Prognostic Effect of Epithelial Cell Adhesion Molecule Overexpression in Untreated Node-Negative Breast Cancer

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Abstract

Purpose: Epithelial cell adhesion molecule (Ep-CAM) recently received increased attention not only as a prognostic factor in breast cancer but also as a potential target for immunotherapy. We examined Ep-CAM expression in 402 consecutive node-negative breast cancer patients with long-term follow-up not treated in the adjuvant setting.

Experimental Design: Ep-CAM expression was evaluated by immunostaining. Its prognostic effect was estimated relative to overexpression/amplification of HER-2, histologic grade, tumor size, age, and hormone receptor expression.

Results: Ep-CAM status was positive in 106 (26.4%) patients. In multivariate analysis, Ep-CAM status was associated with disease-free survival independent of age, pT stage, histologic grade, estrogen receptor (ER), progesterone receptor (PR), as well as HER2 status ($P = 0.028$; hazard ratio, 1.60; 95% confidence interval, 1.05-2.44). Recently, so-called triple-negative (HER-2, ER, and PR) breast cancer has received increased attention. We noticed a similar association of Ep-CAM with disease-free survival in the triple-negative group as for the entire cohort.

Conclusion: In this study of untreated breast cancer patients, Ep-CAM overexpression was associated with poor survival in the entire cohort and in the subgroup of triple-negative breast cancer. This suggests that Ep-CAM may be a well-suited target for specific therapies particularly in HER-2−, ER-, and PR-negative tumors.

Assigning specific treatment to patients according to known tumor-related prognostic factors has long been the holy grail in breast cancer. The St. Gallen Risk Classification proposes histologic grade, tumor size, age, vascular invasion, and HER-2 overexpression and/or amplification to classify patients with lymph node – negative breast cancer in low-risk or intermediate-risk groups (1). In addition, factors predicting the success of a given therapy seem even more important. This led to the premise "first—select the target" (2). Clearly, this underlines the importance of estrogen receptor (ER) and progesterone receptor (PR), which subdivide breast cancers into endocrine-responsive, uncertain endocrine-responsive, and endocrine-nonresponsive categories. In fact, the predictive effect of the hormone receptor status is clearly more important than its prognostic effect. In addition, HER-2 has evolved from a primarily prognostic factor two decades ago (3) into a factor that also predicts response to trastuzumab. The monoclonal antibody trastuzumab remarkably improves survival of HER-2 – positive breast cancer patients not only in the advanced (4) but also in the adjuvant (5) setting. The FinHer trial reported a reduced survival for HER-2 – amplified tumors and showed that trastuzumab virtually abrogates the adverse effect of HER-2 on outcome (6). Based on these findings, it is conceivable that those targets for novel therapies seem more promising, which also carry a prognostic significance.

Epithelial cell adhesion molecule (Ep-CAM) is a transmembrane glycoprotein expressed on most human epithelial cells, functioning as a homophilic cell-cell adhesion molecule (7). Overexpression of Ep-CAM has been reported to correlate with poor disease-free (DFS) and overall survival (OS) in breast cancer (8). In addition to its prognostic importance, Ep-CAM has also been proposed as a potential target for antitumor therapies. Although Ep-CAM is also expressed on nonneoplastic epithelia, a recent study on transgenic mice showed

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Table 1. Clinicopathologic characteristics of all patients (n = 402)

Characteristics	n	%
Age at diagnosis		
<50	111	27.6
≥50	291	72.4
pT stage		
pT_1	263	65.4
pT_2	135	33.6
PT_3	4	1.0
Histological grade		
G I	104	25.9
G II	217	54.0
G III	81	20.1
ER		
Positive	287	71.4
Negative	115	28.6
PR		
Positive	223	55.5
Negative	179	44.5
HER-2 status		
Positive	57	14.2
Negative	345	85.8
Triple-negative		
Yes	70	17.4
No	332	82.6
Ep-CAM score		
Positive (>4)	164	40.8
Negative (1-4)	238	59.2
Ep-CAM status		
Positive (3+)	106	26.4
Negative (0-2+)	296	73.6
Death	109	26.7
Of cancer	55	13.5
Unrelated to cancer	54	13.2
Surviving	300	73.3
Relapse	110	26.9
Regional	47	11.5
Metastasis	84	20.5
Contralateral	7	1.7
No relapse	299	73.1

