Reduced Nuclear Expression of Transcription Factor AP-2 Associates with Aggressive Breast Cancer[1]

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ABSTRACT

Purpose: **We proposed to investigate the expression and prognostic significance of activator protein 2 (AP-2) in breast cancer.**

Experimental Design: **AP-2 was immunohistochemically analyzed in a prospective, consecutive series of 420 breast cancer patients diagnosed and treated between 1990 and 1995 at Kuopio University Hospital, Kuopio, Finland. AP-2 expression was further compared with clinicopathological parameters and patients' survival.**

Results: **Nuclear AP-2 expression was lower in carcinomas compared with normal ductal breast epithelium ($P < 0.001$). Nuclear expression was more often seen in lobular than in ductal or other carcinomas ($P = 0.048$). Cytoplasmic staining was present in 47% of the carcinomas. Low nuclear AP-2 expression level in carcinomas was associated with advanced stage ($P = 0.002$), axillary lymph node positivity ($P = 0.012$), poor differentiation ($P = 0.001$), and recurrences ($P = 0.003$). In univariate survival analyses, low nuclear AP-2 expression ($P = 0.0028$), advanced stage ($P < 0.0001$), lymph node metastases ($P < 0.0001$), and poor differentiation ($P = 0.0498$) predicted shorter recurrence-free survival (RFS). Low nuclear AP-2 staining and/or shift to cytoplasmic expression predicted shorter RFS ($P = 0.0050$) and breast cancer-related survival (BCRS; $P = 0.0314$) in univariate analyses. Cytoplasmic expression alone did not have prognostic value. In multivariate analysis, low nuclear AP-2 expression ($P = 0.0292$) and advanced stage ($P = 0.0001$) were independent predictors of shorter RFS; and stage ($P < 0.0001$) and ER-status ($P = 0.0321$) independently predicted BCRS. In the lymph-node positive patients, RFS was independently predicted by stage ($P = 0.0110$) and nuclear AP-2 status ($P = 0.0151$).**

Conclusions: **AP-2 seems to have a protective role in breast cancer. Low nuclear AP-2 expression was associated with disease progression and increased metastatic capability of the tumor. In addition, reduced nuclear AP-2 expression independently predicted elevated risk of recurrent disease in breast cancer.**

INTRODUCTION

AP-2[3] is a DNA-binding transcription factor (1) that is known to exhibit both activating and repressing effects on target genes (2–6). The protein consists of three highly homologous members, namely AP-2α, AP-2β, and AP-2γ (7, 8), the expression of which is cell-type specific (9, 10). Each of the proteins is encoded by a separate gene (11, 12). AP-2 homo- and heterodimers can activate transcription via GC-rich DNA sequences (1, 7, 13), which is an event modified by several factors (14–19). AP-2 proteins play an important role as regulators of gene expression in development, cell growth, cell differentiation and apoptosis (reviewed in Ref. 20).

Alterations in AP-2 function have been linked with malignant phenotype, and, recently, *AP-2* has been suggested to be a new tumor suppressor gene (4, 21, 22). The tumor suppressive properties are, at least partly, mediated by p21/WAF1 (6). In breast cancer, as well as in some other cancers, reduced AP-2 expression *in vivo* has been associated with disease progression (22–27). In addition, AP-2 seems to be involved in the regulation of genes altered in breast cancer such as *ER* (28) and HER-2/*neu* (7, 29), and, thus, it may have a crucial role in the development and prognosis of breast cancer. However, the function of AP-2 in breast cancer is still unclear, and the prognostic value of the protein has not been assessed thus far.

This large prospective study consisted of 420 breast cancer patients uniformly diagnosed and treated between 1990 and 1995 at Kuopio University Hospital, Kuopio, Finland. The mean follow-up time of the patients was 55.0 months. To investigate the value of AP-2 as a prognostic factor, we analyzed by immunohistochemistry the relationships between AP-2 expression and clinicopathological parameters and patients' survival.

PATIENTS AND METHODS

Patients. The present prospective study consisted of 520 breast cancer patients of the Kuopio Breast Cancer Project (30–33). All of the women with a susceptible breast lump in the project catchment area between April 1990 and December 1995

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[3] The abbreviations used are: AP-2, activator protein 2; BCRS, breast cancer-related survival; RFS, recurrence-free survival; ER, estrogen receptor; PR, progesterone receptor.

