

Distinct spatial expression patterns of AP-2alpha and AP-2gamma in non-neoplastic human breast and breast cancer

Nicolaus Friedrichs¹, Richard Jäger², Ellen Paggen¹, Christian Rudlowski³, Sabine Merkelbach-Bruse¹, Hubert Schorle² and Reinhard Buettner¹

¹Institute of Pathology; ²Department of Developmental Pathology, Institute of Pathology, University of Bonn Medical School, Bonn, Germany and ³Department of Gynecology and Obstetrics, University Hospital of Heidelberg, Heidelberg, Germany

Although transcription factors AP-2alpha and AP-2gamma have been implicated in the control of estrogen receptor (ER) and ErbB-2, their impact for breast cancer is still controversial. To better understand the role of AP-2 proteins in mammary neoplasia, the analysis of their spatial expression pattern in normal breast and breast cancer is required. A total of 51 specimens of female breast cancer patients and a tissue microarray containing 93 additional female breast cancer cases were immunohistochemically stained for AP-2alpha, AP-2gamma, ER and ErbB-2. In 70 cases of the tissue microarray, survival data comprising a period of up to 30 years were present. In non-neoplastic breast tissue, AP-2alpha was expressed in the inner glandular cell layer while AP-2gamma was expressed in the outer myoepithelial cell layer. Ductal carcinoma *in situ* revealed strongly AP-2alpha-positive tumor cells surrounded by a layer of AP-2gamma-positive myoepithelial cells. In invasive carcinoma, expression of AP-2alpha and AP-2gamma was variable. High expression of ER and AP-2alpha showed better survival rates than low expression of these markers. AP-2gamma expression had no effect on survival. These results for the first time reveal a distinct spatial expression pattern of AP-2alpha and AP-2gamma in normal breast and in ductal carcinoma *in situ* with specific AP-2gamma expression in myoepithelium. High ER and AP-2alpha expression in invasive breast cancer showed favorable survival rates. Therefore, AP-2alpha expression seems to be associated with better prognosis of breast cancer. AP-2gamma expression has no influence on survival reflecting that myoepithelial cells are not involved in the neoplastic process.

Modern Pathology (2005) 18, 431–438, advance online publication, 1 October 2004; doi:10.1038/modpathol.3800292

Keywords: AP-2; transcription factor; immunohistochemistry; breast cancer; tumorigenesis

The AP-2 family of transcription factors consists of five homologous 50 kDa proteins, AP-2alpha, AP-2beta, AP-2gamma, AP-2delta,¹ and AP-2epsilon,² which are encoded by separate genes (reviewed in Hilger-Eversheim *et al*³). These transcription factors homo- or heterodimerize via helix-span-helix motifs and transactivate their target genes by binding to GC-rich consensus sequences in the respective promoter regions.^{4,5} AP-2 proteins have been implicated in the regulation of cell type-specific processes based on their expression in neural, neural crest, kidney and epithelial cells during

development. In the adult mammary gland, expression of AP-2alpha, AP-2beta, and AP-2gamma has been documented.^{6,7} Although highlighting the importance of AP-2 genes, the embryonic lethal phenotypes of mice lacking AP-2alpha, AP-2beta, or AP-2gamma^{8–11} have so far precluded the analysis of their role in mammary development and function. Recently published gene-overexpression studies in transgenic mice suggest that both AP-2alpha and AP-2gamma may control proliferation, apoptosis and differentiation of mammary epithelial cells.^{7,12} Disturbances of these processes may lead to mammary carcinoma. In fact, AP-2 transcription factors have been implicated in human breast cancer. Several studies have linked AP-2gamma expression with the expression of the receptor tyrosine kinase ErbB-2 (encoded by the *HER-2/c-neu* proto-oncogene),^{13,14} representing a clinical marker for poor prognosis in breast carcinoma.¹⁵ In mammary tumor