that Ep-CAM on tumor cells is much more easily accessible to antibodies than Ep-CAM expressed in normal tissues (9). Furthermore, Ep-CAM is substantially overexpressed in breast cancer compared with nonneoplastic breast tissue (10). A novel fully human monoclonal IgG1 antibody (MT201, adecatumumab) has shown *in vitro* and *in vivo* activity against Ep-CAM–expressing epithelial tumors (11), likely mediated by cellular and complement-dependent cytotoxicity (12). In a randomized multicenter phase II study, we could recently show a stabilizing effect of adecatumumab treatment on disease progression in patients with Ep-CAM–positive advanced breast cancer (13). To allow a distinction between a pure prognostic effect of Ep-CAM expression and its role as potential treatment target, we have analyzed, in this study, Ep-CAM expression by immunohistochemistry in a cohort of 402 node-negative breast cancer patients from a single institution, who had not received any systemic adjuvant therapy. Besides studying the entire cohort, we additionally analyzed the group of triple-negative breast carcinomas. These tumors are negative for ER, PR, and HER-2. As a result, triple-negative breast cancer is resistant to existing targeted therapies like endocrine treatments or trastuzumab. Therefore, many studies have been published aimed at understanding the biology of triple-negative breast cancer (14–16). The clinical relevance of a possible association of Ep-CAM with worse prognosis also in triple-negative breast cancer would be that this subtype of breast cancer might be treated with adecatumumab, an antibody directed against Ep-CAM.

Patients and Methods

The study cohort consisted of 409 consecutive node-negative breast cancer patients with tumor size $pT1_a$-pT3 and adequate follow-up information who were treated at the Department of Obstetrics and Gynecology of the Johannes Gutenberg University Mainz between the years 1986 and 2000. Of these 409 patients, paraffin blocks with tumor tissue were available for 402 individuals who were analyzed in this study. All patients have been treated by surgical tumor resection but had not received any systemic therapy in the adjuvant setting. The tumor size was collected from the original pathology reports of the Gynecological Pathology Division. Patients had been treated either with modified radical mastectomy (44%) or breast conserving surgery followed by irradiation (56%). We focused on node-negative breast cancer patients with pT_{1-3} tumors without any evidence of metastatic disease at the time of surgery. The median age of the patients at diagnosis was 58.8 y (range, 33-91 y). The study was approved by the ethical review board of the medical association of Rhineland-Palatinate. Follow-up was done by writing letters to patients, phoning, and by checking the patient records at least once a year between surgery (1986-2000) and the last follow-up in October 2007. In this period, we documented (*a*) death from cancer or from other reasons unrelated to cancer and (*b*) recurrence of disease, which included metastasis, local relapse, and secondary tumors. The mean follow-up time was 10 y ± 5 y (SD). Fifty-five patients (13.7%) died of breast cancer and 110 patients (27.4%) relapsed (Table 1). Fifty-four patients (13.4%) died for unrelated reasons. Patients in whom breast cancer could not definitely be ruled out as cause of death were considered as having died from breast cancer. The patients dying of reasons other than breast cancer were censored for the survival analyses at their date of death.

Histology. Histologic grade was assigned according to Elston and Ellis (17) by two of the authors (M.S. and H.A.L.) routinely involved in the histologic diagnosis of breast cases in our certified Breast Cancer Center and unaware of the clinical outcome. Equivocal cases were reassessed on a double-headed microscope and in each case, a consensus grade was reached.

Immunohistochemistry and interpretation of the staining. Immunonohistochemical analyses were done on 4-μm-thick sections according to standard procedures. Serial sections of formalin-fixed and paraffin-embedded tumor tissues were stained with monoclonal ER antibodies (clone 1D5; Dako), monoclonal PR antibodies (clone PgR 636; Dako), as previously described (18), as well as polyclonal HER-2 antibodies (A0485; Dako) and monoclonal Ep-CAM antibodies (clone VU-1D9; NovoCastra). Immunohistochemistry assays were done after a routine staining method. The sections were incubated with a biotin-labeled secondary antibody and streptavidin-peroxidase for 20 min, each, then for 5 min with 0.05% 3′3-diaminobenzidine-tetrahydrochloride and, finally, lightly counterstained with hematoxylin. All series included appropriate positive and negative controls; all controls gave adequate results. The immunohistochemical evaluation was done by two of the authors (M.S., H.P.) trained in histologic and immunohistochemical diagnostics, unaware of the clinical outcome.