were invited to Kuopio University Hospital for further examinations and final diagnosis. Women willing to participate in the project were interviewed and examined by a trained nurse before any diagnostic procedures. The participation rate of the patients with diagnosed breast cancer was 98%. A healthy population control matched by age (±5 years) was drawn for each patient from the Finnish National Population Register covering the same catchment area. Altogether, 479 invasive and 41 noninvasive carcinomas were included in the project. After surgical treatment, the patients were offered adjuvant chemotherapy and/or hormonal therapy and radiotherapy depending on the mode of the surgery, the patient's menopausal status, and the stage of the disease, according to the national guidelines (34). The stage was assessed using UICC classification (35). Patients with noninvasive carcinomas, earlier breast cancer, metastatic disease, or insufficient tumor material were excluded from the present study. Thus, 420 patients with sufficient primary tumors and complete clinical histories were included in the study. The mean age of the 420 patients was 59.1 years and the median 56.7 years (range, 23.3–91.6 years). The mean follow-up time was 55.0 months and the median, 57.3 months (range, 1.2–115.1 months). During the first 5 years of follow-up, 76 patients (18%) had a recurrence, 50 patients (12%) died of breast cancer and 37 patients (9%) died of other causes. The overall 5-year survival rate was 76%. The 5-year RFS rate of the patients was 79% and the BCRS, 85%. The 5-year survival of excluded stage IV patients ($n = 17$) was 24%. The clinicopathological data of the patients are summarized in Table 1.

Table 1 The clinicopathological data of the patients

Characteristic		*n*	%
No. of patients		420	100
Age, yr			
Mean (range)	59.1 (23.3–91.6)		
Tumor size			
T_1		218	52
T_2		168	40
T_3		24	6
T_4		10	2
Lymph node status			
Negative		244	58
Positive		168	40
Unknown		8	2
Stage			
I		160	38
II		213	51
III		39	9
Unknown		8	2
Histological type			
Ductal		270	64
Lobular		68	16
Other		82	20
Histological grade			
I		110	26
II		192	46
III		118	28
ER status			
Positive		326	78
Negative		93	22
Data not available		1	<1
PR status			
Positive		261	62
Negative		157	37
Data not available		2	1
Menopausal status			
Premenopausal		131	31
Postmenopausal		289	69
Recurrence at 5 yr			
No		344	82
Yes		76	18
Cause of death at 5 yr			
Alive		333	79
Breast cancer		50	12
Other		37	9

Histology. The tumor samples were fixed in 10% buffered formalin and embedded in paraffin. The histological diagnosis was confirmed by reviewing one to four original sections of the primary tumor. All of the tumors were simultaneously re-evaluated for histological type and grade by two senior pathologists, who were unaware of the clinical data. The most representative blocks were selected to be cut into new 5-μm thick sections for immunohistochemical analyses.

Immunohistochemistry. The sections were deparaffinized in xylene, rehydrated in ethanol, and washed twice with distilled water. For better antigen retrieval, the samples were boiled in a microwave oven for three times for 5 min in a citrate buffer (pH 6.0). Endogenous peroxidases were blocked by 5% hydrogen peroxidase treatment for 5 min. The samples were washed with PBS (pH 7.2) and incubated in 1.5% normal goat serum for 25 min to prevent nonspecific antigen binding. The samples were incubated with the primary antibody, a rabbit polyclonal antibody for human AP-2α (C-18, specific for AP-2α, AP-2β, and AP-2γ; Santa Cruz Biotechnology 2002, Santa Cruz, CA) used at a working dilution of 1:2000 overnight at 4°C. Before applying the secondary antibody, the samples were washed twice with PBS. The slides were incubated for 35 min with the biotinylated secondary antibody, followed by a wash and 45-min incubation in an avidin-biotinylated peroxidase complex-reagent (Vectastain Rabbit ABC Elite kit; Vector Laboratories, Burlingame, CA). AP-2 expression was visualized with a 5-min diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) treatment. The slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted with DePex (BDH Ltd, Poole, United Kingdom). Each staining series had positive and negative control slides (Fig. 1, *F1* and *F2*), which stained as expected. In addition, strongly positive inflammatory cells in the tumors served as internal controls.

Western Blot. During the surgery a fresh breast cancer specimen was obtained from some of the patients. Immediately after resection, the samples were covered with OCT, cooled in liquid isopentane, cooled in liquid nitrogen, and re-stored at −70°C until analysis. Four tumor samples with high AP-2 expression in immunohistochemical staining were selected for Western blot analyses. The same antibody as in immunohistochemistry was used to detect AP-2.

