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Original Article

Cytoplasmic Trop-1/Ep-CAM Overexpression is Associated with a Favorable Outcome in Node-positive Breast Cancer

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Objective: Trop-1/Ep-CAM modulates growth and survival of transformed cells, and it is highly expressed in most carcinomas including breast cancer. Only membranous staining is typically considered in evaluating Trop-1/epithelial cell adhesion molecule (Ep-CAM) expression in tumor cells. However, there is evidence of retention of Trop-1/Ep-CAM, as functionally incompetent molecules, in intra-cytoplasmic vesicles. Hence, we investigated whether cytoplasmic immunostaining may have an independent clinical significance with respect to membranous staining.
Methods: Membranous and cytoplasmic Trop-1/Ep-CAM expression was immunohistochemically investigated in 642 unilateral breast cancers from patients with a 99-month median follow-up. Multiple correspondence analysis was used to investigate the association between Trop-1/Ep-CAM and other biological variables. The impact of Trop-1/Ep-CAM expression on the patient's outcome was evaluated as event-free survival by the Kaplan–Meier method and proportional hazard Cox model.
Results: While tumors with intermediate/strong membranous staining were mostly associated with concomitant cytoplasmic Trop-1/Ep-CAM expression (97%), tumors with weak-to-nil membranous staining showed intermediate/high cytoplasmic expression in 23% of cases. Cytoplasmic overexpression was associated with a favorable outcome, especially in node-positive patients, regardless of the adjuvant therapy received.
Conclusion: Trop-1/Ep-CAM expression may have different clinical implications according to its subcellular localization.

Key words: cytoplasmic Trop-1/Ep-CAM – breast cancer – node-positive – prognosis

INTRODUCTION

Trop-1/epithelial cell adhesion molecule (Ep-CAM) (known under many different names including ESA and GA733-2) is a highly overexpressed carcinoma-associated antigen encoded by the *TACSTD1/EPCAM* gene (1). It is a transmembrane adhesion molecule that transduces a calcium signal (2) and modulates cell growth and survival. Accordingly, Trop-1/Ep-CAM is mostly expressed by less

differentiated and proliferating cells (3–5). In normal epithelial tissues including the luminal epithelium of mammary gland (6), Trop-1/Ep-CAM localizes to the basolateral membrane, whereas in carcinomas (including breast cancer) its expression pattern shifts to an intense membranous overexpression, frequently associated with cytoplasmic staining (7,8).

The prognostic relevance of Trop-1/Ep-CAM has been demonstrated in several human carcinomas (9,10) including breast cancer, in which membranous overexpression of Trop-1/Ep-CAM has been reported to correlate with poor disease-free and overall survival (11,12). Recently, Trop-1/Ep-CAM/ESA has also been identified as a marker for cancer-initiating stem cells (13,14), making it an interesting target for cancer therapy. In fact, since its discovery (15), Trop-1/Ep-CAM has been exploited as target for antibody-mediated immunotherapy with murine or humanized monoclonal antibodies ((16), and unpublished observations) and also for gene therapy (17,18). Adecatumumab, an antibody directed against Trop-1/Ep-CAM, has recently been found to have a stabilizing effect on disease progression in patients with Trop-1/Ep-CAM-positive advanced breast cancer (19).

Routinely, Trop-1/Ep-CAM expression is evaluated by immunohistochemistry (IHC) for cell surface staining. However, previous experimental findings showed that, in addition to the membrane staining, a specific intra-cellular immunostaining can also be detected (20). Immunofluorescence and electron-microscopy analysis demonstrated that Trop-1/Ep-CAM can accumulate in membranous intra-cellular compartments that include endoplasmic reticulum, Golgi apparatus and other vesicles (Supplementary data, Figs S1 and S2), raising the issue that intra-cellular accumulation may affect Trop-1/Ep-CAM activity in cell adhesion.

To elucidate whether such a specific cytoplasmic staining may have an independent clinical significance, we considered a consecutive series of unilateral primary breast cancers in which Trop-1/Ep-CAM expression was evaluated in parallel at the membranous and cytoplasmic level. Our findings indicate that cytoplasmic Trop-1/Ep-CAM overexpression is associated with a favorable outcome, especially in node-positive patients, regardless of the adjuvant treatment received. The results suggest a different clinical implication of Trop-1/Ep-CAM expression according to its subcellular localization that can be exploited for a best patient prognosis definition.

PATIENTS AND METHODS

Seven hundred consecutive patients treated for a primary breast cancer between January 1989 and December 1993 at the Surgical Units of Ferrara S. Anna Hospital-University or at Surgical Units of the Ferrara province's hospitals were retrospectively included in this study. Informed written consent was obtained from all patients and the University of Ferrara Research Ethics Committee approved the study. Eligible criteria were pathologic stage T1–T3, availability of at least 10 resected axillary lymph nodes, the absence of synchronous bilateral tumors or any other malignancy before breast cancer diagnosis and up to 6 months after surgery, the absence of distant metastases at diagnosis and up to 6 months after surgery and no neo-adjuvant therapy. At diagnosis, 392 patients were classified as node-negative (pN−) and 308 as node-positive (pN+).

According to the treatment protocols applied, 303 of them received an adjuvant therapy. Clinical baseline and patient's follow-up data (date and site of relapse, last follow-up time and date and cause of death) were extracted from the Ferrara Cancer Registry. Data on patient age, tumor histologic type, pathological stage (pT), grading and estrogen receptor (ER) status were also collected. After assessment of routine biological markers, for 642 patients (Table 1), a residual paraffin-embedded tissue material of the primary tumor was available for the immunohistochemical evaluation of Trop-1/Ep-CAM expression. The protocol of this study was approved by the board of the Ministry of the University and Research ('Identification and validation of new markers of metastasizing phenotype of breast cancer', prot. MM06095812_006, year 2000). The article was prepared in agreement with the recommendations for tumor marker reporting studies (21).

