specific staining, the slides were incubated in appropriate serum (Lab Vision, Westinghouse Drive, Fremont, CA) for ten minutes at room temperature, followed by incubation with the primary antibody (anti c-Jun, rabbit polyclonal, cat.# sc-1694, dilution of 1:150; anti p-c-Jun, mouse monoclonal, Cat.# sc-822; dilution of 1:150; anti JunB, mouse monoclonal, Cat# sc-8051, dilution of 1:300; anti JunD, rabbit polyclonal, Cat# sc-74, dilution of 1:500; anti Fra1, rabbit polyclonal, Cat# sc-605, dilution of 1:400; anti Fra2, rabbit polyclonal, Cat# sc-604, dilution of 1:200; all from Santa Cruz Biotechnology and anti c-Fos, Cat# PC05, dilution of 1:10, Oncogene Science) in a wet chamber at 4°C overnight. Following this, slides were rinsed in PBS, incubated with biotinylated secondary antibody and subsequently conjugated to streptavidin-HRP (Lab Vision, Westinghouse Drive, Fremont, CA). Bound antibodies were detected using 3-amino-9-ethylcarbazole (AEC) (Lab Vision, Fremont, CA). Subsequently after staining was visible, the slides were washed in pre-warmed distilled water, counterstained with hematoxylin and cover-slipped.

Immunohistochemical evaluation and statistical analysis

The tissue array sections were scored semiquantitatively for nuclear staining by light microscopy done by two independent pathologists. The staining intensity was graded on the following scale: 0 = negative, 1 = weak, 2 = moderate, 3 = strong staining intensity. Results of the immunohistochemical analysis include a variable for the staining intensity and a variable for the percentage of positive tumor cells. The percentage of positive tumor cells ranged from 0% to 100% and was graded as follows: $0 \leq 10\% = 1$, 10-50% = 2, 51-80% = 3, 81-100% = 4 points. For final immunohistochemical evaluation these two results were multiplied; 0-2 points were designated as negative staining (0), 3-5 points as weak (1+), 6-8 points as moderate (2+) and 9-12 points as strong (3+) staining[21]. The scores of multiple tissue cores from the same breast cancer specimens were averaged and spots with discrepant scores were re-examined to obtain a final consensus score.

Statistical correlation of the AP-1 protein expression was performed by applying Kendall's Tau test. For comparison of AP-1 expression levels between nodal negative and positive tumors the Mann Whitney U-test was employed. To determine associations of expression of a certain AP-1 protein and the overall or metastasis-free survival of a patient, Cox regression analysis

was applied. To enhance the power of statistical comparison, the following categories were pooled: strong/moderate vs weak/negative or strong vs moderate/weak/negative staining, respectively. Correlation of clinocopathological parameters with AP-1 expression was performed by employing the 2-tailed Spearman correlation test.

Results

Jun and Fos family protein expression

A total of 55 primary breast cancer specimens, 29 nodal metastases and four tumor-free tissue samples were available for analysis. Tissue cores that were not analyzed were excluded because of loss of tissue cores, loss of cellular components within the spot or a too small sample size.

With the exception of two AP-1 family members, JunB and Fra1, the majority of breast tumors, as well as the lymph node metastases showed high expression levels of the Jun and Fos family members. Table 1a shows an overview of the AP-1 protein expression. Highest expression levels were seen for c-Jun and c-Fos. For c-Jun strong or moderate staining was observed in 51% or 47% of the tumors, respectively. C-Fos was detectible in all cases, exhibiting predominantly strong (46%) or moderate (49%) staining, resulting in 95% of the tumors showing high immunoreactivity. To analyze the activation state of c-Jun a specific antibody binding to the phosphorylated and activated form of c-Jun (p-c-Jun) was applied. Staining of p-c-Jun was strong/moderate in 26%/44% of the tumors. In summary 91% of the c-Jun positive tumor cells showed positive staining for p-c-Jun, although with a lower staining intensity. For c-Jun 98% of the tumors showed moderate/strong (51%/47%) staining, whereas for p-c-Jun the majority of the tumors were moderately/weakly (44%/22%) stained. Considerable immunoreactivity was also seen for JunD and Fra2. JunD was strong or moderately expressed in 24% or 58% of the tumors, respectively. Fra2 was similarly expressed, showing strong or moderate staining in 20% or 67% of the cases. Interestingly, the expression pattern of JunB and Fra1 differed from the pattern observed for the majority of AP-1 proteins. Strong staining for JunB was observed in only two tumors, whereas the majority of the tumors exhibited weak (66%) or no labeling (15%). Fra1 expression was weak (51%) or negative (4%) in more than the half of the tumors, with only three tumors showing strong staining.