The frozen tissue samples were homogenized, incubated on ice for 30 min, and centrifuged at 13,000 rpm for 15 min at 4°C. Homogenization was performed using lysis homogenization buffer [0.02 M Tris-HCl, 0.002 M EDTA, 0.1 M NaCl (pH 8.0)] with protease inhibitors (1 mmol VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mmol phenylmethylsulfonyl fluoride). After centrifugation, the samples were either refrigerated and stored at −70°C until analysis or prepared immediately for gel

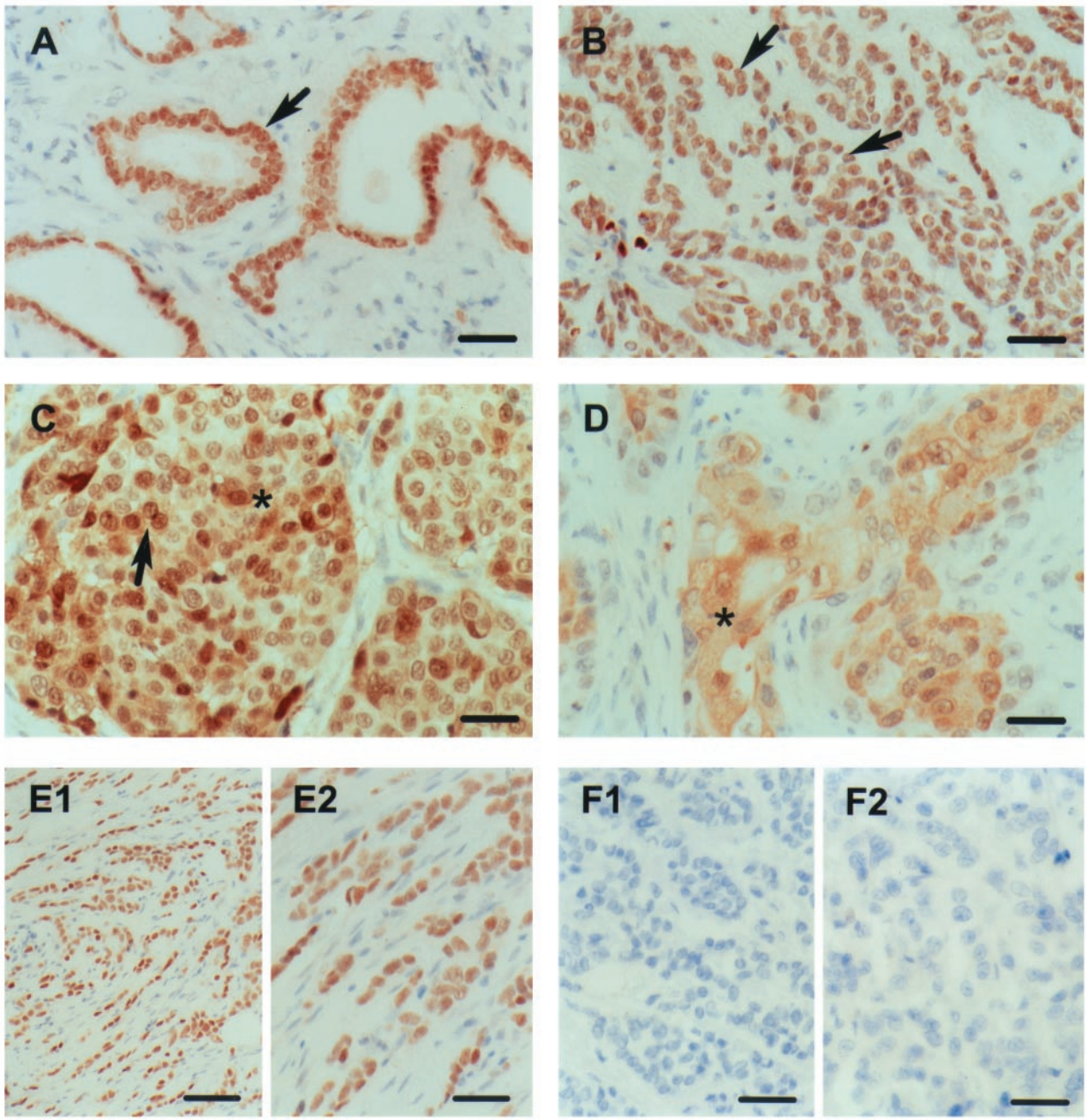

Fig. 1 *A*, ductal carcinoma, grade I. Nuclear staining (*arrow*). *B*, strong nuclear (*arrows*) AP-2 staining in ductal carcinoma, grade II. *C*, ductal carcinoma, grade III. Nuclear (*arrow*) and cytoplasmic (*asterisk*) expressions. *D*, cytoplasmic expression (*asterisk*) with a few positive nuclei in ductal carcinoma, grade III. *E1–E2*, lobular carcinoma, nuclear staining. *F1–F2*, negative control slides of samples *B* and *C*, respectively. *A–D*, *E2–F2*: *bar*, 40 μm; *E1*: *bar*, 80 μm.