Correspondence: Dr N Friedrichs, MD, Institute of Pathology, University of Bonn Medical School, Sigmund-Freud-Strasse 25, 53127 Bonn, Germany.
E-mail: Nicolaus.Friedrichs@ukb.uni-bonn.de
Received 24 May 2004; revised and accepted 30 June 2004; published online 1 October 2004

cell lines, high expression of AP-2alpha and AP-2gamma correlated with the expression of the *ErbB-2* gene; moreover, the *ErbB-2* promoter was shown to contain AP-2 binding sites and was transactivated by AP-2 proteins in cell transfection studies.¹⁴ Furthermore, the *AP-2gamma* gene maps at the genomic locus 20q13.2, which is frequently amplified in breast cancer cell lines and breast carcinomas.¹⁶ Immunohistochemical analyses revealed a correlation between AP-2alpha and AP-2gamma expression and ErbB-2 expression in breast tumor specimens, whereas AP-2alpha alone was associated with positivity for estrogen receptor (ER) expression, a clinical marker for a favorable prognosis.⁶ The *estrogen receptor* gene had previously been shown to be controlled by AP-2.¹⁷ These results, however, were questioned by a different study, which found AP-2 expression to be higher in normal breast epithelia than in invasive tumors,¹⁸ confirming a previous study, which had suggested that AP-2 might represent a novel tumor-suppressor gene because AP-2alpha/beta inversely correlated with ErbB-2 in invasive breast carcinoma.¹⁹ To our knowledge, none of the studies addressed the spatial expression patterns of the different AP-2 isoforms on either normal breast tissue or breast tumor specimens. However, this issue may be of crucial importance to resolve the controversial findings concerning the roles of AP-2 proteins in breast cancer. We therefore analyzed the spatial distribution of AP-2alpha and AP-2gamma in breast tumor specimens using specific antibodies. Our results for the first time show distinct spatial patterns of these AP-2 isoforms in non-neoplastic breast epithelia and breast tumors.

Material and methods

Patients

Samples from 51 female patients treated for invasive breast cancer in 1999 to 2002 were analyzed in the Department of Pathology, University of Bonn Medical School, Germany. The study collective has been intensively characterized previously with respect to tumor stage, tumor grade and HER2/neu status.²⁰

In addition, a tissue microarray containing 93 additional cases collected at the Department of Gynecology and Obstetrics, University of Heidelberg, Germany was analyzed. To account intratumoral heterogeneity two tissue samples per case have been mounted on the tissue microarray. Therefore, a total number of 186 staining intensity values have been analyzed. A total of 70 cases on this tissue microarray included detailed follow-up data comprising a period of up to 30 years.

Combining both patient collectives, 144 patients have been evaluated concerning AP-2alpha-, AP-2gamma-, ER- and ErbB-2-(HER2/neu) staining intensities.

Hematoxylin–eosin (H&E) staining was performed for routine pathological evaluation followed by staining and subsequent evaluation of AP-2alpha, AP-2gamma, ER, ErbB-2, cytokeratin 18 and smooth muscle actin.

Immunohistochemistry

Staining procedure for AP-2alpha and AP-2gamma
Tissue sections were incubated overnight at 60°C, deparaffinized for 2 × 10 min in xylene followed by 5 min in 100% ethanol and 20 min in methanol/H₂O₂. Then slides were put for 5 min in 96% and subsequently in 70% ethanol and for another 5 min in distilled water. For better antigen retrieval, samples were boiled in a microwave oven in citrate buffer pH 6.0 (AP-2alpha 5 × 5 min and AP-2gamma 3 × 5 min). Then samples were washed in PBS for 5 min and incubated for 15 min in 10% normal horse-serum (Vector Laboratories, Burlingame, USA). The samples were incubated for 1 h at 37°C with rabbit anti-human AP-2alpha antibody (Cat. No.: 1600003, Geneka Biotechnology Inc., USA) used at a dilution of 1:200 or with rabbit anti-human AP-2gamma antibody (H-77, Santa Cruz, USA) at a dilution of 1:50, respectively. The samples were washed twice for 5 min in PBS and then incubated at 20°C for 30 min with the biotinylated secondary antibody in an avidin-biotinylated peroxidase complex-reagent (Vectastain Rabbit ABC elite kit; Vector Laboratories), followed by washing in PBS and subsequent visualization with diaminobenzidine tetrahydrochloride (DAB, Sigma, Munich, Germany). After a final washing step, slides were counterstained with Mayer's hematoxylin, dehydrated and mounted.

Staining procedure for cytokeratin 18, smooth muscle actin, ER and ErbB-2

Immunohistochemical stainings for cytokeratin 18, smooth muscle actin and ER were performed by indirect immunoperoxidase as described in detail previously.²¹ Immunohistochemical staining of ErbB-2 was performed using HercepTest (DAKO, Glostrup, Denmark) as described before.²²

Evaluation of immunohistochemical staining

Evaluation of HercepTest was performed observing the staining pattern of cell membranes as set in the criteria specified by Dako for interpretation. According to the intensity of membrane staining a score was established.