Tissue Microarrays and Trop-1/Ep-CAM IHC

Tissue microarray (TMA) blocks were assembled as follows. A Tru-Cut needle (4 mm in internal diameter) was used to punch 3 mm-spaced holes in the recipient block. Donor blocks of formalin-fixed, paraffin-embedded archival primary tumor samples were retrieved after re-evaluation of hematoxylin and eosin-stained sections. Representative tumor areas were identified; 4 mm diameter cores of tumor tissues were removed from each donor block and transferred in the recipient block (24 samples per slide). The TMA was then incubated for 15 min at 37°C to allow the tumor cores to firmly adhere to the recipient block. Consecutive 5 μm-thick sections were cut from the TMA and mounted on polarized slides. Slides were deparaffinized, rehydrated and treated with 3% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. The slides were processed in a microwave oven in a TEC buffer (Tris-citrate-EDTA), pH 7.8, to unmask antigenic sites after formalin fixation. IHC was performed with an automated immunostainer (Ventana NEXES, Medical System, Tucson, AZ, USA). Slides were stained for Trop-1/Ep-CAM using the VU-1D9 antibody (NovoCastra Laboratories Ltd, Newcastle upon Tyne, UK) and Vectastain ABC peroxidase kit (Vector Laboratories, DBA Italia, Segrate, Italy) was used to reveal antibody binding. Slides treated with isotype-matched antibody were used as negative controls. Endogenous biotin was saturated with a biotin-blocking kit (Vector Laboratories). Figure 1 shows some representative examples for specific membranous and cytoplasmic immunostaining.

Table 1. Clinicopathological characteristics of breast cancer patients with available leftover material for Trop-1/Ep-CAM evaluation

Categorical variables	Overall		Node-positive		Node-negative	
	n	Percentage	*n*	Percentage	*n*	Percentage
Age						
34–40	46	7.2	25	8.8	21	5.8
41–50	134	20.9	63	22.3	71	19.8
51–55	77	12.0	43	15.2	34	9.5
56–70	253	39.4	95	33.6	158	44.0
71–90	132	20.5	57	20.1	75	20.9
Total	642	100.0	283	100.0	359	100.0
Histologic type						
Ductal	483	75.2	234	82.7	249	69.4
Lobular	100	15.6	38	13.4	62	17.3
Other types	59	9.2	11	3.9	48	13.3
Total	642	100.0	283	100.0	359	100.0
pT stage						
pT1	413	64.5	143	50.7	270	75.4
pT2	214	33.4	129	45.7	85	23.8
pT3	13	2.1	10	3.6	3	0.8
Total	640	100.0	282	100.0	358	100.0
Histological grade						
G1	390	18.9	31	11.0	90	25.1
G2	130	60.8	178	62.9	212	59.2
G3	641	20.3	74	26.1	56	15.7
Total	121	100.0	283	100.0	358	100.0
Estrogen receptor						
≤10%	113	21.2	63	26.1	50	17.1
>10%	421	78.8	178	73.9	243	82.9
Total	534	100.0	241	100.0	293	100.0
PR						
≤10%	159	30.0	80	33.5	79	27.2
>10%	370	70.0	159	66.5	211	72.8
Total	529	100.0	239	100.0	290	100.0
HER2/neu						
≤10%	435	68.8	181	64.9	254	72.0
>10%	197	31.2	98	35.1	99	28.0
Total	632	100.0	279	100.0	353	100.0
Membranous Ep-CAM score						
0	426	66.5	186	65.7	240	67.2
1+	99	15.5	41	14.5	58	16.2
2+	60	9.4	28	9.9	32	9.0
3+	55	8.6	28	9.9	27	7.6
Total	640	100.0	283	100.0	357	100.0
Cytoplasmic Ep-CAM score						
0	196	30.5	91	32.2	105	29.2
1+	215	33.5	84	29.7	131	36.5
2+	147	22.9	66	23.3	81	22.6
3+	84	13.1	42	14.8	42	11.7
Total	642	100.0	283	100.0	359	100.0
Adjuvant therapies						
Chemotherapy	89	17.2	65	29.5	24	8.0
Hormone therapy	187	36.0	108	49.1	79	26.4
Chemotherapy plus Hormone therapy	27	5.2	25	11.4	2	0.7
No therapy	216	41.6	22	10.0	194	64.9
Total	519	100.0	220	100.0	299	100.0

pT stage, pathological stage.

Two pathologists (R.L. and M.P.) independently examined all TMA sections. For each tumor at least 400 cells were counted, and membranous (Trop-1/Ep-CAMm) and cytoplasmic (Trop-1/Ep-CAMc) expression were recorded. In both cases, the staining intensity was scored as 0, 1, 2 or 3 corresponding to the presence of negative, weak, intermediate and strong staining, respectively. The total number of cells and the number of cells stained were counted; the percentage was calculated and categorized according to a positivity score: 0, no colored cells; 1, 1–9%; 2, 10–49%; 3, 50–79%; 4, 80–100%. The total score was calculated by multiplying intensity score by positivity score, and categorized as follows: 0, negative total score; 1+, total score 1–4; 2+, total score 5–8; 3+, total score 9–12. According to Spizzo et al. (22,23), who defined a total score >4 as Trop-1/Ep-CAM overexpression, in the statistical analysis Trop-1/Ep-CAM expression was dichotomized in low-to-nil (i.e. categories 0 and 1+, corresponding to a total score ≤4) and intermediate/high (i.e. categories 2+ and 3+, corresponding to a total score >4). Additional biological variables were categorized according to conventional cut-offs (Table 1).