transfer. In the latter case, samples were heated at 100°C for 5 min with equal amount of SDS sample buffer [0.125 M Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol (pH 6.8)]. Next, the centrifuged samples were separated on an SDS-polyacrylamide gel (100 V for 3 h at room temperature) and transferred onto a nitrocellulose membrane (Hybond ECL nitrocellulose membrane; Amersham Pharmacia Biotech United Kingdom Limited, Little Chalfant, Buckinghamshire, United Kingdom) by electrophoresis (100 V for 2 h at 4°C). Filters were blocked with 0.1% soy buffer solution [0.137 M NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 0.1% Tween, and 0.1% soy (pH 7.4)] and then incubated with the primary antibody diluted 1:5000 for 30 min. After a wash with PBS-Tween buffer, the horseradish peroxidase-conjugated secondary anti-

Table 2 Relationships between nuclear AP-2 expression and clinicopathological variables

Variable	Sample *n*	AP-2 expression (%) Low[a]	AP-2 expression (%) High[a]	χ^2	df	*P*
Stage	412			12.4	2	0.002
I	160	39	61			
II	213	57	43			
III	39	59	41			
Lymph node status	412			6.4	1	0.012
Negative	244	45	55			
Positive	168	58	42			
Histological type	420			6.1	2	0.048
Ductal	270	52	48			
Lobular	68	37	63			
Other	82	55	45			
Histological grade	420			14.1	2	0.001
I	110	39	61			
II	192	48	52			
III	118	64	36			
ER-status	419			<0.1	1	0.927
Positive	326	50	50			
Negative	93	51	49			
PR-status	418			<0.1	1	0.860
Positive	261	51	49			
Negative	157	50	50			
Recurrence at 5 yr	420			9.0	1	0.003
No	344	47	53			
Yes	76	66	34			

[a] Median as a cutoff value.

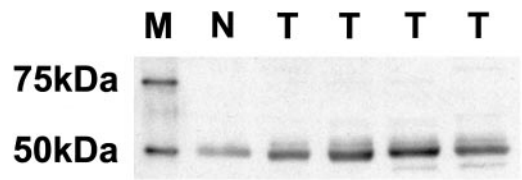

Fig. 2 Western blot analysis for AP-2. Four representative AP-2-positive tumor samples (*T*) and one benign AP-2 positive breast sample (*N*) were included in the analysis. All of the samples showed a band M_r about 50,000, which is the same as given for AP-2α. Trial Mix Protein marker (*M*) was used as a size marker. *kDa*, M_r in thousands.

body (Santa Cruz Biotechnology) was applied onto the filter diluted 1:12,000 and was incubated for 45min. After a wash, the signals were visualized using a commercial substrate kit (Supersignal West Pico Trial kit; Pierce, Perbio Science Deutschland GmbH, Bonn, Germany). A prestained marker (Bio-Rad Laboratories, Hercules, CA) and Trial Mix marker (Novagen, CN Bioscience, Ltd., Beeston, Nottingham, United Kingdom) were run in parallel to the samples. A benign breast tissue sample served as a positive control.

Scoring of Immunoreactivity. The specimens were analyzed by three observers (J. P., K. R., V-M. K.) unaware of the patients' clinical outcome. Discrepancies between the observers were found in less than 10% of the slides examined, and consensus was reached on a further review. All of the tumor cells with detectable nuclear staining were considered as positive. For statistical analyses, the percentage distribution of stained tumor cell nuclei in the sample was divided into low (<80%) or high (≥80%) expression groups using the median as a cutoff value. Other cutoff values were also tested (*e.g.*, mean, and values between the 40th and 50th percentiles), but the median value was chosen because it does not introduce a bias that could be caused by the use of a minimum *P* approach (36). Cytoplasmic expression was considered positive if >10% of cells in the tumor area were stained.

Statistical Analyses. The statistical analyses were carried out by using the SPSS for Windows 9.0 program (SPSS Inc., Chicago, IL). Differences between benign and malignant tissues were examined with a nonparametric Mann-Whitney U test, in which the variables were considered as continuous. The associations between AP-2 immunohistochemistry and clinicopathological parameters were tested with contingency tables and a χ^2 test. The univariate survival analyses were performed using the Kaplan Meier's log-rank analysis, and the independent prognostic value of variables was further examined with Cox's regression analysis. *P*s ≤ 0.05 were considered as significant in the analyses. In the Cox's multivariate analysis, the Enter-method was used with an additional removal limit of $P < 0.10$. Both BCRS and RFS were examined. The recurrence-free time was defined as a time between the diagnosis and the date of the first local recurrence or a distant metastasis, whichever appeared first. Patients who remained healthy or died without breast cancer during the follow-up were censored at the time of the last control examination or death.