Staining intensity of AP-2alpha and AP-2gamma was evaluated by two different observers. Also immunohistochemistry for ER and ErbB-2 was scored independently by two different pathologists. Evaluation of AP-2alpha, AP-2gamma and ER was performed using a score according to Remmele and Stegner²³ in order to achieve comparable values. Nuclear AP-2alpha staining of the inner glandular cell layer and nuclear AP-2gamma staining of the

outer myoepithelial cell layer in normal breast tissue within the slide was serving as internal control. Score value was 0 if no staining was present. Score 1 was used if $\leq 10\%$ of cells were stained, score 2 if 10–50%, score 3 if 51–80% and score 4 if more than 80% of cells showed immunohistochemical staining reaction. The result of this score was multiplied with the staining intensity score value. Staining intensity was set to score value 0 if no staining was present. Score 1 was used if weak staining of cells was observed, score 2 moderate and score 3 if strong staining of cells was present. Thus, final scores ranged between a minimum of 0 point and a maximum of 12 points.

In Vitro Translation of Proteins and Western Blot

In vitro transcription and translation of *AP-2alpha* or *AP-2gamma* were carried out using the TNT Quick Coupled Transcription/Translation System (Promega, Madison, USA) following the manufacturer's instructions. Briefly, 1 μg of plasmid DNA containing the human *AP-2alpha* or *AP-2gamma* cDNA, respectively, was *in vitro* transcribed using T7 RNA polymerase, and transcripts were translated into protein using rabbit reticulocyte lysate in a one-tube reaction. Plasmids used were pCMX-*AP-2alpha*,²⁴ pT7 β NcoAP-2gamma (kindly provided by Dr D Taverna, Institute for Cancer Research and Treatment, Candiolo, Italy) and pCMX-pL1 as empty control vector. In all, 13 μl of translated protein was mixed with 20 μl 8 \times sample buffer (600 mM Tris pH 6.8, 8% (w/v) SDS, 20% (v/v) β -mercapto-ethanol) and heated to 100°C for 10 min. A volume of 11 μl of this solution was separated using a 10% SDS-polyacrylamid gel²⁵ and transferred to a PVDF membrane by electroblotting. After blocking in 10% (w/v) dry milk in PBS, the membrane was incubated with the same anti-AP2 antibodies as described for immunohistochemistry and a peroxidase-conjugated anti-rabbit antibody and detected using the ECL system (Amersham, Little Chalfont, UK).

Statistical Analysis

Spearman's rank correlation analysis was applied to examine relationships between immunohistochemical staining intensities of AP-2alpha, AP-2gamma, ER and ErbB-2 using SPSS 12.0.1 software (SPSS Inc., Chicago, USA). A *P*-value of ≤ 0.01 was considered significant.

Prognostic value of immunohistochemical AP-2alpha-, AP-2gamma- and ER- staining intensity was analyzed with regard to overall survival using Wilcoxon (Gehan) test.

Results

We studied a collective of 51 female breast cancer cases that were described in detail previously.²⁰ In

addition, a tissue microarray containing 93 cases of invasive female breast cancer with detailed follow-up data of 70 cases comprising 30 years was included in our study. All 144 cases were analyzed immunohistochemically using commercially available antibodies directed against AP-2alpha and AP-2gamma. Specificity of these antibodies for the particular AP-2 isoforms was first evaluated by Western blot analyses using *in vitro*-translated AP-2 proteins. As shown in Figure 1, the AP-2alpha-specific antibody did not crossreact with AP-2gamma protein, and, vice versa, the AP-2gamma-specific antibody H-77 did not bind to AP-2alpha protein whereas each of the antibodies detected its respective antigen. As a control, a nonselective AP-2 antibody reacted both with AP-2alpha and AP-2gamma.

In non-neoplastic breast, immunohistochemistry revealed nuclear staining for AP-2alpha in differentiated ductal and lobular epithelia analogous to the staining pattern of the antibody cytokeratin 18 (Figure 2a and b). We detected exclusively nuclear AP-2 staining, but no cytoplasmic immunosignals. Only in a few cases weak cytoplasmatic staining was observed, which we considered nonspecific as these signals were not reproducible at higher antibody dilution (data not shown). Remarkably, AP-2gamma was not expressed in the cytokeratin 18-positive epithelia but was specifically detected in the nuclei of myoepithelial cells of the basal ductal and lobular epithelia, which were also positive for the myoepithelial marker smooth muscle actin (Figure 2c and d).