Table 1a. — *AP-1 protein expression in primary breast tumors (A); B and C show separately evaluated AP-1 protein expression levels for nodal negative primary tumors (B) and nodal positive breast tumors (C). An analysis of AP-1 expression in lymph node metastases and the tumors from which they derived are shown in D and E, respectively.*

	c-Jun		p-c-Jun		JunB		JunD		c-Fos		Fra1		Fra2		p53	
	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n
A: Primary breast cancers																
Strong	51	28/55	26	14/55	4	2/55	24	13/55	46	25/55	6	3/55	20	11/55	18	9/49
Moderate	47	26/55	44	24/55	16	9/55	58	32/55	49	27/55	40	22/55	67	37/55	18	9/49
Weak	2	1/55	22	12/55	66	36/55	18	10/55	6	3/55	51	28/55	13	7/55	22	11/49
Negative	0	0/55	9	5/55	15	8/55	0	0/55	0	0/55	4	2/55	0	0/55	41	20/49
B: LN- primary breast cancers																
Strong	50	7/14	29	4/14	14	2/14	36	5/14	50	7/14	7	1/14	21	3/14	27	3/11
Moderate	50	7/14	43	6/14	29	4/14	50	7/14	43	6/14	57	8/14	57	8/14	27	3/11
Weak	0	0/14	21	3/14	57	8/14	14	2/14	7	1/14	36	5/14	21	3/14	18	2/11
Negative	0	0/14	7	1/14	0	0/14	0	0/14	0	0/14	0	0/14	0	0/14	27	3/11
C: LN+ primary breast cancers																
Strong	51	21/41	24	10/41	0	0/41	20	8/41	44	18/41	5	2/41	20	8/41	16	6/38
Moderate	46	19/41	44	18/41	12	5/41	61	25/41	51	21/41	34	14/41	71	29/41	16	6/38
Weak	2	1/41	22	9/41	68	28/41	20	8/41	5	2/41	56	23/41	10	4/41	24	9/38
Negative	0	0/41	10	4/41	20	8/41	0	0/41	0	0/41	5	2/41	0	0/41	45	17/38
D: Lymph node metastases																
Strong	38	11/29	7	2/29	0	0/29	38	11/29	28	17/29	0	0/29	17	5/29	18	5/25
Moderate	55	16/29	41	12/29	3	1/29	31	9/29	45	10/29	34	10/29	76	22/29	11	3/25
Weak	7	2/29	41	12/29	55	16/29	28	8/29	28	2/29	66	19/29	7	2/29	29	7/25
Negative	0	0/29	10	3/29	41	12/29	3	1/29	0	0/29	0	0/29	0	0/29	43	10/25
E: Tumors to LN-metastases																
Strong	52	15/29	21	6/29	0	0/29	24	7/29	34	10/29	7	2/29	24	7/29	15	4/25
Moderate	48	14/29	45	13/29	10	3/29	62	18/29	41	12/29	41	12/29	72	21/29	19	5/25
Weak	0	0/29	24	7/29	62	18/29	14	4/29	24	7/29	48	14/29	3	1/29	15	4/25
Negative	0	0/29	10	3/29	28	8/29	0	0/29	0	0/29	3	1/29	0	0/29	50	12/25

Comparison of AP-1 protein expression in nodal negative and positive tumors (Mann Whitney's test).

Table 1b)

	p value							
	c-Jun	p-c-Jun	JunB	JunD	c-Fos	Fra1	Fra2	p53
LN- versus LN+ tumors	1.000	0.735	0.004*	0.280	0.777	0.099	0.608	0.195

* statistically significant ($p < 0.05$).