RESULTS

AP-2 Expression. To compare the expression of AP-2 in malignant tumors and in benign ductal breast epithelium, an additional set of 24 samples containing benign epithelium were stained for AP-2. The nuclear expression was significantly re-

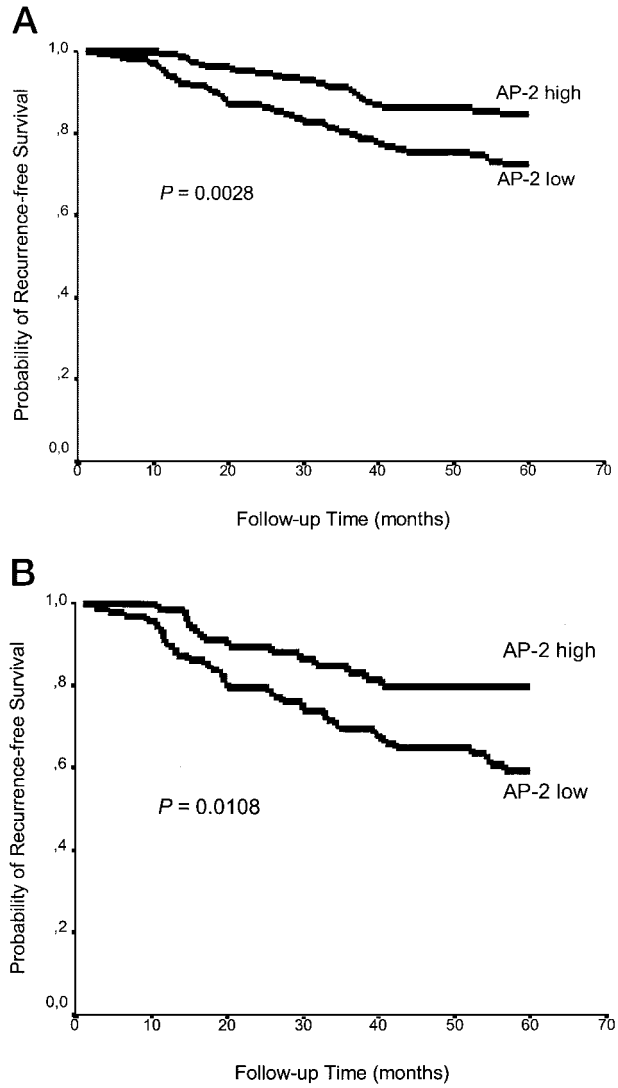

Fig. 3 The *P* of RFS according to nuclear AP-2 expression (median as a cutoff value). *A*, in all of the patients; *B*, in the lymph node-positive patients.

duced in carcinomas ($P < 0.001$) compared with that in benign breast epithelium. The staining pattern for AP-2 in carcinomas was mainly nuclear (Fig. 1, *A* and *B*) although cytoplasmic expression was detectable in 47% of the cases (Fig. 1, *C* and *D*). Lobular carcinomas expressed more often AP-2-positive nuclear staining (63% of the cases; Fig. 1, *E1* and *E2*) than ductal (48% of the cases; Fig. 1, *A–C*) or other types of (45% of the cases) carcinomas ($P = 0.048$; Table 2).

Western Blot Analysis. In Western blotting, the primary antibody recognized a protein M_r of about 50,000, which has been reported to be the size of AP-2α. The results of Western blotting are shown in Fig. 2.

Relationship of AP-2 Expression to the Clinicopathological Data. Low nuclear AP-2 expression was significantly associated with advanced stage ($P = 0.002$), axillary lymph node positivity ($P = 0.012$), poor differentiation of the tumor ($P = 0.001$), and recurrences ($P = 0.003$). Nuclear AP-2 expression was not associated with ER and PR receptor status. The results are summarized in Table 2. Cytoplasmic positivity, instead, was more often present in ER- ($P = 0.005$) and PR- ($P = 0.043$) negative tumors and was, in addition, associated with ductal carcinoma type ($P = 0.001$) and poor differentiation ($P = 0.012$).

Univariate Survival Analysis. In univariate analyses, low nuclear AP-2 expression was a significant predictor of shorter RFS (Fig. 3*A*). The 5-year RFS in patients with high nuclear AP-2 expression was 85% compared with 73% in the low expressing group ($P = 0.0028$). Similarly, in patients with axillary lymph node metastases, low nuclear AP-2 expression was related to shorter RFS ($P = 0.0108$; Fig. 3*B*). A nonsignificant trend was observed between reduced nuclear AP-2 expression and poor BCRS ($P = 0.0964$). The other prognostic factors of shorter RFS and BCRS were advanced stage ($P < 0.0001$ for both), lymph node positivity ($P < 0.0001$ for both), and poor differentiation ($P = 0.0314$ for BCRS and $P = 0.0498$ for RFS; Table 3). ER ($P < 0.0001$) and PR ($P = 0.0012$) negativity were also related to poor BCRS (Table 3).