In ductal carcinoma *in situ*, a similar staining pattern of AP-2alpha and AP-2gamma was noticed. An outer wall of AP-2gamma-positive myoepithelial cells was separating AP-2alpha-positive, polymorphic tumor cells from the stroma (Figures 3a–e). Often staining reaction of AP-2alpha was more intensive in ductal carcinoma *in situ* than in invasive tumor (Figure 3b). There was no difference in AP-2alpha and AP-2gamma staining intensities among tumor cells of pure DCIS cases (see Figure 7 in Supplementary information).

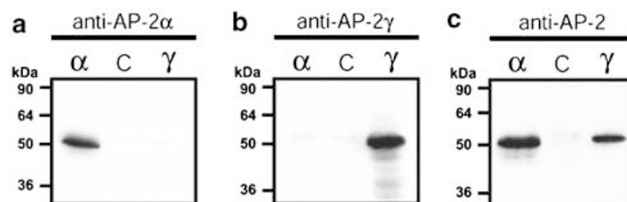


Figure 1 Specificity of AP-2 isoform-specific antibodies. *In vitro*-translated AP-2alpha (α), AP-2gamma (γ) proteins or control reaction (C) were gelelectrophoretically separated and after Western blotting reacted with antibodies specific for AP-2. (a) Detection using the AP-2alpha-specific antibody. (b) Detection using the AP-2gamma-specific antibody H-77. (c) Detection using the pan-reactive antibody C-18 that recognizes both AP-2alpha and AP-2gamma.