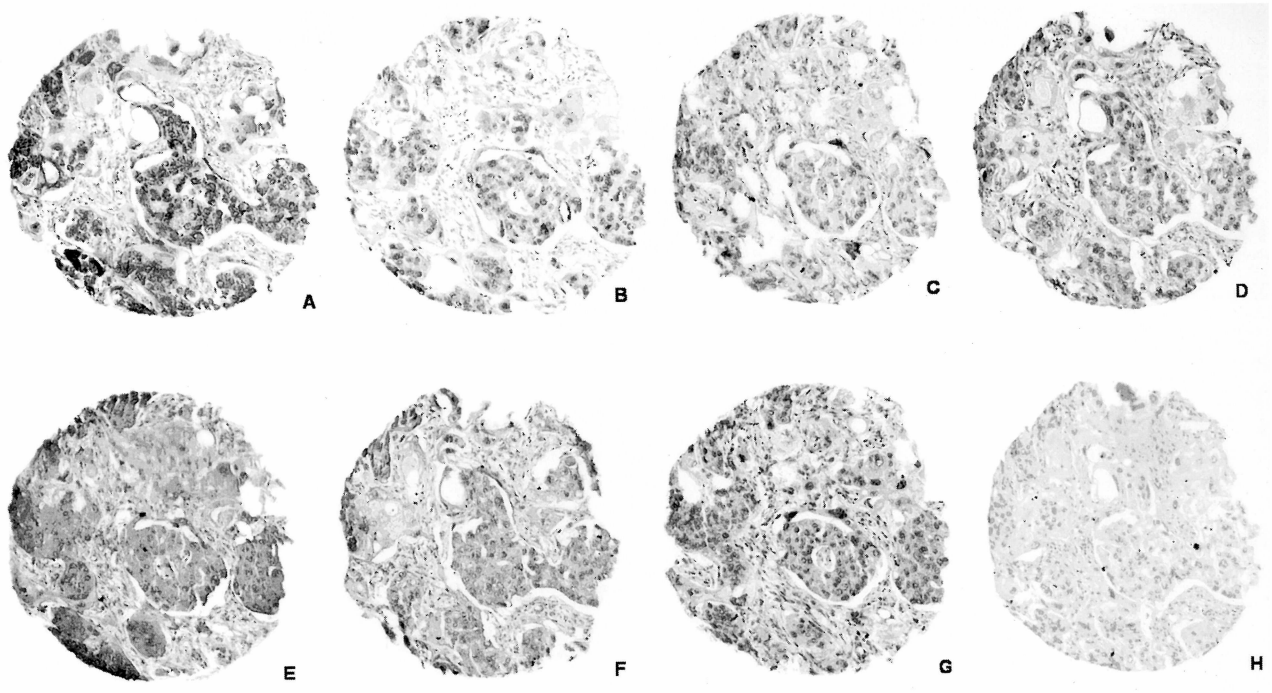

Figure 1. — Immunohistochemical AP-1 protein staining patterns of a human breast carcinoma on consecutive tissue microarray sections. A: c-Jun protein expression B: phosphorylated-c-Jun protein expression; C: JunB protein expression; D: JunD protein expression; E: c-Fos protein expression;, F: Fra1 protein expression; G: Fra2 protein expression and H: p53 protein expression.

Interestingly, almost no significant differences were observed between the expression patterns of breast cancer specimens, that did or did not develop nodal metastases. Figure 3 represents AP-1 expression levels in a diagramed format. Both showed highly similar expression patterns for almost all AP-1 proteins examined, with the exception of JunB, which showed a significantly lower expression score ($p = 0.004$, Mann Whitney test) in lymph node positive tumors than in lymph node negative tumors (Table 1b and Figure 3).

The immunohistochemical staining pattern of the AP-1 proteins was predominantly nuclear. While tumor cells were the primary source of AP-1 protein expression, adjacent stromal and endothelial cells occasionally also showed weak to strong immunolabeling on each tested sample (data not shown). Interestingly, tumor-free epithelial breast tissue taken from the region of fibrocystic mastopathy surrounding the tumor also showed expression of the diverse AP-1 proteins which typically was at least as strong as in the tumor cells (Figure 1). Taken together, the majority of the tumors exhibited strong/moderate staining, with the exception of JunB and Fra1, showing predominantly weaker labeling, whereby the number of tumors expressing high levels of AP-1 proteins ranged from 20-98%.

Correlations of Jun and Fos family proteins with each other

Correlation analysis of the expression levels of AP-1 family members was performed by applying Kendall's tau test. Results of the statistical analysis are shown in Table 2.

Table 2. — *Correlation of AP-1 protein expression in human breast tumors (Kendall's tau test).*

Paired	Variables	Tau	p value
c-Jun	p-c-Jun	0.341	0.001*
c-Jun	JunB	-0.150	0.114
c-Jun	JunD	0.241	0.050
c-Jun	c-Fos	0.097	0.444
c-Jun	Fra1	0.279	0.007*
c-Jun	Fra2	0.207	0.116
c-Jun	p53	0.304	0.005*
p-c-Jun	JunB	-0.187	0.147
p-c-Jun	JunD	0.154	0.180
p-c-Jun	c-Fos	0.223	0.041*
p-c-Jun	Fra1	0.056	0.616
p-c-Jun	Fra2	0.087	0.398
p-c-Jun	p53	0.141	0.269
JunB	JunD	0.188	0.035*
JunB	c-Fos	0.182	0.098
JunB	Fra1	0.033	0.772
JunB	Fra2	-0.159	0.067
JunB	p53	-0.067	0.636
JunD	c-Fos	0.436	< 0.001*
JunD	Fra1	0.327	0.001*
JunD	Fra2	0.157	0.238
JunD	p53	0.132	0.173
c-Fos	Fra1	0.381	< 0.001*
c-Fos	Fra2	0.150	0.153
c-Fos	p53	0.179	0.122
Fra1	Fra2	0.268	0.011*
Fra1	p53	0.180	0.152
Fra2	p53	0.062	0.565

* statistically significant ($p < 0.05$ in 2-sided test).