For further evaluation, nuclear AP-2 staining was combined with cytoplasmic expression of the protein. Interestingly, not only low nuclear expression of AP-2 but also cytoplasmic involvement was associated with decreased survival when compared with high, strictly nuclear AP-2 expression. High nuclear expression without cytoplasmic involvement predicted longer RFS ($P = 0.0050$; Fig. 4*A*) and BCRS ($P = 0.0314$) in the whole cohort and in the lymph node-positive patients [$P = 0.0030$ for RFS (Fig. 4*B*), and $P = 0.0411$ for BCRS]. Cytoplasmic expression alone did not have prognostic value for BCRS or RFS.

Multivariate Survival Analysis. Complete data were available for 409 patients for recurrence-free and 403 for BCRS analyses. The Cox's multivariate analyses included statistically significant variables (stage, histological grade, and AP-2 expression in RFS analysis; stage, histological grade, and ER- and PR-status in BCRS analysis) derived from the univariate analyses. The independent predictors of short RFS were low nuclear AP-2 expression ($P = 0.0292$) and advanced stage ($P = 0.0001$). The BCRS was independently predicted by stage ($P < 0.0001$) and ER status ($P = 0.0321$). Similarly, in the lymph node-positive patients ($n = 166$), RFS was independently predicted by stage ($P = 0.0110$) and nuclear AP-2 status ($P = 0.0151$), which were, in addition, the only significant variables derived from univariate survival analysis in this group. The results are presented in Table 4. In the lymph node-negative patients ($n = 244$), the number of events was too low (27 recurrences and 14 breast cancer deaths) to have statistical power in the survival analysis.

DISCUSSION

AP-2 is a tissue type-specific transcription factor hypothesized to be involved in the development of breast cancer (7, 29, 37, 38). Previously, AP-2 has been reported to possess tumor suppressive properties in breast cancer (24). Until now, however, the prognostic significance of AP-2 has not been defined. We demonstrate here, in a large prospective series of breast

Table 3 Statistically significant variables of BCRS and RFS in univariate analysis

Variable	Surviving breast cancer at 5 years			Recurrence-free at 5 yr		
	n	%	*P*	*n*	%	*P*
Stage	412		<0.0001	412		<0.0001
I	160	92		160	86	
II	213	86		213	77	
III	39	55		39	55	
Lymph node status	412		<0.0001	412		<0.0001
Negative	244	92		244	86	
Positive	168	77		168	68	
Histological grade	420		0.0314	420		0.0498
I	110	92		110	85	
II	192	85		192	78	
III	118	80		118	73	
ER status	419		<0.0001	419		0.0910
Positive	326	89		326	80	
Negative	93	71		93	74	
PR status	418		0.0012	418		0.1884
Positive	261	89		261	80	
Negative	157	78		157	76	
Nuclear AP-2	420		0.0964	420		0.0028
Low < 80%	211	83		211	73	
High ≥ 80%	209	88		209	85	

cancer patients, that low nuclear AP-2 expression associates with disease progression and increased metastatic capability of the tumor cells and, in addition, independently predicts increased risk of recurrent disease in breast cancer. Still, however, the traditional prognostic factors such as stage, lymph node status, and ER status remained the strongest predictors of RFS and BCRS in the survival analyses.

To divide the continuous values of nuclear AP-2 staining into two expression categories, we chose the median as the cutoff value. Similarly, Gee *et al.* (24) have used median value as a cutoff for AP-2 proteins in breast cancer. The benefit of using median is that it divides the material into two equal-sized groups and can be used without introducing a statistical bias (36). Dichotomizing continuous variables concerning biological phenomena into different groups according to a single more-or-less arbitrary percentile may be artificial and may discard important relationships. In addition, in benign breast epithelia, the AP-2 expression level was predominantly high (*i.e.*, >80% of the cells stained positive). Similar results were also obtained when the mean value (40th percentile) and values between the 40th and the 50th percentile were used as a cutoff, thus confirming the biological reliability of the used cutoff and the value of the results (data not shown).