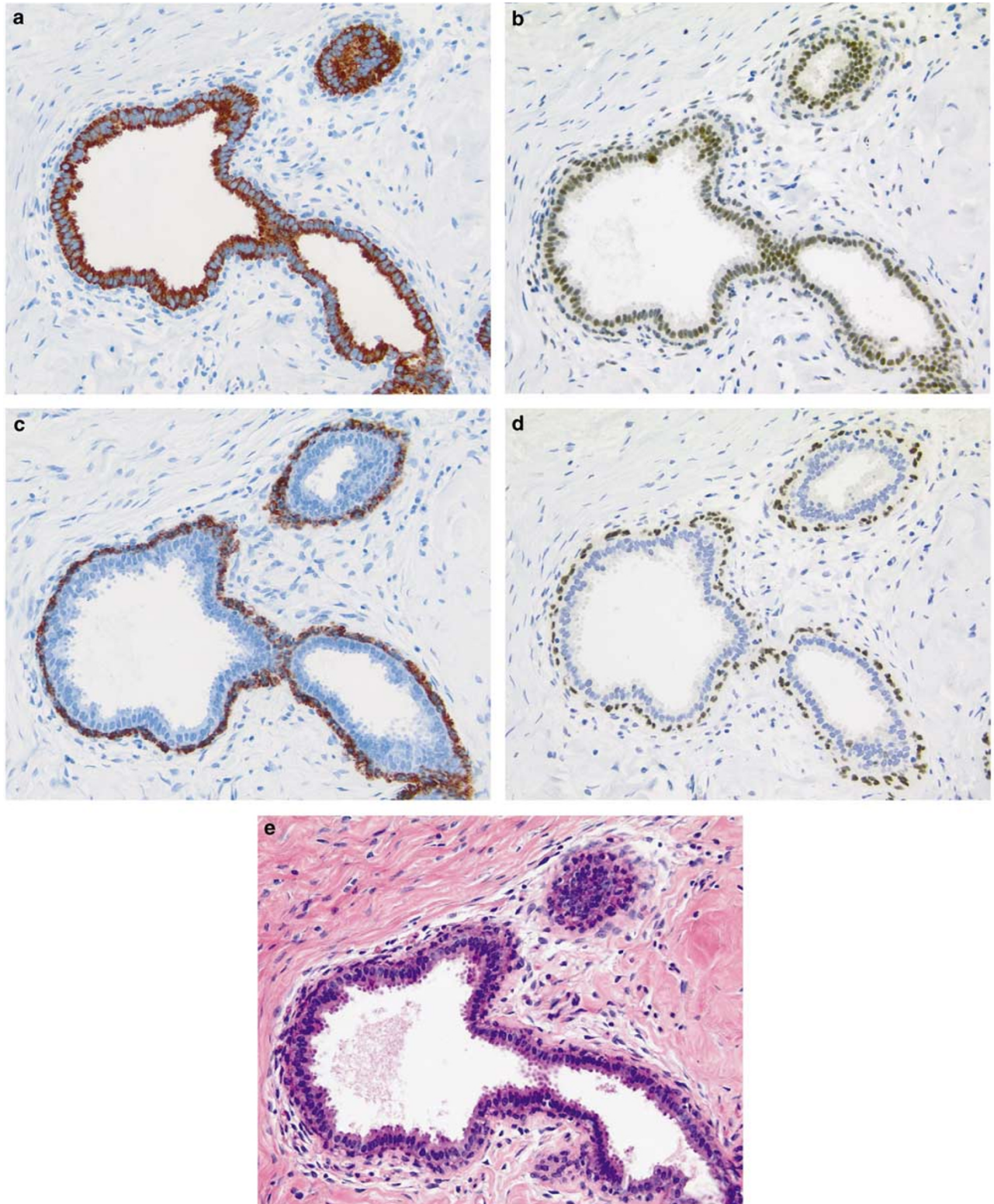


Figure 2 Staining pattern of cytokeratin 18 (a), AP-2alpha (b), smooth muscle actin (c), AP-2gamma (d) and hematoxylin–eosin (e) in non-neoplastic breast tissue ($\times 200$).

In invasive carcinoma variable AP-2alpha (Figure 4) and AP-2gamma staining of the tumor cells was observed. Undifferentiated carcinomas occasionally expressed AP-2alpha and AP-

2gamma simultaneously. In all cases, nonatypical AP-2gamma-reacting cells with myoepithelial morphology were absent in invasive tumor sections.