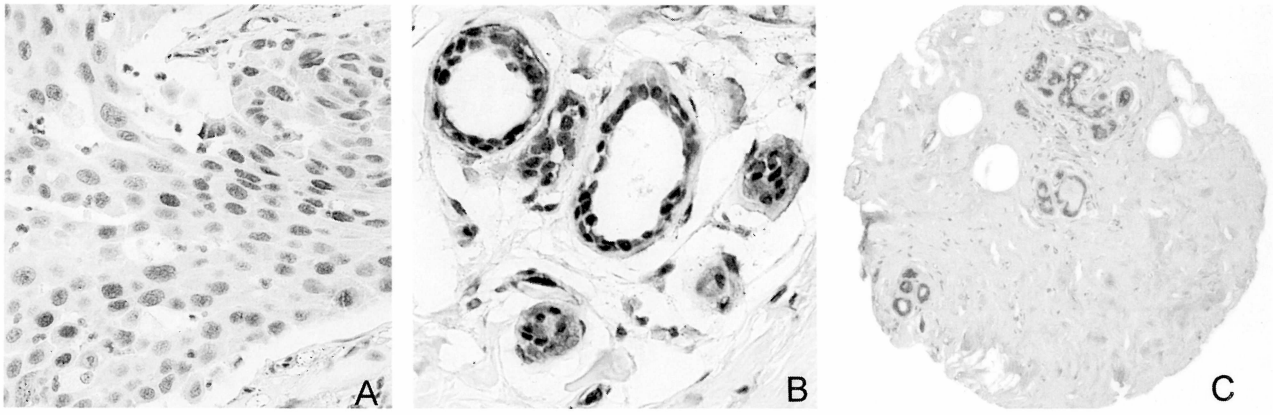

Figure 2. — Expression of activated, phosphorylated c-Jun in a ductal mammary carcinoma (A) and mastopathic breast tissue (B) (x400 magnification); staining pattern of c-Fos protein in mastopathic tissue (C) (x100 magnification).

Table 3. — *Correlation analysis of AP-1 and p53 protein expression and overall and metastasis-free survival in human breast cancer patients (Cox regression analysis).*

Parameter	Score	Overall survival RR (95% CI)	p value	Metastasis-free survival RR (95% CI)	p value
c-Jun	3 vs 0-2	0.977 (0.498 to 1.91)	0.945	0.655 (0.281 to 1.53)	0.328
p-c-Jun	0-1 vs 2-3	0.862 (0.419 to 1.77)	0.687	2.17 (0.636 to 7.42)	0.216
JunB	0-1 vs 2-3	0.465 (0.180 to 1.21)	0.115	0.544 (0.159 to 1.86)	0.332
JunD	0-1 vs 2-3	0.835 (0.345 to 2.02)	0.689	2.18 (0.726 to 6.53)	0.165
JunD	3 vs 0-2	0.802 (0.363 to 1.78)	0.587	1.17 (0.445 to 3.06)	0.754
c-Fos	0-1 vs 2-3	0.659 (0.286 to 1.52)	0.328	1.50 (0.551 to 4.09)	0.427
c-Fos	3 vs 0-2	0.796 (0.398 to 1.59)	0.518	0.740 (0.323 to 1.70)	0.476
Fra1	0-1 vs 2-3	0.534 (0.266 to 1.07)	0.077	0.555 (0.231 to 1.34)	0.189
Fra2	0-1 vs 2-3	1.78 (0.543 to 5.83)	0.342	2.52 (0.567 to 11.2)	0.224
Fra2	3 vs 0-2	0.682 (0.282 to 1.65)	0.396	1.24 (0.447 to 3.43)	0.682
p53	0-1 vs 2-3	0.675 (0.320 to 1.42)	0.303	1.18 (0.477 to 2.91)	0.721
patient age		1.02 (0.990 to 1.04)	0.256	0.993 (0.950 to 1.04)	0.748

Highly significant coexpression was observed for c-Fos with JunD ($p < 0.001$) and Fra1 ($p < 0.001$), respectively. Fra1 expression was related furthermore to expression of Fra2 ($p = 0.011$) and c-Jun ($p = 0.007$). For JunD a significant relationship to JunB ($p = 0.035$), Fra1 ($p = 0.001$) and a trend towards an association with c-Jun ($p = 0.050$) expression could be observed. Activation of c-Jun was associated with c-Fos ($p = 0.041$). All other AP-1 protein expression levels were not significantly associated.

Correlation of AP-1 protein expression and clinicopathological parameters

AP-1 protein expression and known clinicopathologically relevant parameters, such as tumor size, histological grade, tumor stage, development of distant metastases, and age and overall survival of the patient were then correlated. Overall and metastasis-free survival was analyzed by Cox regression analysis (Table 3). No significant association between the expression of a distinct AP-1 component and the overall survival and metastasis-free survival of the patient was found. However, high Fra1 expression was associated with increased overall survival ($p = 0.077$, RR = 0.534, Cox regression analysis).