The present study strengthens the theory that AP-2 may act as a tumor suppressor in breast cancer, because low nuclear expression was associated with tumor progression and metastatic behavior of the disease. Patients with low AP-2 positivity had poorly differentiated tumor, lymph node metastases, and recurrences more often than patients expressing AP-2 highly. This is well in line with the results of Gee *et al.* (24), who described a connection between high AP-2 positivity and high differentiation in breast cancer, including a low mitotic count and low (I-II) grade. In other malignancies, similar findings have been found, also (22, 23, 25, 27). Accordingly, it seems obvious that functional AP-2 protein is needed to maintain cellular growth balance.

Importantly, we show here that a reduction of nuclear AP-2 independently indicated poor RFS. The 5-year RFS rates were 85 and 73% in the AP-2 high and low expressing groups, respectively. The difference was even greater for lymph node-positive patients whose 5-year RFS decreased from 80 to 60% in the AP-2-low-expressing group. This result is supported by previous studies done with smaller breast cancer materials, in which nonsignificant trends between high nuclear AP-2 expression and better prognoses have existed (24, 38). A similar association was found in a study of cutaneous malignant melanoma, in which the loss of nuclear AP-2 was associated with malignant transformation and tumor progression *in vivo* and independently predicted shorter RFS (22). In experiments done with the highly metastatic A375SM melanoma cells, re-expression of AP-2 was observed to decrease the tumorigenicity and the metastatic potential of the cells in nude mice (4). In addition, nuclear AP-2 expression has also been shown to decrease in poorly differentiated cervical intraepithelial neoplasms (25).

Interestingly, in our series, a shift from nuclear into cytoplasmic expression of AP-2 was observed along increasing cellular atypia. This finding supports the value of AP-2 for normal growth because the phenomenon impaired the patients' prognosis compared with those with a strictly nuclear expression of the protein. Both RFS and BCRS were shorter for all of the patients and for lymph node-positive patients if the nuclear AP-2 expression was low and/or the protein was present in the cytoplasm. However, cytoplasmic expression alone did not have prognostic value. Because nuclear localization of a transcription factor is essential for its function, probably both the reduced nuclear AP-2 expression and the accumulation in the cytoplasm indicate less functionally active protein. In cultured keratinocytes, AP-2α expression was nuclear in basal cells and cytoplasmic in the superficial cell layers, which indicated a down-regulation in the function of AP-2α by the relocalization of the protein to the cytoplasmic compartment (39). In addition, the accumulation of AP-2α in the nucleus was seen after a medium

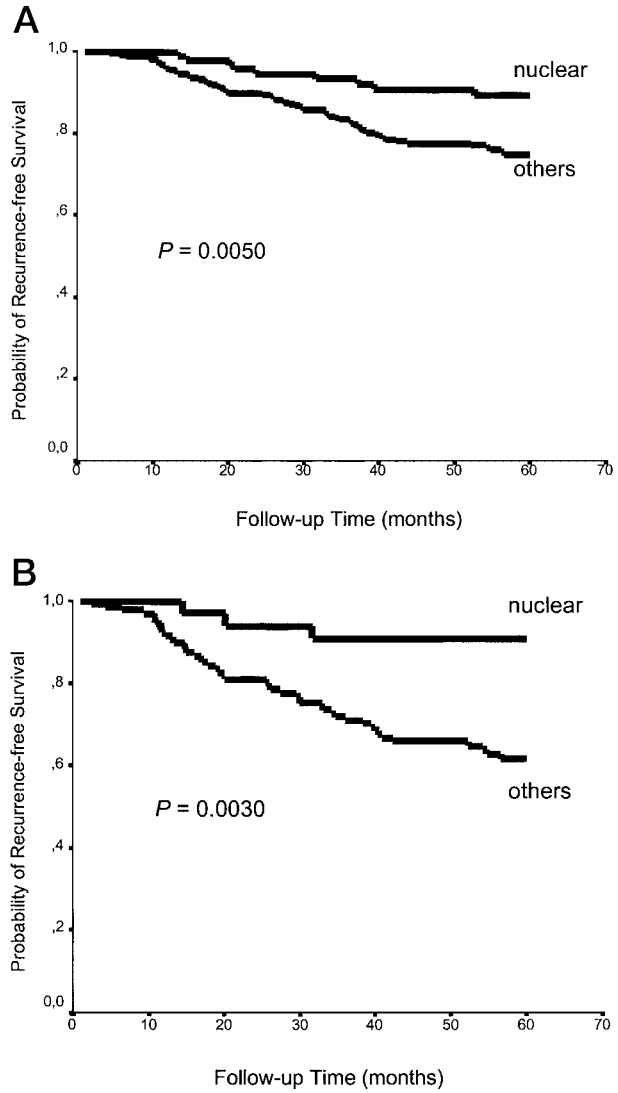

Fig. 4 Probability of RFS according to combined (nuclear/cytoplasmic) expression of AP-2. The group "*nuclear*" contains those patients with high nuclear AP-2 expression without cytoplasmic involvement. The group titled as "*others*" contains all of the patients with low nuclear AP-2 expression and those with positive cytoplasmic staining. *A*, all of the patients; *B*, the lymph node-positive patients.