Statistical Analysis

The association among Trop-1/Ep-CAMc, Trop-1/Ep-CAMm and clinicopathological characteristics was evaluated by means of the odds ratio (OR) with exact 95% confidence interval (CI) (function Fisher's test of the stats package of R) (24). The agreement between Trop-1/Ep-CAMc and Trop-1/Ep-CAMm levels was assessed by kappa statistic (κ).

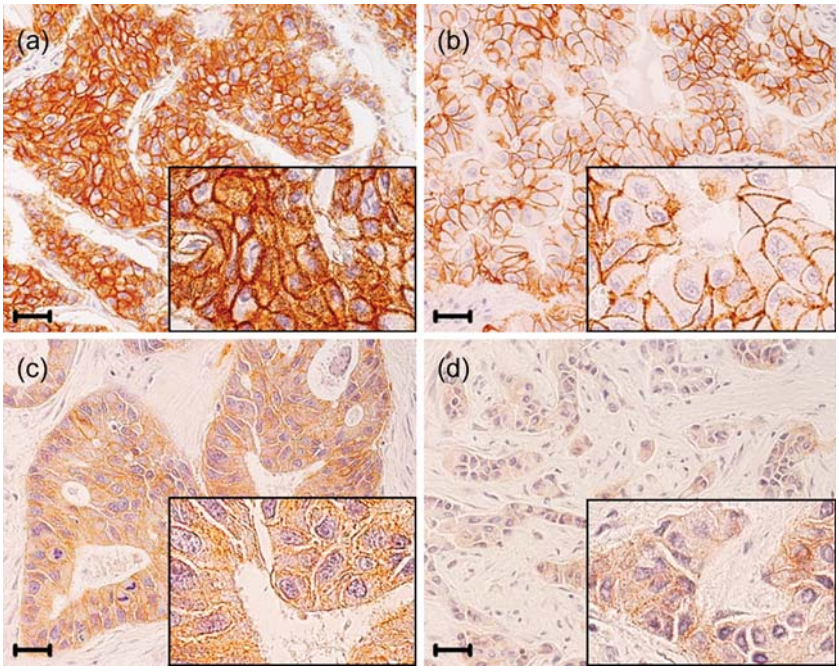

Figure 1. Immunohistochemical analysis of Trop-1/Ep-CAM expression in breast cancer. (a) Strong membranous and cytoplasmic expression. (b) Strong membranous and weak cytoplasmic expression. (c) Weak membranous and strong cytoplasmic expression. (d) Weak membranous and cytoplasmic expression. The insert provides details of Trop-1/Ep-CAM expression patterns. Original magnification 40× (scale bars = 20 μm)

The κ value was interpreted as follows: $\kappa < 0$ when the observed agreement was less than that expected by chance (disagreement); $0 \leq \kappa \leq 0.2$ slight agreement; $0.21 \leq \kappa \leq 0.4$ fair agreement; $0.41 \leq \kappa \leq 0.6$ moderate agreement; $0.61 \leq \kappa \leq 0.8$ substantial agreement; $0.81 \leq \kappa \leq 1.0$ almost perfect agreement.

The associations among Trop-1/Ep-CAMc and Trop-1/Ep-CAMm expression and other biological variables were investigated and visualized through multiple correspondence analysis (MCA) that visualizes on a bi-dimensional plot the association of both categorical and continuous variables (25). MCA has the advantage of implying neither linearity nor specific distribution characteristics, and of visualizing association between markers and tumors. Markers are labeled according to their category, whereas the points representing the tumors are not shown to improve figure readability. Points close to each other correspond to tumors with similar characteristics, whereas close marker labels correspond to associated marker categories. The use of a bi-dimensional plot, easy to interpret, is possible at the expense of losing some information on the pattern of associations. The distance between points is based on a χ^2 metric, whereas the measure on the axes does not have any physical meaning.

The effect of Trop-1/Ep-CAMc or Trop-1/Ep-CAMm expression on the patient outcome was evaluated by survival analysis using as endpoint the time elapsed from surgery to the occurrence of the first adverse event (e.g. local relapse, distant metastasis, contralateral tumor, a second tumor and death without evidence of neoplastic disease). Event-free survival curves were plotted by the Kaplan–Meier method. A proportional hazard multivariable Cox model was used to estimate the Trop-1/Ep-CAM effect adjusted for age, ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2), pT, grading and the number of metastatic lymph nodes. To evaluate the appropriateness of the proportional hazard Cox model assumption, Schoenfeld residuals were analyzed (26). Hazard ratios (HRs) with 95% CI were used to quantify the prognostic impact of variables. R software (http://www.r-project.org) was utilized throughout this study. The median follow-up was estimated by the reversed Kaplan–Meier method (27).