Furthermore a correlation analysis for AP-1 protein expression and parameters of the TNM-classification-system and tumor-grading was performed by applying the two-tailed Spearman correlation test (Table 4). Statistical analyses revealed a significant negative correlation of JunB expression with tumor stage ($p = 0.039$) and a negative trend towards association with tumor size ($p = 0.061$). For Fra1 a tendentious negative relationship between the dedifferentiation of a tumor ($p = 0.057$) was observed. p53 protein expression was negatively correlated to patient age ($p = 0.021$) and tumor stage ($p = 0.022$).

Discussion

AP-1 expression can be modulated by a wide range of various stimuli and can, in turn, induce an enormous variety of cellular functions. Since AP-1 was one of the first trancription factors identified and the two most prominent family members, c-Jun and c-Fos, are cellular homologues of viral oncogenes, AP-1 is regarded to be proto-oncogene. However, until now its role in tumorigenesis and promotion of malignancy is still poorly understood. The distinct AP-1 proteins can function as tumor promoters as well as tumor suppressors, with regard to cell growth, transformation, invasion and angiogenesis. Furthermore AP-1 can induce or inhibit apoptosis. Since

Table 4. — *Correlation analysis of AP-1 and p53 protein expression and clinicopathological parameters in human breast cancer patients (2-tailed Spearman correlation test).*

Parameter		c-Jun	p-c-Jun	JunB	JunD	c-Fos	Fra1	Fra2	p53
Age	Correlation Coefficient	-0.077	-0.206	0.038	0.021	0.105	0.174	-0.039	-.330*
	p value	0.577	0.131	0.781	0.877	0.446	0.205	0.777	0.021
Grading	Correlation Coefficient	0.019	-0.011	-0.184	-0.141	-0.196	-0.258	0.177	-0.052
	p value	0.893	0.936	0.178	0.306	0.15	0.057	0.197	0.722
Tumor size	Correlation Coefficient	-0.011	-0.182	-0.254	-0.036	-0.232	-0.145	-0.09	-0.142
	p value	0.939	0.184	0.061	0.792	0.088	0.291	0.514	0.33
Staging	Correlation Coefficient	-0.06	-0.168	-.293*	-0.081	-0.234	-0.232	0.036	-.341*
	p value	0.677	0.242	0.039	0.574	0.103	0.104	0.806	0.022

* statistically significant ($p < 0.05$ in 2-sided test).