HER-2 was scored from 0 to 3+ according to the well-published manufacturer's instructions.

ER, PR, and Ep-CAM were assessed using a immunoreactive score defined by the product of a proportion score (0, none; 1, <10%; 2,

10-50%; 3, 51-80%; 4, >80% positive cells) and an intensity score (0, no staining; 1, weak; 2, moderate; 3, strong). Only nuclear staining was considered for ER and PR, and only specific surface membrane staining was considered for Ep-CAM. ER and PR were analyzed with score (proportion stained) 0 and 1 versus >1. Patients with a score of >1 were considered positive for ER or PR, respectively. To allow for comparison of Ep-CAM staining results, we used the Ep-CAM score (intensity × proportion score) as described by Gastl and coworkers (8). An Ep-CAM score of 0 to 4 was considered negative, a score of >4 as positive. Furthermore, a novel Ep-CAM status similar to the established

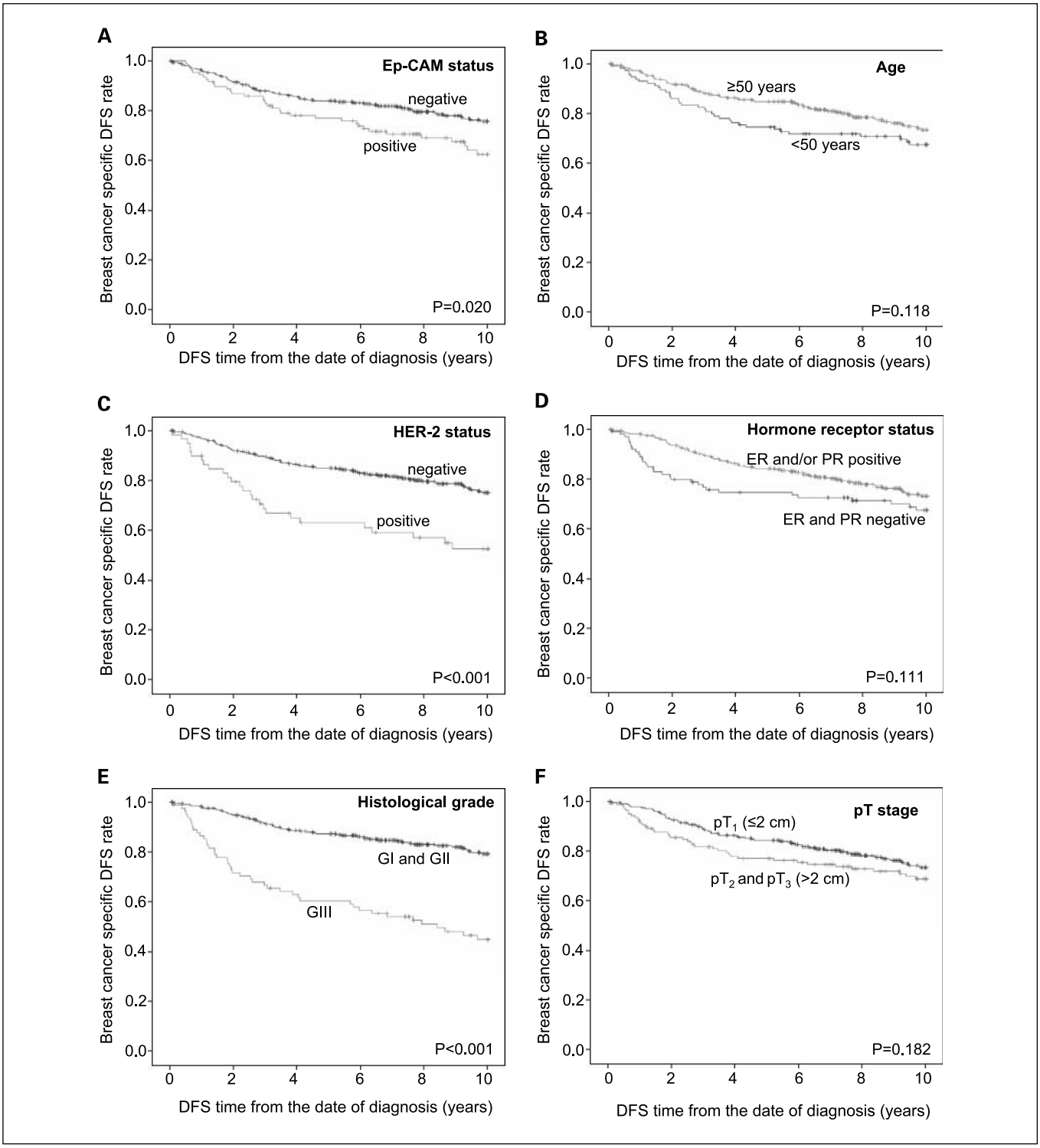

Fig. 1. Association of potential factors of influence with breast cancer – specific DFS time in 402 patients with node-negative breast cancer *A,* Ep-CAM status; *B,* age; *C,* HER-2 status; *D,* hormone receptor status; *E,* histologic grade; *F,* pT stage.

Table 2. Multivariate analysis for breast cancer–specific DFS in the entire cohort of breast cancer patients (n = 402)

Prognostic factors	P	HR	95% CI
Age (<50 vs ≥50 y)	0.097	0.70	0.46-1.07
pT stage (≤2 cm vs >2 cm)	0.397	1.20	0.79-1.81
Histological grade (Grade 3 vs grade 1 and 2)	<0.001	3.92	2.54-6.06
ER and PR (negative vs positive)	0.061	1.59	0.98-2.58
HER-2 status (positive vs negative)	0.001	2.23	1.39-3.58
Ep-CAM status (positive vs negative)	0.028	1.60	1.05-2.44

Abbreviations: HR, hazard ratio; 95% CI, 95% confidence interval.

HER-2 scoring system was used to classify Ep-CAM overexpression. Only tumors with strong specific surface membrane staining (intensity score 3) in >10% of the tumor cells were considered as Ep-CAM 3+ overexpressors and were thus assigned a positive Ep-CAM status.