Table 4 Results of Cox's multivariate analysis for BCRS and RFS

Category (variable)	β (SE)	Relative risk (95% CI)[a]	*P*
BCRS			
Stage			<0.0001
I	Reference		
II	0.88 (0.41)	2.41 (1.08–5.39)	0.0323
III	2.25 (0.45)	9.45 (3.92–22.77)	<0.0001
ER status			
Negative	0.87 (0.41)	2.39 (1.08–5.32)	0.0321
Positive	Reference		
RFS			
Stage			0.0001
I	Reference		
II	0.56 (0.29)	1.74 (0.99–3.08)	0.0560
III	1.53 (0.36)	4.60 (2.26–9.37)	<0.0001
Nuclear AP-2			
Low < 80%	0.54 (0.25)	1.71 (1.06–2.78)	0.0292
High ≥ 80%	Reference		
RFS in the lymph node-positive patients			
Stage			
II	Reference		
III	0.78 (0.31)	2.17 (1.19–3.95)	0.0110
Nuclear AP-2			
Low < 80%	0.79 (0.32)	2.20 (1.16–4.14)	0.0151
High ≥ 80%	Reference		

[a] CI, confidence interval.

change, which, thus, suggests that the cells restored AP-2α transcriptional activity by redistributing the protein to the nucleus from the cytoplasm (39).

In previous studies, the expression pattern of AP-2 proteins has been mainly nuclear in breast cancer (24, 38). However, both nuclear and cytoplasmic AP-2 expression has been previously described in several malignancies (22, 23, 25, 26, 40, 41). Three of these studies examined the prognostic value of cytoplasmic AP-2 expression. Indeed, in ovarian cancer, high cytoplasmic AP-2α expression was a favorable sign, whereas nuclear expression with low cytoplasmic expression increased the risk of dying of ovarian cancer (23). However, in colorectal and prostate carcinomas, cytoplasmic AP-2 had no prognostic value (26, 41). In breast cancer, the combining of cytoplasmic and nuclear AP-2 expressions may provide important additional information on the prognosis and behavior of the disease.

AP-2 proteins can activate target genes but, on the other hand, are also known to negatively regulate several genes (2, 4, 5, 42). The growth-repressive properties of AP-2 are at least partly mediated by cell cycle inhibitor p21/WAF1 (6). In SB-2 melanoma cells, the inactivation of AP-2 by a dominant-negative AP-2 caused an increase in matrix metalloproteinase-2 expression, microvessel density, and angiogenesis *in vivo* (43). In breast cancer, the tumors' malignant potential that was gained by a loss of AP-2 may be explained by the reduced suppression of growth-inducing genes and/or by the insufficient activation of growth-suppressing genes. Even though the possible consequences of decreasing the amounts of functional AP-2 to other cellular factors were not investigated in this study, the large number of uniformly treated patients strengthens the value of the current findings and significantly increases the current knowledge in this area.

In the present study, we failed to statistically demonstrate the previously reported association between nuclear AP-2 expression and ER positivity *in vivo* (24, 38). One reason may be that the connection between AP-2 and ER has never been studied in a large clinical material before. On the other hand, the antibody used in the present study recognizes all of the three AP-2 proteins, thus reducing the value of possible association between certain AP-2 isoforms and ER. It is also possible that AP-2 is not essential for ER transcription *in vivo*, even though both AP-2α and AP-2γ have been reported to activate ER promoter in an ER-negative cell line (28). The increased cytoplasmic AP-2 expression in ER-negative carcinomas supports the role of functional AP-2 in ER regulation. On the other hand, this connection could be secondary because of the cells' poor differentiation. Finally, AP-2γ has been identified as an impor-

tant regulatory element of ER in hormone-sensitive breast cancer (37, 44). Therefore, the role of different AP-2 proteins in ER regulation *in vivo* needs to be investigated.

As a conclusion, we state that low nuclear AP-2 expression independently predicted shorter RFS for all of the patients and for lymph node-positive patients. AP-2 expression status may be a useful tool in selecting lymph node-positive patients with increased risk of recurrence. However, the molecular mechanisms involved in the regulation of AP-2 and the events leading to decreasing amounts of the protein in breast cancer remain to be investigated. Understanding of the function of AP-2 in malignant transformation may reveal new potential prospects in the management of breast cancer.

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