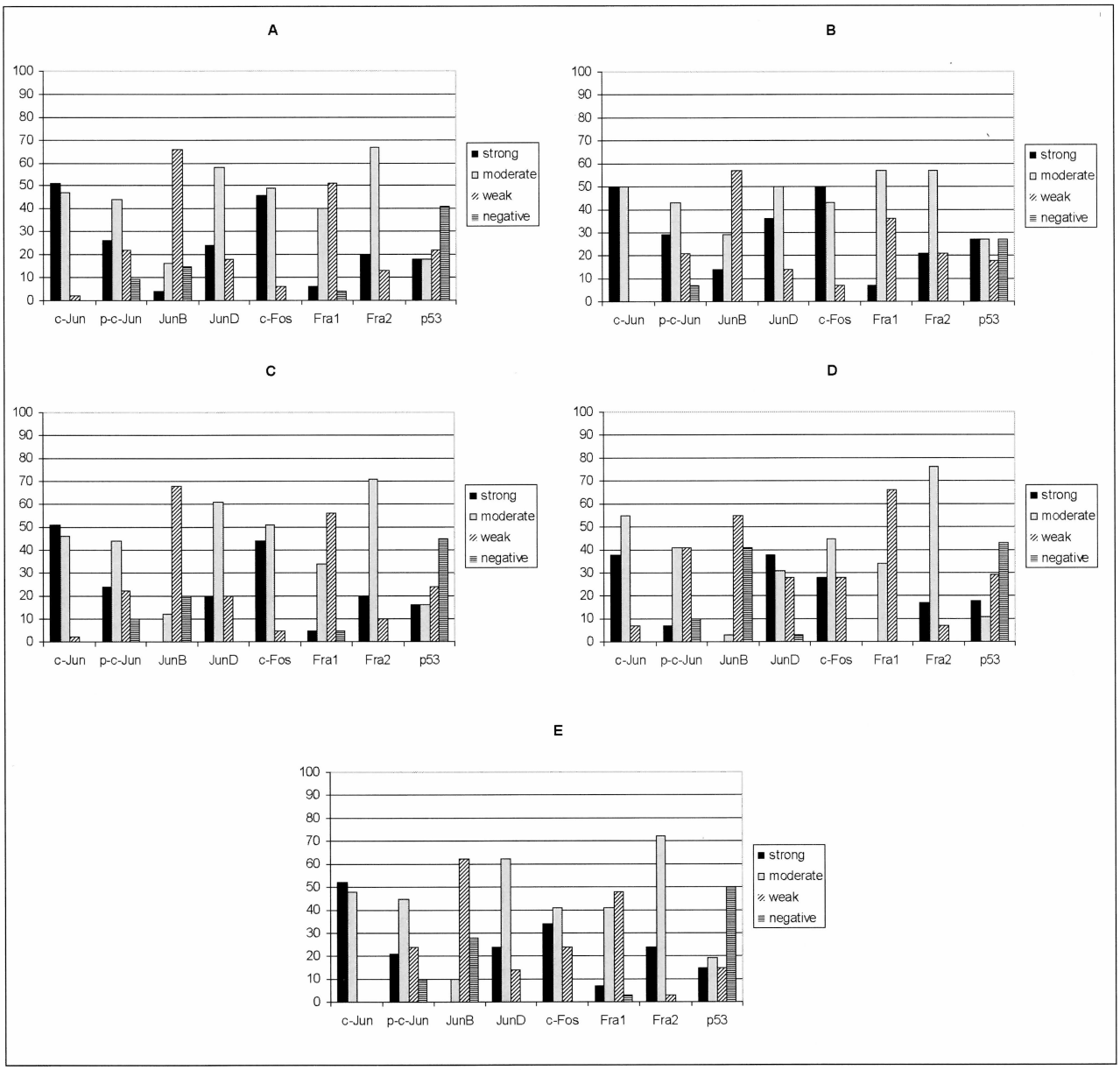

Figure 3. — AP-1 protein expression in primary breast tumors (A); B and C show separately evaluated AP-1 protein expression levels for nodal negative primary tumors (B) and nodal positive breast tumors (C). An analysis of AP-1 expression in lymph node metastases and the tumors from which they derived are shown in D and E, respectively.

functional AP-1 acts as a dimer, which can be composed of several distinct family members, it is hardly manageable to commit the AP-1 transcrition factor complex on a single consensus function. Besides the high number of possible dimer-compositions, relative expression levels, tumor stage, genetic background and several other biological settings greatly influence its activity.

This study focusses on the expression of AP-1 components of the Jun (c-Jun, JunB, JunD) and Fos (c-Fos, Fra1, Fra2) family members. The expression levels of these proteins were investigated by immunohistochemistry on primary breast carcinomas and corresponding lymph node metastases, and additionally on a small number of benign adjacent breast tissues. Our results demonstrate that the majority, (70-98%) of the breast cancer specimens showed high expression levels of the AP-1 proteins c-Jun, p-c-Jun, JunD, c-Fos and Fra2, whereas for JunB and Fra1 weaker staining intensities were observed. It is rather unusual to find oncogenes that are expressed in almost 100% of the examined tumors. These results, however, indicate that AP-1 proteins certainly play an important role in development and maintenance of growth in human breast cancer. Studies on breast cancer cell lines showed that c-Jun overexpression increases growth rate of normal breast and malignant MCF7 cells, whereas other breast cancer cell lines seem to become independent on c-Jun growth signals the more they become malignant, probably by using other pathways, as multiple genetic alterations arise during carcinogenesis [22, 23]. Some studies even suggest higher c-Jun or c-Fos expression rates in normal breast than in breast cancer cells, whereas others indicate that expression of these AP-1 proteins is increased only in malignant cells [24-26]. However, cell lines can never represent the identical biological behavior of breast carcinomas in vivo, which could explain the differences. The AP-1 transcription-factor complex is certainly a key regulator of cell proliferation in several tissues. Since tissue samples derived from tumor surrounding mastopathic tissue also showed positive immunostaining for all tested Jun and Fos proteins, AP-1 could contribute to atypical growth patterns. This hypothesis is also supported by immunohistochemical analyses on lung carcinomas, which demonstrated that normal lung epithelium exhibited no staining, premalignant lesions strong and malignant carcinoma cells reduced c-Jun staining [27]. Furthermore, analysis of activation of c-Jun by a specific antibody binding to the phosphorylated and activated form of c-Jun revealed that expression significantly correlates with activation, indicating that most of the c-Jun proteins are activated, when expressed.

Of all AP-1 family proteins, only JunB and Fra1 showed weaker staining. This result supports the role of JunB as a tumor supressor. Several studies are consistent in reporting that JunB antagonizes proliferative activity, and attenuates transformation of REFs by c-Jun [16, 28, 29]. Only in the absence of c-Jun, does JunB substitute for the proliferation defect by acting as a growth promoter, which further clearly demonstrates the ambivalent role of AP-1 independent of various cellular settings [17]. Once a tumor is established, JunB together with c-Jun can revert into an aggressive protein by increasing angiogenesis, as was demonstrated in a culture model of fibrosarcoma [30]. Our study revealed a significant association between increased JunB expression and decreased tumor stage and a trend towards reduced tumor size. Furthermore, JunB expression was significantly higher in nodal negative tumors compared to nodal positive specimens. These results emphasize the role of JunB as a tumor suppressor by contributing to inhibitory growth signals and less aggressive tumor behavior. A significant negative correlation of high p53 expression and low tumor stage seems to confirm the ubiquitous accepted role for p53 as a tumor-supressive acting protein. The second AP-1 protein, which showed rather weak staining was Fra1. Interestingly, our study revealed a trend ($p = 0.057$) towards dedifferentiation of a tumor and negative or weak Fra1 expression. Recent studies suggest Fra1 to suppress the via c-Jun and c-Fos mediated AP-1 tumor promoting activity, whereas others demonstrated Fra1 to contribute to Ha-Ras induced oncogenic transformation and to increased invasiveness of tumor cells [31-35]. Therefore, the role of Fra1 protein certainly needs to be further elucidated.