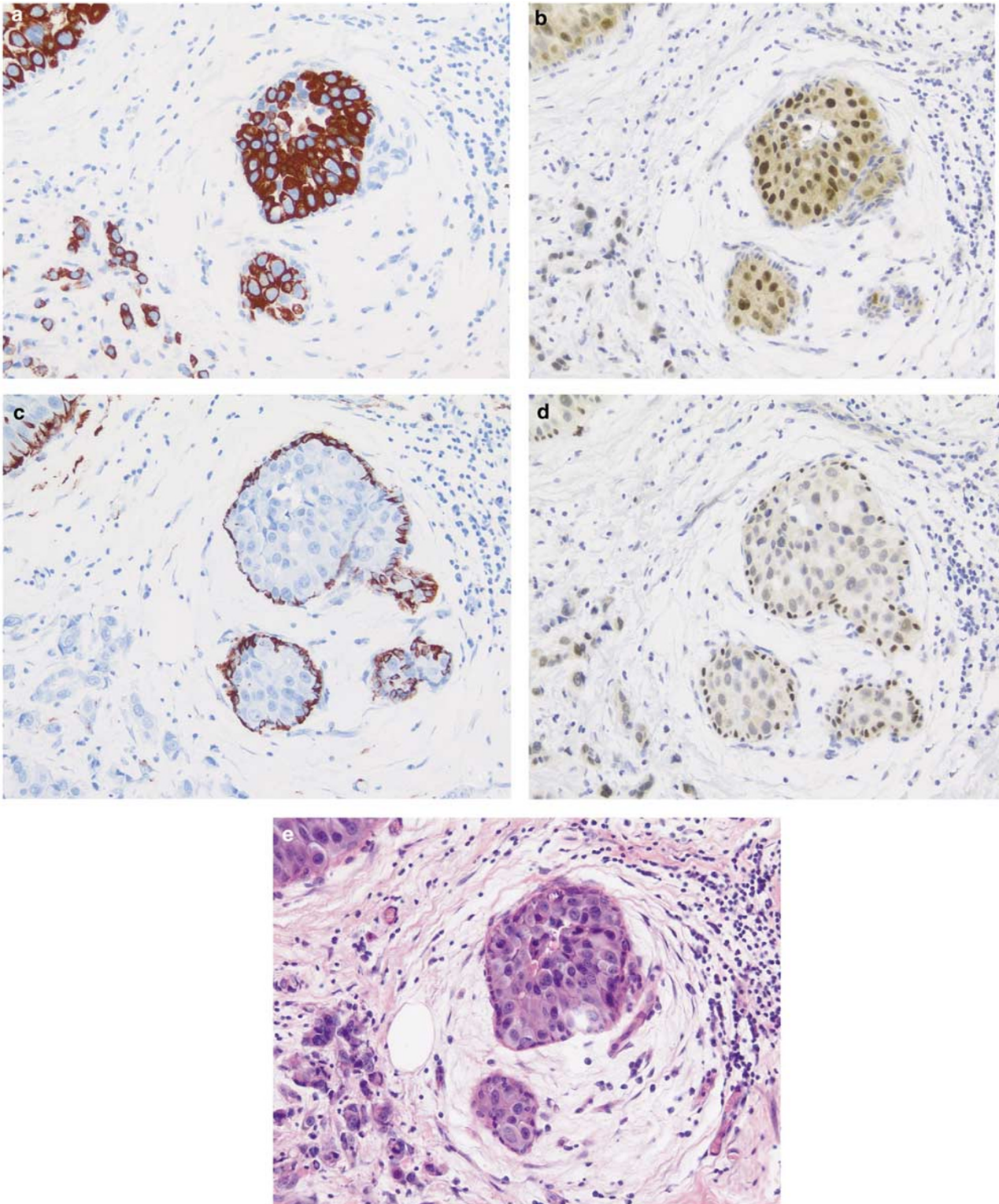


Figure 3 Staining pattern of cytokeratin 18 (a), AP-2alpha (b), smooth muscle actin (c), and AP-2gamma (d) and hematoxylin-eosin (e) in ductal carcinoma *in situ* ($\times 200$).

In Spearman's rank correlation analysis, of all 144 cases the intensity of AP-2alpha expression showed a weak but significant correlation to ER ($P < 0.001$, $r = 0.237$) (see Figure 6 in Supplementary information), AP-2gamma ($P < 0.001$, $r = 0.448$)

and ErbB-2 ($P < 0.001$, $r = 0.332$). AP-2gamma did not show a correlation to ER ($P = 0.963$, $r = 0.003$) or ErbB-2 ($P = 0.043$, $r = 0.138$). ER staining correlated inversely with tumor malignancy grade ($P = 0.016$, $r = -0.313$). With regard to T- and

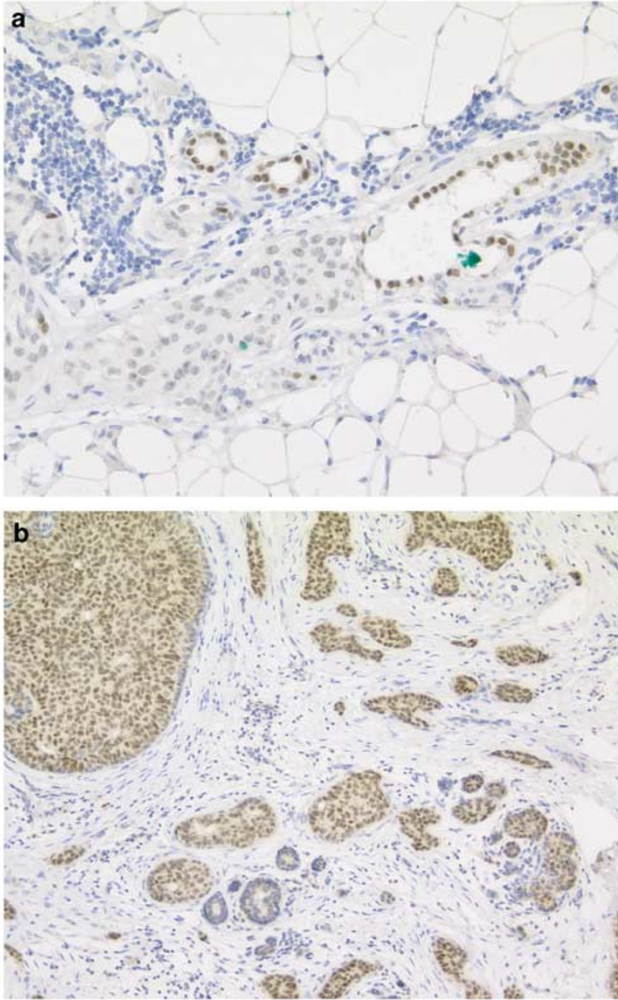


Figure 4 Staining intensity of AP-2alpha in two different cases of invasive ductal carcinoma: a case with weak AP-2alpha staining in invasive tumor (a) and a second case with intensive AP-2alpha staining in invasive carcinoma (b). Note intensive staining of AP-2alpha in adjacent non-neoplastic breast epithelia serving as internal staining control ($\times 100$).

N-stage no correlation could be found with staining results.