Fluorescence* in situ *hybridization. All HER-2 2+ cases were confirmed by fluorescence *in situ* hybridization using a dual-color probe (DakoCytomation) containing a spectrum orange–labeled *HER-2* gene (17q11.2-q12) probe and a spectrum green–labeled centromere control for chromosome 17 (17p11.1-q11.1). Appropriate positive controls were included in each staining run. Analysis was done with a Zeiss fluorescence microscope (Axioskop 2) by one of the authors (C.C.) trained in histologic diagnostics and fluorescence microscopy, again blinded to the clinical outcome. A minimum of 80 nonoverlapping nuclei were evaluated, and the ratio of *HER-2* signals per nuclei relative to chromosome 17 centromere signals were calculated. Ratios of two and more were classified as *HER-2* amplification. HER-2 2+ tumors with *HER-2* amplification were finally considered HER-2 positive.

Statistical analysis. Survival rates were calculated according to the Kaplan-Meier method. DFS was computed from the date of diagnosis to the date of recurrence of disease or death from cancer if there was no earlier recurrence. Breast cancer–specific OS was computed from the date of diagnosis to the date of death from breast cancer. Patients who died of an unrelated cause were censored at the date of death. Survival was compared with the Log-rank test. Multivariate Cox survival analyses were done with inclusion. Dichotomization was done as follows: Ep-CAM status in positive and negative, age in ≥50 y and <50 y, HER-2 status in positive and negative, hormone receptor status in positive (ER and/or PR positive) and negative (both, ER, and PR negative), histologic grade in GIII versus GI and GII, and pT stage in pT1 (≤2 cm) versus pT_2 and pT_3 (>2 cm). For all survival analyses, a follow-up period of 10 y was used (which corresponds to the mean follow-up period), and the prognostic importance of Ep-CAM was studied restricted to events during the 10 y after diagnosis. Correlations between Ep-CAM status, age, HER-2 status, hormone receptor status, histologic grade, and pT stage were analyzed using the χ^2 test (likelihood quotient). All P values are two sided. As no correction for multiple testing was done, they are descriptive measures. All analyses were done using SPSS15.0.