Interestingly, with the exception of JunB, no correlation was found between the expression of c-Jun and c-Fos protein family members and metastatic behavior, neither in respect to development of lymph node metastases nor to distant metastases, although several studies have shown an influence of distinct AP-1 proteins, such as c-Fos and c-Jun in tumor invasion and angiogenesis [36, 37]. This may have to do with the ubiquitous expression of AP-1 proteins in tumor tissue.

Conclusions

Taken together these results further confirm the role of JunB as a tumor suppressor protein and suggest a similar role for Fra1. Since AP-1 is suggested to participate in early events during carcinogenesis, it would be an interesting target for prevention of malignant carcinogenesis and perhaps a prognostic factor for clinical outcome. However, before considering AP-1 as a potential therapeutic target, further research is certainly needed, especially investigations that contribute to the functional diversity of these proteins.

References

[1] Angel P., Karin M.: "The role of Jun, Fos and the AP-1 complex in cell proliferation and transformation". *Biochim. Biophys. Acta,* 1991, *1072,* 129.

[2] Chimenov Y., Kerppola T.K.: "Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specifity". *Oncogene*, 2001, *6,* 533.

[3] Shaulian E., Karin M.: "AP-1 as a regulator of cell life and death". *Nature Cell Biol.*, 2002, *4,* E131.

[4] Shaulian E., Karin M.: "AP-1 in cell proliferation and survival". *Oncogene,* 2001, *20,* 2390.