In the survival analysis of patients with tumors that were immunostained on the tissue microarray, scoring results of AP-2alpha, AP-2gamma and ER were separated into two groups: low expression representing score values from 0 to 6 and high expression showing values from 7 to 12. Patients showing high expression of ER had a better outcome than those with low expression (Figure 5a) ($P=0.009$). High AP-2alpha expression also showed a favorable outcome concerning survival, while low AP-2alpha expression was linked to worse prognosis (Figure 5b) ($P=0.131$). The AP-2alpha overexpressing cohort showed a mean survival rate of 15.0 years and the ER overexpressing group a value of 15.5 years. In contrast, patients with low AP-2alpha expression in breast cancers showed a mean

survival rate of 11.0 years while patients with low ER expression reached a value of 11.0 years. Immunohistochemical expression of AP-2gamma had no effect on survival (Figure 5c) ($P=0.562$). The AP-2gamma high expressing group had a value of 12.0 years and the low expressing group a mean survival rate of 11.5 years.

Discussion

Using AP-2alpha- and AP-2gamma-specific antibodies, we here demonstrate distinct spatial distribution of both AP-2 isoforms in non-neoplastic human breast epithelia. AP-2alpha-positive cells represent cytokeratin 18-positive glandular epithelial cells and AP-2gamma-positive cells represent SMA-positive myoepithelia. In early and well-differentiated breast cancer, the expression of AP-2 seems to reflect the epithelial compartment contributing to the tumor. Therefore, in Ductal carcinoma *in situ* both AP-2alpha and AP-2gamma are found expressed in a spatial distribution resembling normal epithelia with AP-2gamma representing the SMA-positive myoepithelial barrier surrounding the AP-2alpha-positive tumor cells. Invasive areas lack myoepithelia and hence AP-2gamma-positive cells with myoepithelial morphology disappear while AP-2alpha-positive tumor cells remain. Undifferentiated tumors may also show AP-2gamma-positive, atypical tumor cells that either reflect the acquisition of myoepithelial markers as a consequence of losing normal epithelial differentiation, or may also reflect aberrant AP-2 expression patterns.

Previous studies did not report the nonoverlapping spatial expression patterns of AP-2alpha and AP-2gamma in normal breast tissue, most likely due to applying antibodies with partly overlapping specificity for the highly conserved AP-2 isoforms. For instance, the antibody used by Gee *et al*¹⁹ only detected AP-2alpha and AP-2beta, and thus AP-2gamma could not be analyzed. The AP-2 antibody employed in the study of Pellikainen *et al*¹⁸ has a much higher sensitivity for AP-2alpha and AP-2beta than for AP-2gamma and thus the latter isoform most likely was not stained appropriately. In the study of Turner *et al*⁶ no benign tissue was shown, and it is not mentioned whether the antibodies were employed to discriminate between the two epithelial compartments.

In ductal carcinoma, *in situ* staining reaction of AP-2alpha was often more intensive than in invasive tumor. Therefore, a larger collective of pure DCIS should be studied to clarify the question whether AP-2alpha-overexpressing tumor cells reflecting a 'less malignant' tumor cell clone are generally located in DCIS while invasive tumor cells with low AP-2alpha expression reflect higher malignancy.

Expression of ER is a well-established prognostic marker in breast cancer. High ER expression is