RESULTS

Overall, 525 (82%) tumors showed low-to-nil (0, 1+ total score) Trop-1/Ep-CAMm expression and 411 (64%) had low-to-nil (0, 1+ total score) Trop-1/Ep-CAMc expression (Table 1). However, while tumors with intermediate/high (2+, 3+ total score) Trop-1/Ep-CAMm expression were mostly associated with concomitant intermediate/high Trop-1/Ep-CAMc expression (111/115, 97%), those with low-to-nil Trop-1/Ep-CAMm expression showed intermediate/high Trop-1/Ep-CAMc expression in a non-negligible number of cases (120/525, 23%) (Supplementary data, Table S1). The overall agreement (i.e. membrane and cytoplasm both with low-to-nil or intermediate/high Trop-1/Ep-CAM expression) accounted for 81% of cases (516/640). The disagreement was distributed as follows: low-to-nil Trop-1/Ep-CAMm expression was associated with

intermediate/high Trop-1/Ep-CAMc expression in 120 cases (19% of the total), whereas intermediate/high Trop-1/Ep-CAMm expression was associated with low-to-nil Trop-1/Ep-CAMc expression in only 4 cases (0.6% of the total) (Supplementary data, Table S1). The OR for association between Trop-1/Ep-CAMc and Trop-1/Ep-CAMm was 93 (95% CI 34–354). The agreement between Trop-1/Ep-CAMc and Trop-1/Ep-CAMm was moderate ($\kappa = 0.53$).

When we considered the pN status, 123 (34%) node-negative tumors showed intermediate/high Trop-1/Ep-CAMc expression, associated with concomitant intermediate/high Trop-1/Ep-CAMm expression in about half of the cases (59/123, 48%). Similarly, 108 (38%) node-positive tumors showed intermediate/high Trop-1/Ep-CAMc, associated with concomitant intermediate/high Trop-1/Ep-CAMm expression in 56 cases (52%), suggesting the lack of association between nodal involvement and Trop-1/Ep-CAM expression of the primary tumor as supported by statistical analysis (OR 1.2; 95% CI 0.8–1.7).

As regards the hormone steroid receptor status, 13% (55/421) of ER-positive (>10%) and 32% (36/113) of ER-negative (≤10%) tumors showed intermediate/high Trop-1/Ep-CAMm expression (OR 0.3; 95% CI 0.2–0.5), whereas 32% (136/421) of ER-positive and 51% (58/113) of ER-negative tumors showed intermediate/high Trop-1/Ep-CAMc expression (OR 0.5; 95% CI 0.3–0.7). Similarly, 13% (48/368) of PR-positive (>10%) and 25% (39/159) of PR-negative (≤10%) tumors had intermediate/high Trop-1/Ep-CAMm expression (OR 0.5; 95% CI 0.3–0.8), whereas 34% (125/370) of PR-positive and 41% (65/159) of PR-negative tumors showed intermediate/high Trop-1/Ep-CAMc expression (OR 0.7; 95% CI 0.5–1.1). Overall, the findings seem to suggest an inverse relationship between ER or PR status and Trop-1/Ep-CAM overexpression. Conversely, no association was found between HER2 and Trop-1/Ep-CAM expression. In fact, 19% (38/197) of HER2-positive (>10%) and 18% (77/433) of HER2-negative (≤10%) tumors showed intermediate/high Trop-1/Ep-CAMm expression (OR 1.1; 95% CI 0.7–1.7), whereas 35% (69/197) of HER2-positive (>10%) and 37% (160/435) of HER2-negative (≤10%) tumors showed intermediate/high Trop-1/Ep-CAMc expression (OR 0.9; 95% CI 0.6–1.3).

Association Analysis

The associations between Trop-1/Ep-CAM and other clinico-biological variables, namely patient age, histologic type, tumor grading, pT, ER and PR status and HER2 were studied through MCA, which allows one to visualize, on an easy to interpret bi-dimensional plot, the associations of both categorical and discretized continuous variables (25). Although MCA provides much more information than conventional contingency tables, for assisting the non-familiar MCA reader, a supplementary contingency table, which details case series description according to different categories of the Ep-CAM score, is also provided (Supplementary data, Table S1).

MCA indicated that the two first axes explained the 57.7% of the total variability (respectively, 44.7% the first axis and 13.0% the second axis). As shown in Fig. 2, the first axis separates ER ≤10, PR ≤10, HER2 >10, high Trop-1/Ep-CAM scores, G3 (on the left) from ER >10, PR >10, HER2 ≤10, low Trop-1/Ep-CAM scores, G1 (on the right). The second axis mostly separates low Trop-1/Ep-CAM scores and G3 (below) from high Trop-1/Ep-CAM scores and G1 (up). As expected, age category ≤40 appears remarkably associated with unfavorable prognostic variable categories (namely, pT >1, G3, HER2 >10 and N+).

Event-Free Survival Analysis

The follow-up of the study was closed on 31 December 2002. The median follow-up of the 642 patients was 99 months (range, 1–157 months) even though it was curtailed at 8 years when the probability of patients of being in follow-up was ∼56%. During follow-up, 96 patients developed distant metastases, 48 a local relapse, 13 a contralateral tumor, 30 another malignancy and 91 dead as the first event. The estimated crude cumulative incidences at 8 years are, respectively, 15.3, 7.7, 2.1, 4.8 and 14.7%.