[5] Miller D.A., Curran T., Verma I.M.: "c-Fos protein can induce cellular transformation: a novel mechanism of a cellular oncogene". *Cell,* 1984, *36,* 51.
[6] Maki Y., Bos T., Davis C., Starbuck M., Vogt P.: "Avian sarcoma virus 17 carries the jun oncogene". *Proc. Natl. Acad. Sci. USA*, 1987, *84,* 2848.
[7] Ransone L.J., Verma I.M.: "Nuclear proto-oncogenes fos and jun". *Ann. Rev. Cell Biol.*, 1990, *6*, 539.
[8] Vogt P.K.: "Fortuitous convergences: the beginnings of JUN". *Nature Rev. Cancer,* 2002, *2,* 465.
[9] Eferl R., Wagner E.F.: "AP-1: a double edged sword in tumorigenesis". *Nature Rev. Cancer,* 2003, *3,* 859.
[10] Van Dam H., Castellazzi M.: "Distinct roles of Jun: Fos and Jun: ATF dimers in oncogenesis". *Oncogene,* 2001, *20*, 2453.
[11] Smith L.M., Wise S.C., Hendricks D.T., Sabichi A.L., Bos T., Reddy P. *et al.*: "c-Jun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype". *Oncogene,* 1999, *18,* 6063.
[12] Liu Y., Ludes-Meyers J., Zhang Y., Munoz-Medellin D., Kim H.T., Lu C. *et al.*: "Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth". *Oncogene*, 2002, *21,* 7680.
[13] Marconcini L. *et al.*: "c-Fos-induced growth factor/vascular endothelial growth factor D induces angiogenesis in vivo and in vitro". *Proc. Natl. Acad. USA,* 1999, *96,* 9671.
[14] Chang L., Karin M.: "Mammalian MAP kinase signalling cascades". *Nature,* 2001, *410*, 37.
[15] Schutte J., Viallet J., Nau M., Segal S., Fedorko J., Minna J.: "JunB inhibits and cFos stimulates the transforming and transactivating activities of cJun". *Cell,* 1989, *59*, 987.
[16] Passegue E., Jochum W., Behrens A., Ricci R., Wagner E.F.: "JunB can substitute for Jun in mouse development and cell proliferation". *Nat. Gent.,* 2002, *30,* 158.
[17] Castellazzi M., Spyrou G., La Vista N., Dangy J.P., Piu F., Yaniv M., Brun G.: "Overexpression of cjun, junB and junD affects cell growth differentially". *Proc. Natl. Acad. Sci. USA,* 1991, *88,* 8890.
[18] Agarwal S.K, Novotny E.A., Crabtree J.S., Weitzman J.B., Yaniv M., Burns A.L. *et al.*: "Menin interacts with the AP1 transcription factor JunD and represses JunD activated transcription". *Cell,* 1999, *96,* 143.
[19] Weitzmann, J.B., Flette, L., Matsuo, K., Yaniv, M.: "JunD protects cells from p53 dependent senescence and apoptosis". *Mol. Cell,* 2000, *6,* 1109.
[20] Schreiber M., Kolbus A., Piu F., Szabowski A., Mohle-Steinlein U., Tian J. *et al.*: "Control of cell cycle progression progression by c-jun is p53 dependent". *Genes Dev.,* 1999, *13,* 607.
[21] Remmele W., Schicktanz K.H.: "Immunohistochemical determination of estrogen and progesterone receptor content in human breast cancer. Computer-assisted image analysis (QIC score) vs. subjective grading (IRS)". Pathol. Res. Pract., 1993, 189, 862.
[22] Ludes-Meyers J., Zhang Y., Munoz-Medellin D., Kim H.T., Lu C., Ge G. *et al.*: "AP-1 blockade inhibits the growth of normal and malignant breast cells". *Oncogene,* 2001, *20,* 2771.
[23] Liu Y., Ludes-Meyers J., Zhang Y., Munoz-Medellin D., Kim H.T., Lu C. *et al.*: "Inhibition of AP-1 transcription factor causes blockade of multiple signal transduction pathways and inhibits breast cancer growth". *Oncogene,* 2002, *21,* 7680.
[24] Smith L.M., Birrer M.J., Stampfer M.R., Brown P.H.: "Breast cancer cells have lower activating protein 1 transcription factor activity than normal mammary epithelial cells". *Cancer Res.,* 1997, *57,* 3046.
[25] Tiniakos D.G., Scott L.E., Corbett I.P., Piggott N.H., Horne C.H.: "Studies of c-jun oncogene expression in human breast using a new monoclonal antibody, NCL-DK4". *J. Pathol.,* 1994, *172*, 19.
[26] Linardopoulos S., Malliri A., Pintzas A., Vassilaros S., Tsikkinis A., Spandidos D.A.: "Elevated expression of AP-1 activity in human breast tumors as compared to normal adjacent tissue". *Anticancer Res.,* 1990, *10*, 1711.
[27] Szabo E., Riffe M.E., Steinberg S.M., Birrer M.J., Linnoila R.I.: "Altered cJUN Expression: An early event in human lung carcinogenesis". *Cancer Res.,* 1996, *56,* 305.
[28] Passegue E., Wagner E.F.: "JunB represses cell proliferation by transcriptional activation of p16(INK4a) expression". *EMBO J.,* 2000, *19*, 2969.
[29] Bakiri L., Lallemand D., Bossy-Wetzel E., Yaniv M.: "Cell-cycle dependent variations in c-Jun and JunB phosphorylation: a role in the control of cyclinD expression". *EMBO J.,* 2000, *19*, 5056.
[30] Toft D.J., Rosenberg S.B., Bergers G., Volpert O., Linzer D.I.: "Reactivation of proliferin gene expression is associated with increased angiogenesis in a cell culture model of fibrosarcoma tumor progression". *Proc. Natl. Acad. Sci. USA,* 2001, *98,* 13055.
[31] Yoshioka K., Deng T., Cavigelli M., Karin M.: "Antitumor promotion by phenolic antioxidants: Inhibition of AP-1 activity through induction of Fra1 expression". Proc. Natl. Acad. Sci. USA, 1995, *92,* 4972.
[32] Philips A., Teyssier C., Galtier F., Rivier-Covas C., Rey J.M., Rochefort H., Chalbos D.: "Fra1 Expression level modulates regulation of activator protein-1 activity by estradiol in breast cancer cells". *Mol. Endokrinol.,* 1998, *12,* 973.
[33] Mechta F., Lallemand D., Pfarr C.M., Yaniv M.: "Transformation by ras modifies AP1 composition and activity". *Oncogene*, 1997, *14,* 837.
[34] Kustikova O., Kramerov D., Grigorian M., Berezin V., Bock E., Lukanidin E., Tulchinsky E.: "Fra1 induces morphological transformation and increases in vitro invasiveness and motility of epitheloid adenocarcinoma cells". *Mol. Cell. Biol.,* 1998, *18,* 7095.
[35] Bamberger A.-M. Methner C., Lisboa B.W., Stadtler C., Schulte H.M., Loning T., Milde-Langosch K.: "Expression pattern of the AP-1 family in breast cancer: association of FosB expression with a well-differentiated, receptor-positive phenotype". *Int. J. Cancer,* 1999, *84*, 533.
[36] Hennigan R.F., Hawker K.L., Ozanne B.W.: "Fos-transformation activates genes associated with invasion". *Oncogene,* 1994, *9,* 3591.
[37] Smith L.M., Wise S.C., Hendricks D.T., Sabichi A.L., Bos T., Reddy P., Brown P.H., Birrer M.J.: "cJun overexpression in MCF-7 breast cancer cells produces a tumorigenic, invasive and hormone resistant phenotype". *Oncogene,* 1999, *18,* 6063.

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