Results

Distribution of prognostic factors. Established pathologic and clinical variables were assessed, including age, tumor size (pT stage), histologic grade, ER, PR, and HER-2 status. Ep-CAM expression was determined by immunostaining. Using a scoring system, 164 (40.8%) patients were classified as positive, whereas 238 (59.2%) were negative. The novel more stringent Ep-CAM status is based on the same principle as the HercepTest and showed strong expression in only 106 patients (26.4). The remaining 296 patients (73.6%) were scored Ep-CAM status negative. Because of its similarity to the successful and already clinically applied HER-2 scoring system (0 to 3+), we only used the Ep-CAM status for further analysis of the prognostic relevance of Ep-CAM. Seventy (17.4%) patients were negative for ER, PR, and HER-2 expression. Among these patients with triple-negative breast cancer, 25 (35.7%) were Ep-CAM status positive. The estimated DFS was 70%, and breast cancer–specific OS was 84% after a follow-up period of 10 years for the entire patient cohort.

Ep-CAM overexpression is associated with breast cancer specific DFS. Kaplan Meier analysis visualized a difference in DFS time between patients with positive and negative Ep-CAM status in the cohort of 402 patients with node-negative breast cancer (P = 0.020, log-rank test; Fig. 1A). Similarly, HER-2 status (P < 0.001; Fig. 1C) and histologic grade (P < 0.001; Fig. 1E) were associated with DFS. In contrast, no significant influence of age (P = 0.118; Fig. 1B), ER, and PR status (P = 0.111; Fig. 1D) as well as pT stage (P = 0.182, Fig. 1F) were observed. In principle, a similar scenario was obtained when OS was studied (Supplementary Fig. S1; Supplementary Table S1). Ep-CAM status (P = 0.045), HER-2 status (P = 0.007), histologic grade (P < 0.001), as well as ER and PR status (P = 0.031) were associated with OS time in Kaplan Meier analysis, whereas age (0.998) and pT stage (P = 0.181) were not.

Conducting a multivariate Cox regression analysis adjusted for age, pT stage, histologic grade, ER and PR, as well as HER-2 status, Ep-CAM status was associated with DFS (P = 0.028; hazard ratio, 1.60; 95% confidence interval, 1.05-2.44; Table 2). Besides Ep-CAM, also histologic grade (P < 0.001; hazard ratio, 3.92; 95% confidence interval; 2.54-6.06) and HER-2 status (P = 0.001; hazard ratio, 2.23; 95% confidence interval, 1.39-3.58) were explanatory.

Role of Ep-CAM in HER-2, ER, and PR triple-negative patients. Triple-negative breast cancer is resistant to targeted therapies, such as trastuzumab or endocrine treatments. Therefore, it is of particular interest, whether Ep-CAM is also associated with prognosis in HER-2–, ER-, and PR-negative patients. DFS and OS of triple-negative breast cancer tended to be slightly shorter than that from receptor-positive breast cancer; however, no statistically detectable difference could be observed in our patient cohort (P = 0.216 for OS and 0.818 for DFS; Supplementary Fig. S2). Interestingly, Ep-CAM status was also associated with prognosis in the triple-negative patients (Fig. 2). Kaplan Meier analysis resulted in shorter DFS time (P = 0.010)

and in shorter OS time ($P = 0.044$) in patients with positive Ep-CAM status. Besides Ep-CAM status, only histologic grade was associated with DFS ($P = 0.047$), whereas age and pT stage did not result in statistically detectable differences (Supplementary Figs. S3 and S4). Thus, the effect of Ep-CAM on prognosis seems to be qualitatively similar in the triple-negative subgroup compared with the entire cohort. Multivariate Cox regression analysis is difficult to interpret because the triple negative subgroup comprised only 70 patients. Multivariate Cox regression analysis adjusted for age, pT stage, and histologic grade showed a trend for an association of Ep-CAM status with DFS ($P = 0.060$; hazard ratio, 2.54; 95% confidence interval, 0.96-6.72; Supplementary Table S2). However, also histologic grade was only borderline significantly associated with DFS ($P = 0.055$; hazard ratio, 2.54; 95% confidence interval, 0.98-12.95), illustrating the limitation due to small case numbers.

Correlation of Ep-CAM with other clinically relevant variables. Ep-CAM status was inversely correlated with ER status ($P = 0.004$; likelihood ratio) and showed a positive correlation with histologic grading ($P = 0.017$; Supplementary Table S3).