The Kaplan–Meier survival estimates, according to Trop-1/Ep-CAMm or Trop-1/Ep-CAMc expression, are shown in Figs 3–5. It is noteworthy that Trop-1/Ep-CAMm and Trop-1/Ep-CAMc expression were differently associated

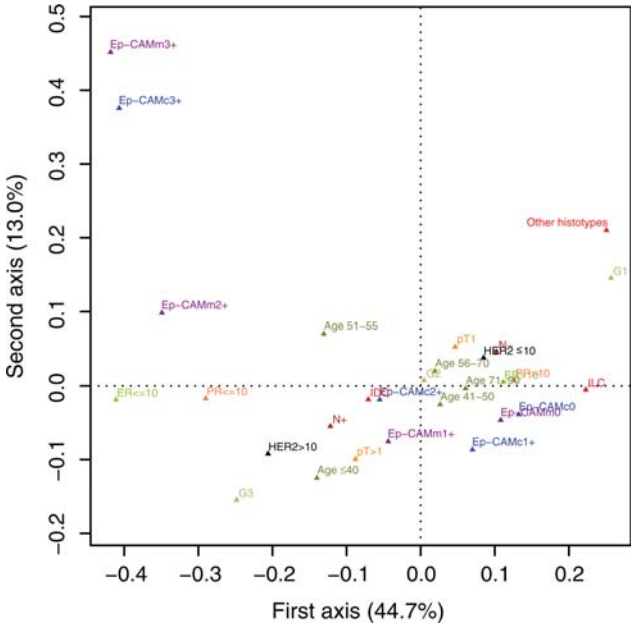

Figure 2. Association between membranous (Trop-1/Ep-CAMm) and cytoplasmic (Trop-1/Ep-CAMc) Trop-1/Ep-CAM expression and other clinico-biological variables. The association was evaluated by multiple correspondence analysis. The triangles represent the categories. The distance between the labels, based on a χ^2 metric, is a measure of the dissimilarity of the corresponding categories.

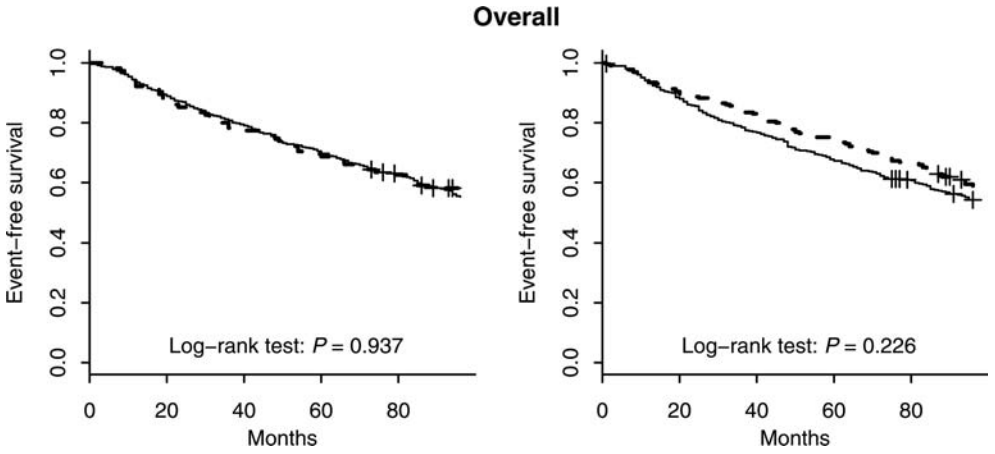

Figure 3. Kaplan–Meier event-free survival curves stratified according to membranous (Trop-1/Ep-CAMm, on the left) and cytoplasmic Trop-1/Ep-CAM expression (Trop-1/Ep-CAMc, on the right) in the overall case series. Solid line: low-to-nil expression; thick dashed line: intermediate/high expression.

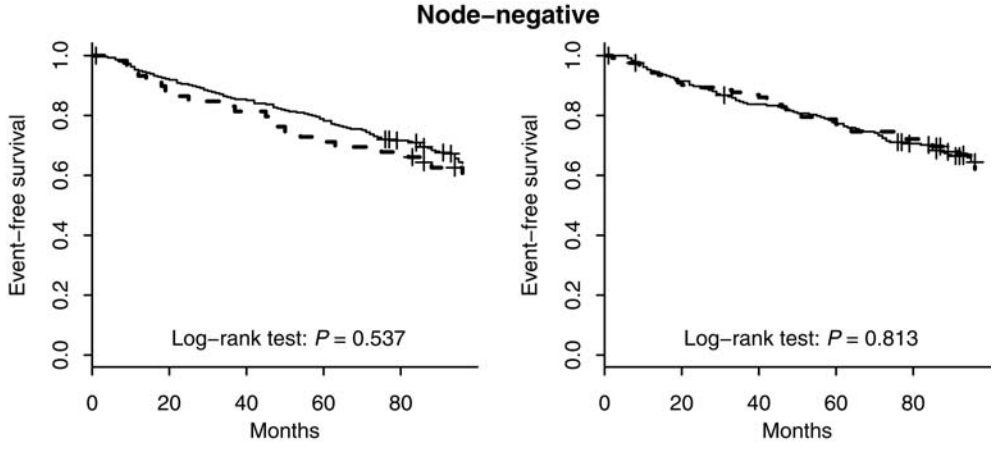

Figure 4. Kaplan–Meier event-free survival curves stratified according to membranous (Trop-1/Ep-CAMm, on the left) and cytoplasmic Trop-1/Ep-CAM expression (Trop-1/Ep-CAMc, on the right) in the node-negative breast cancer subset. Solid line: low-to-nil expression; thick dashed line: intermediate/high expression.