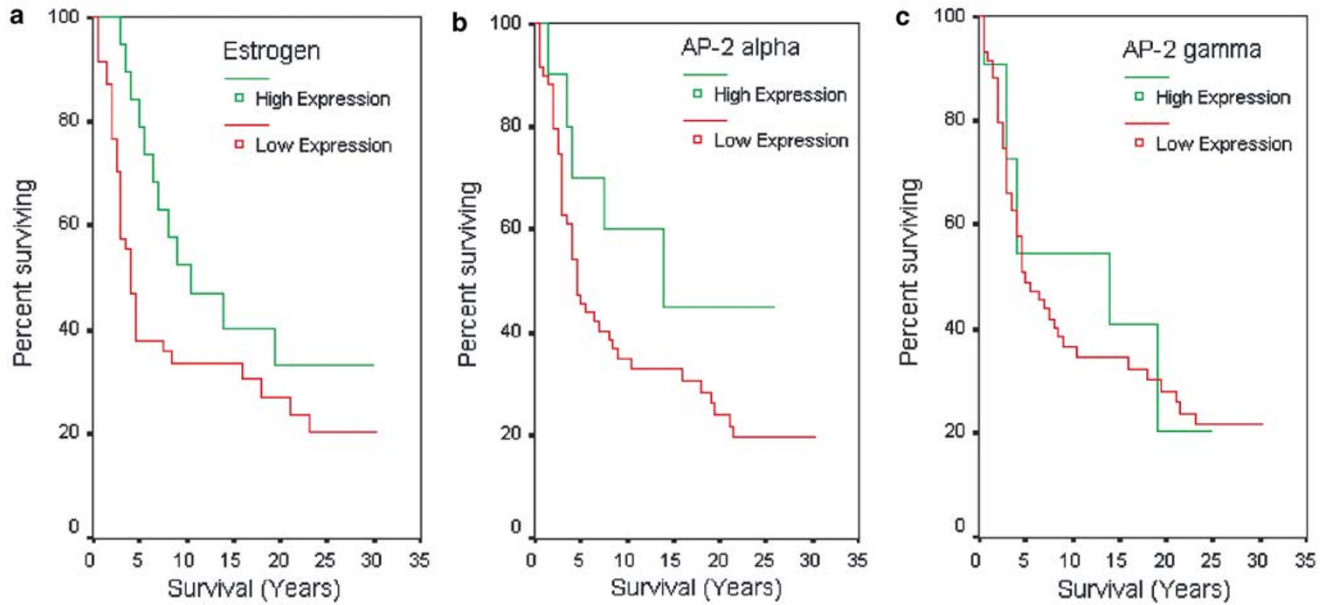


Figure 5 Survival data of 70 female patients with breast cancer in relation to expression intensities of ER (a), AP2alpha (b) and AP-2gamma (c).

linked to a favorable prognosis as shown by Henderson and Patek.²⁶ The present study confirmed this finding detecting a better survival rate of ER-overexpressing patients than patients with low or absent ER staining. Furthermore, a weak correlation of ER and AP-2alpha was found; a fact that has also been described by other authors.^{6,19} These findings indicate a comparable diagnostic value of ER- and AP-2alpha-expression in invasive breast cancer.

Analyzing AP-2 expression in invasive breast cancer, Turner *et al*⁶ found a significant upregulation of AP-2gamma. In contrast, Pellikainen *et al*¹⁸ described a significantly lower expression of AP-2 isoforms alpha, beta and gamma in invasive carcinoma compared with normal ductal breast epithelium. According to our results, the expression of the different AP-2 isoforms should be evaluated separately as they represent different histological components contributing to the tumor. In invasive carcinoma, AP-2alpha expressing ductal epithelia seem to be involved in the tumor process in the first line. In contrast, a primary involvement of the myoepithelial-derived protein AP-2gamma in ductal neoplasia seems unlikely. Nevertheless, an extensive AP-2gamma staining of invasive tumor cells was occasionally found in undifferentiated carcinoma. The latter observation could be explained as an adoption of myoepithelial tumor cell characteristics with progression of breast cancer.

Concerning the prognostic value of AP-2 expression in invasive breast cancer, Gee *et al*¹⁹ found that loss of AP-2alpha and beta expression was associated with disease progression. The authors concluded that AP-2alpha and AP-2beta serve as a

tumor suppressor in breast cancer. Nevertheless, these findings were not supported by long-term follow-up data. In the present study, the AP-2alpha overexpressing cohort showed favorable mean survival rates confirming the preceding findings. This fact could indicate a potential role of AP-2alpha as a protective factor in breast tumorigenesis. AP-2gamma expression did not have influence on survival rates, reflecting the different histopathological role of myoepithelial cells in breast cancer.

The distinct compartments of mammary epithelium expressing AP-2alpha or AP-2gamma, respectively, point to specific biological functions of the different AP-2 isoforms. The identification of cytokeratin 5-positive mammary stem cells giving rise to both glandular cells and myoepithelial cells²⁷ raises the possibility that along these developmental fates, the distinct AP-2 isoforms may behave as genetic switches that control sets of differentiation-specific genes. It will therefore be of interest to identify the particular AP-2 isoform(s) expressed in the mammary stem cell compartment. As breast cancer may arise from these stem cells or CK18/SMA-positive progenitor cells,²⁸ this issue is not only of relevance for understanding breast tumorigenesis but may also be important for a refined diagnosis of subtypes of breast cancer.

Acknowledgements

Grants from the Deutsche Forschungsgemeinschaft (Scho 503, BU672/3-5) to HS and RB supported this work. This work is dedicated to Karl Heinz Friedrichs.

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/mpath>).