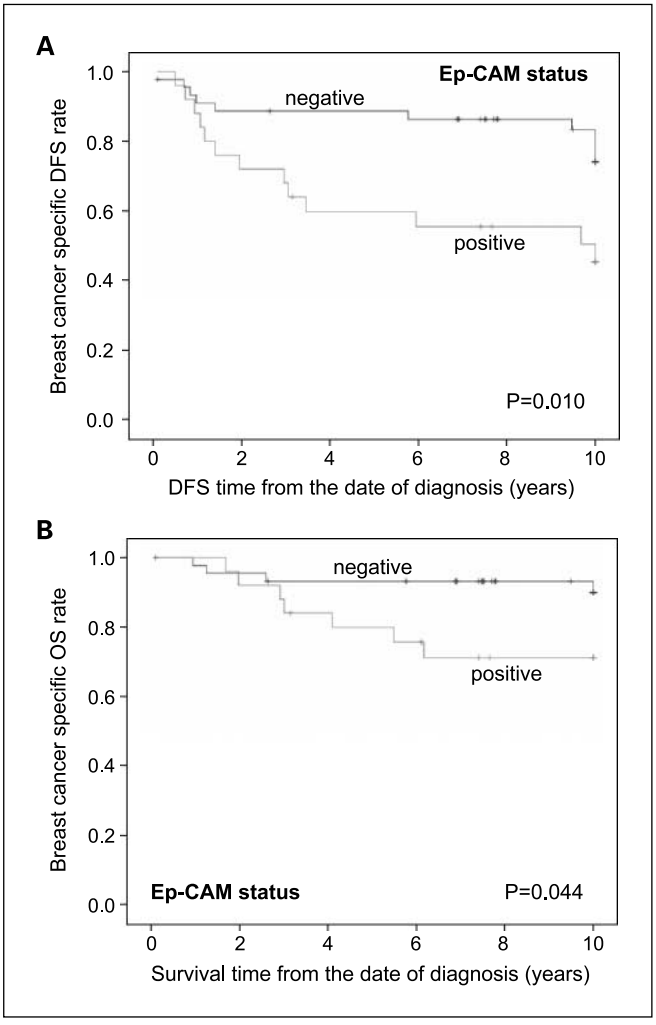

Fig. 2. Association of Ep-CAM status with DFS (*A*) and OS (*B*) in 70 patients with triple-negative breast cancer.

In contrast, Ep-CAM status was not significantly associated with age, pT stage, PR status, HER-2 status, and the triple-negative status (Supplementary Table S3). ER status and PR status were positively correlated with each other ($P < 0.001$), whereas HER-2 status was inversely correlated with ER status ($P = 0.003$) and PR status ($P = 0.001$; Supplementary Table S3). In a next step, we tried to reconstruct a sequence of events considering loss of ER as well as PR, acquisition of positive HER-2 status as well as positive Ep-CAM status (Supplementary Table S4). The loss of PR clearly represents an early event that often occurs before acquisition of a positive HER2 status, before the ER receptor is lost and before a tumor progresses to grade 3. In contrast, associations considering acquisition of a positive Ep-CAM status were only moderate (Supplementary Table S4).