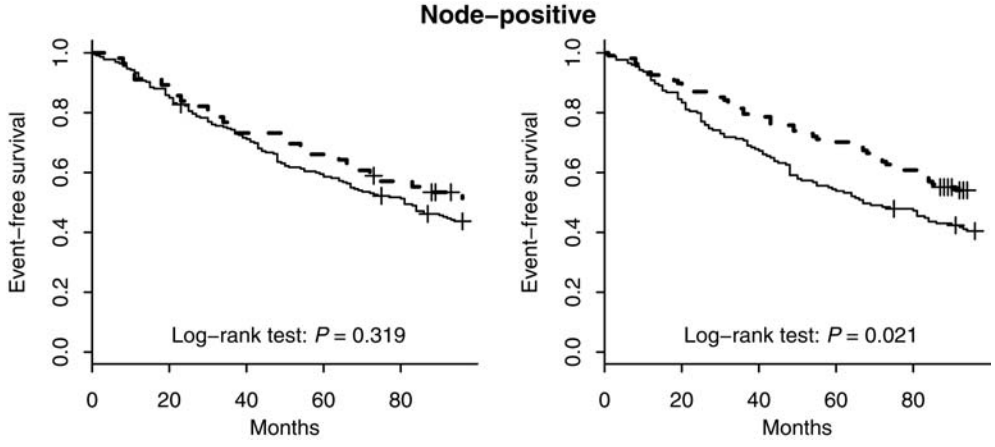

Figure 5. Kaplan–Meier event-free survival curves stratified according to membranous (Trop-1/Ep-CAMm, on the left) and cytoplasmic Trop-1/Ep-CAM expression (Trop-1/Ep-CAMc, on the right) in the node-positive breast cancer subset. Solid line: low-to-nil expression; thick dashed line: intermediate/high expression.

with prognosis. In fact, while Trop-1/Ep-CAMm expression did not affect the patient outcome, Trop-1/Ep-CAMc was associated with a favorable prognosis, particularly in patients with a node-positive tumor (Fig. 5) where intermediate/high Trop-1/Ep-CAMc expression levels provided a lower hazard with respect to low-to-nil expression level (HR 0.67; CI 0.48–0.94, $P = 0.021$).

The favorable association between Trop-1/Ep-CAMc expression and the outcome was evident also when the membranous status was concomitantly considered. In fact, as shown in Fig. 6, intermediate/high Trop-1/Ep-CAMc and low-to-nil Trop-1/Ep-CAMm expression levels were associated with a favorable prognosis with respect to low-to-nil Trop-1/Ep-CAMc and low-to-nil Trop-1/Ep-CAMm expression (HR 0.65; $P = 0.05$). Furthermore, intermediate/high Trop-1/Ep-CAMc and Trop-1/Ep-CAMm expression levels were associated with a favorable prognosis with respect to low-to-nil Trop-1/Ep-CAMc and Trop-1/Ep-CAMm expression (HR 0.71; $P = 0.12$), although not significantly.

Since most node-positive patients received an adjuvant therapy, we explored the prognostic impact of Trop-1/Ep-CAMc overexpression according to the treatment. As shown in Fig. 7, intermediate/high Trop-1/Ep-CAMc levels were associated with a favorable outcome regardless of the treatment modalities.

When we explored the association between Trop-1/Ep-CAMc expression and ER status (Fig. 8), we found that in the node-positive subset, Trop-1/Ep-CAMc overexpression was able to better define patients with a favorable prognosis especially within the ER-positive subgroup.

In the multivariable regression model, some relevant prognostic factors (tumor grade, the number of metastatic lymph nodes, ER, PR and HER2 status) were included for adjusting the Ep-CAM effect. The results are reported in Table 2. Patients whose tumor had intermediate/high expression of cytoplasmic Trop-1/Ep-CAM had a favorable outcome when compared with patients with low-to-nil Trop-1/Ep-CAMc expression (HR 0.60; CI 0.40–0.89).

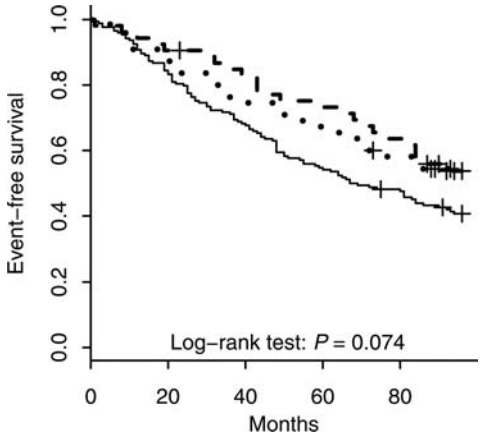

Figure 6. Kaplan–Meier event-free survival curves stratified according to membranous (Trop-1/Ep-CAMm) and cytoplasmic (Trop-1/Ep-CAMc) expression in node-positive patients. Solid line: low-to-nil Trop-1/Ep-CAMm and low-to-nil Trop-1/Ep-CAMc; thick dashed line: low-to-nil Trop-1/Ep-CAMm and intermediate/high Trop-1/Ep-CAMc; dots line: intermediate/high Trop-1/Ep-CAMm and intermediate/high Trop-1/Ep-CAMc. The class low-to-nil Trop-1/Ep-CAMc and intermediate/high Trop-1/Ep-CAMm included only one patient who relapsed at 25 months.

DISCUSSION

Trop-1/Ep-CAM overexpression on neoplastic tissues is correlated with cellular proliferation and de-differentiation. For this putative involvement in cancer progression, in the last decade Trop-1/Ep-CAM expression has received increasing attention as a prognostic factor and potential target of therapy in many malignancies. In breast cancer, in particular, Trop-1/Ep-CAM overexpression, immunohistochemically evaluated, has been reported to correlate with poor prognosis in node-positive as well as in node-negative patients (11, 12, 22, 23) although there is no consensus regarding the prognostic significance of the molecule. That is principally because the real biological role of Trop-1/Ep-CAM remains unclear as well demonstrated by its recurring ‘discovery’ and the plethora of names used to identify it. Some studies have shown that loss of Trop-1/Ep-CAM expression is required

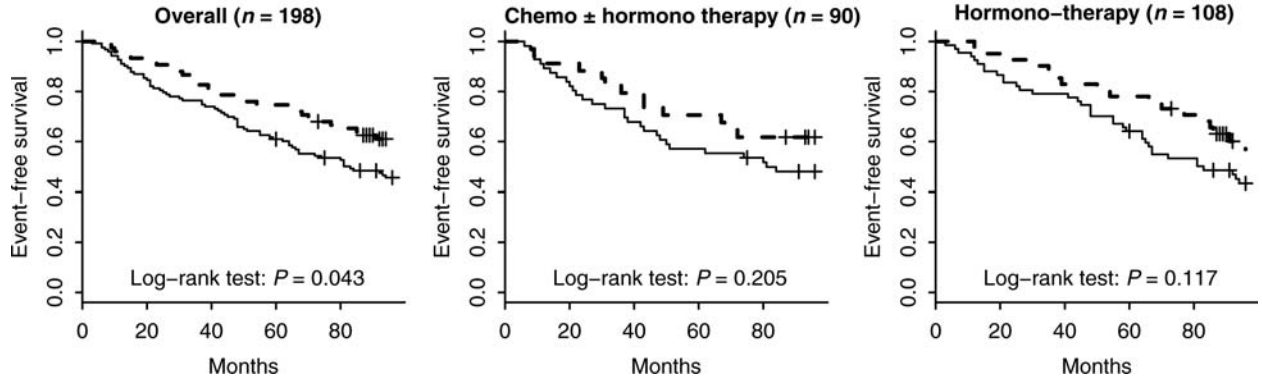

Figure 7. Kaplan–Meier event-free survival curves stratified according to cytoplasmic Trop-1/Ep-CAM expression and adjuvant therapy (tamoxifen alone versus chemotherapy with or without tamoxifen) in node-positive patients. Solid line: low-to-nil expression; thick dashed line: intermediate/high expression.

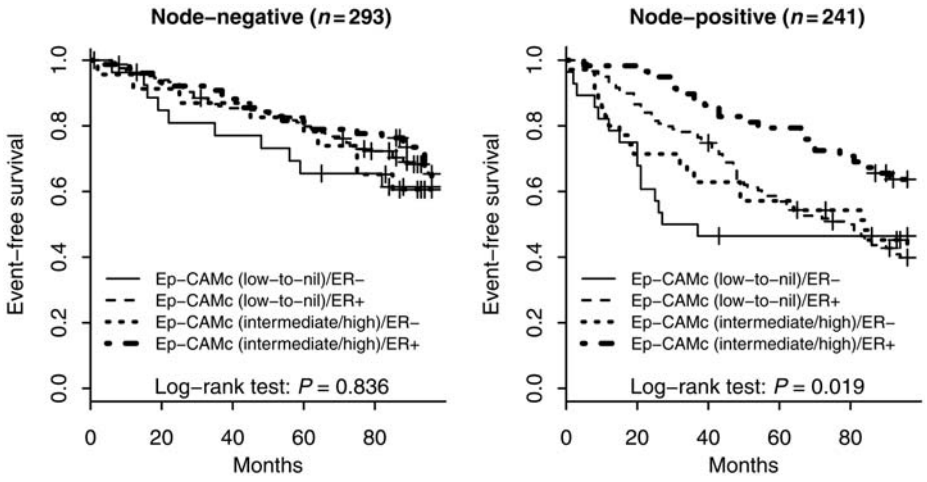

Figure 8. Kaplan–Meier event-free survival curves stratified according to cytoplasmic Trop-1/Ep-CAM expression and the estrogen receptor (ER) status (cut-off value = 10%).

Table 2. Risk analysis for event-free survival in a multivariate Cox model in node-positive patients

Variable	Coefficient estimate	HR	95% CI	*P* value
Ep-CAMc intermediate/high versus low-to-nil	−0.51	0.60	0.40–0.89	0.012
pT>1 versus pT1	0.39	1.48	1.02–2.15	0.040
G2 versus G1	−0.32	0.73	0.40–1.31	0.288
G3 versus G1	−0.18	0.84	0.43–1.64	0.601
Age	0.01	1.01	0.99–1.02	0.299
ER+ versus ER−	−0.20	0.82	0.50–1.35	0.432
PR+ versus PR−	−0.35	0.70	0.46–1.09	0.114
HER2+ versus HER2−	−0.23	0.79	0.53–1.19	0.262
4–9 nodes versus 1–3 nodes	0.17	1.19	0.76–1.85	0.454
>9 nodes versus 1–3 nodes	0.93	2.53	1.60–3.99	<0.0001

HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

for tumor cell migration because of a decrease in the cytoskeleton-anchored fraction of E-cadherin, thereby leading to a reduction in the intercellular adhesion (28). On the contrary, it has been reported that Trop-1/Ep-CAM overexpression induces oncogene upregulation and cell proliferation (29). In addition, in all clinical studies only membranous Trop-1/Ep-CAM expression was considered, thus not considering the potential biological significance of other subcellular protein localizations in comparison with the cytoplasmic membrane.