Discussion

Ep-CAM (17-1a antigen) was the first human tumor–associated antigen identified with monoclonal antibodies (19). Since then, Ep-CAM has been extensively studied as a prognostic factor and as a potential antitumor target in a variety of cancer entities (20). In breast cancer, Ep-CAM expression was found to be associated with poor prognosis (8). Silencing *Ep-CAM* gene expression with *Ep-CAM* short interfering RNA resulted in a substantial decrease in the rate of cell proliferation, migration, and invasion in breast cancer cell lines (10). In the present study of 402 consecutive node-negative breast cancer patients, Ep-CAM status was associated with DFS independent from age, pT stage, histologic grade, ER, PR, as well as HER2 status. Previously, the prognostic effect of Ep-CAM has been examined in a large database of 1705 patients (21). Similar to our study, Spizzo et al. (21) have observed that Ep-CAM overexpression was associated with worse prognosis. In this study, Ep-CAM expression predicted poor OS but was not an independent prognostic marker by multivariate analysis. In addition, the prognostic effect of Ep-CAM was confined to the node-positive patients. However, this is not a real discrepancy to our data and may be explained by differences in the study design. First, Spizzo and coworkers analyzed only OS. Also in our study, no significant association has been obtained for OS in multivariate analysis (Supplementary Table S1), in contrast to DFS. This may be explained by the higher number of events for DFS ($n = 110$) compared with OS ($n = 55$). A second difference to Spizzo's study is the use of the Ep-CAM status in our work. Because staining patterns for Ep-CAM are similar to those of HER-2, we have also applied a scoring system ranging from 0 to 3+ to assess Ep-CAM overexpression. Similar to the guidelines for the evaluation of HER-2-immunohistochemistry, we defined tumors with strong complete staining of the membranes of >10% of the tumor cells as Ep-CAM 3+ and, thus, as Ep-CAM status positive. This more stringent Ep-CAM score decreased the number of tumors rated Ep-CAM positive from 164 (40.8%), which is similar to the 41.7% described by Spizzo and coworkers (18) to 106 (26.4%). A third deviation from Spizzo's study is the difference in the distribution of grading. In Spizzo's study, 26.4%, 40.1%, and 33.4% of patients showed histologic grading I, II, and III, respectively, compared with our study with 25.9% (grade I), 54.0% (grade II), and 20.1% (grade III). This may be relevant because histologic

grade is associated with Ep-CAM and, on the other hand, also represents a strong prognostic factor.

Besides Ep-CAM, only histologic grade and HER-2 had prognostic significance in our cohort of node-negative breast cancer patients. In multivariate analysis, histologic grading was the only important prognostic factor for both DFS and OS. We have previously shown in a smaller and nonoverlapping cohort of breast cancer patients that *HER-2* was an independent factor for prognosis only when its amplification was assessed using fluorescence *in situ* hybridization (22). However, because the commonly accepted testing algorithm for HER-2 uses fluorescence *in situ* hybridization only in equivocal cases with an immunohistochemical score of 2+ (23), we only used fluorescence *in situ* hybridization in the 2+ cases in the present study. A similar algorithm has recently been proposed by Peiro and coworkers (24). However, these authors forwent to test for its prognostic significance. The finding of a significant association of HER-2 with DFS in our study further supports the inclusion of HER-2 into the current St. Gallen Risk Classification (1).

Recently, so-called triple-negative breast cancer has received increased attention (14–16). These tumors are negative for ER, PR, and HER-2. As a result, triple-negative breast cancer is resistant to existing targeted therapies like endocrine treatments or trastuzumab. We noticed a similar association of Ep-CAM with DFS in the triple-negative group as for the entire cohort. Unfortunately, the number of only 70 triple-negative patients in 402 (17.4%) was relatively small. Therefore, further studies are needed to confirm this observation. A prognostic role of Ep-CAM in triple-negative breast cancer is of clinical relevance because it may be a basis for treating this subgroup of patients with adecatumumab, an antibody directed against Ep-CAM. Because of resistance to approved targeted therapies, i.e., endocrine treatment and trastuzumab, it is of increasing importance to examine the prognostic significance of alternative targets such as Ep-CAM in this group of patients.

In our cohort of triple-negative patients, only a slight trend toward reduced breast cancer–specific DFS and OS was observed compared with the receptor-positive patients. Indeed, several authors (25–28) have described a poor survival associated with triple-negative breast cancer. An explanation might be that the studies of triple-negative breast cancer mentioned before included mixed cohorts of node-positive as well as node-negative patients and thus allowed for systemic treatment. In contrast, our patients were all node-negative and did not receive systemic therapies in the adjuvant setting. Obviously, the absent usefulness of endocrine therapies and/or trastuzumab in the group of triple-negative breast cancer will only lead to poorer survival when compared with patients receiving these therapies. Thus, our patient cohort provides the unique opportunity to study the actual effect of potential markers on the natural course of breast cancer exclusively treated by local therapies (surgery and radiation). Under this premises, the group of triple-negative breast cancer, which comprised 17.4% of all patients, exhibited a similar outcome as receptor-positive patients. The percentage of triple-negative breast cancers in our cohort was comparable with published results, which range between 11.2% (28) and 24.3% (25).

In conclusion, we have shown in a cohort of 402 node-negative breast cancer patients that Ep-CAM, histologic grade, and HER-2 are associated with DFS. A similar role of Ep-CAM in the subgroup of triple-negative breast cancer patients is plausible.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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