Our previous experimental findings showed that Trop-1/Ep-CAM may also accumulate in membranous intra-cellular compartments, i.e. endoplasmic reticulum, Golgi apparatus and other vesicles (Supplementary data, Figs S1 and S2), raising the issue that intra-cellular accumulation may affect Trop-1/Ep-CAM function as it may prevent activity at the cell membrane (20). Hence, we assessed both membranous and cytoplasmic Trop-1/Ep-CAM expression on a large breast cancer case series. We found that cytoplasmic immunostaining was present in ∼70% of cases and that intermediate/high expression levels were associated with a favorable outcome, evaluated as event-free survival, in node-positive patients irrespective of the adjuvant therapy (cytotoxic or hormonal) administered. Remarkable also was the finding that cytoplasmic Trop-1/Ep-CAM overexpression was able to identify patients with an unfavorable outcome within the ER-positive group, usually associated with a good prognosis. As a whole, the present findings indicate that cytoplasmic expression provided useful information on node-positive primary tumors, hence allowing an important prognostic refinement. The finding that, in our case series, neither membranous nor cytoplasmic Trop-1/Ep-CAM expression was predictive in node-negative patients is not surprising because, while Schmidt et al. (12) suggested the usefulness of EpCAM as an independent marker in overall survival, Tandon et al. (30) did not find any correlation.

From a biological point of view, the presence of intermediate/high levels of Trop-1/Ep-CAM in the cytoplasm could be explained by its functional accumulation in the membranous intra-cellular compartments with the aim to regulate the protein localization at the cell membrane where it may affect cell proliferation and cell–cell adhesion. Indeed, Trop-1/Ep-CAM negatively modulates E-cadherin-mediated adhesion by disrupting the link between α-catenin and F-actin (31,32). Therefore, the intra-cellular accumulation of Trop-1/Ep-CAM may represent a way to circumvent its oncogenic potential and/or maintain epithelial cells in a differentiated state. It should be noted that subcellular delocalization is a phenomenon recently observed in several other proteins involved in cell adhesion and polarity including, for example, Lgl and Scribble proteins (33–35).

Because of the cross talk among the different proteins involved in epithelial cell polarity and adhesion, it is evident that such a mislocalization induces an overall functional inactivation of polarity pathways resulting in an altered cell polarization and epithelial tissue assembly and actually promoting cancer cell motility and invasion (36–38). Our findings indicate that Trop-1/Ep-CAM may have a similar behavior with diverse clinical implications according to subcellular localization.

Supplementary data

Supplementary data are available at http://www.jjco.oxfordjournals.org.

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Conflict of interest statement

None declared.

References

1. Calabrese G, Crescenzi C, Morizio E, Palka G, Guerra E, Alberti S. Assignment of TACSTD1 (alias TROP1, M4S1) to human chromosome 2p21 and refinement of mapping of TACSTD2 (alias TROP2, M1S1) to human chromosome 1p32 by *in situ* hybridization. *Cytogenet Cell Genet* 2001;92:164–5.
2. Ripani E, Sacchetti A, Corda D, Alberti S. The human Trop-2 is a tumor-associated calcium signal transducer. *Int J Cancer* 1998;76:671–6.
3. Klein CE, Hartmann B, Schön MP, Weber L, Alberti S. Expression of 38-kD cell-surface glycoprotein in transformed human keratinocyte cell lines, basal cell carcinomas, and epithelial germs. *J Invest Dermatol* 1990;95:74–82.
4. Schön MP, Schön M, Mattes MJ, et al. Biochemical and immunological characterization of the human carcinoma-associated antigen MH 99/KS 1/4. *Int J Cancer* 1993;55:988–95.
5. Zanna P, Trerotola M, Vacca G, et al. Trop-1 is a novel cell growth stimulatory molecule that marks early stages of tumor progression. *Cancer* 2007;110:452–64.
6. MacDougall JR, Matrisian LM. Targets of extinction: identification of genes whose expression is repressed as a consequence of somatic fusion between cells representing basal and luminal mammary epithelial phenotypes. *J Cell Sci* 2000;113:409–23.
7. Gosens MJ, van Kempen LCL, van de Velde CJH, van Krieken JHJM, Nagtegaal ID. Loss of membranous Ep-CAM in budding colorectal carcinoma cells. *Mod Pathol* 2007;20:221–32.
8. Yanamoto S, Kawasaki G, Yoshitomi I, Iwamoto T, Hirata K, Mizuno A. Clinicopathologic significance of EpCAM expression in squamous cell carcinoma of the tongue and its possibility as a potential target for tongue cancer gene therapy. *Oral Oncol* 2007;43:869–77.
9. Went PT, Lugli A, Meier S, et al. Frequent EpCAM protein expression in human carcinomas. *Hum Pathol* 2004;35:122–8.
10. Went P, Dirnhofer S, Schöpf D, Moch H, Spizzo G. Expression and prognostic significance of EpCAM. *J Cancer Mol* 2008;3:169–74.
11. Gastl G, Spizzo G, Obrist P, Dünser M, Mikuz G. Ep-CAM over-expression in breast cancer as a predictor of survival. *Lancet* 2000;356:1981–2.
12. Schmidt M, Hasenclever D, Schaeffer M, et al. Prognostic effect of epithelial cell adhesion molecule over-expression in untreated node-negative breast cancer. *Clin Cancer Res* 2008;14:5849–55.
13. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003;100:3983–8.
14. Visvader JE, Lindeman GJ. Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nat Rev Cancer* 2008;8:755–68.
15. Herlyn M, Steplewski Z, Herlyn D, Koprowski H. Colorectal carcinoma-specific antigen: detection by means of monoclonal antibodies. *Proc Natl Acad Sci USA* 1979;76:1438–42.
16. Riethmuller G, Holz E, Schlimok G, et al. Monoclonal antibody therapy for resected Dukes' C colorectal cancer: seven-year outcome of a multicenter randomized trial. *J Clin Oncol* 1998;16:1788–94.
17. Haisma HJ, Pinedo HM, Rijswijk A, et al. Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM. *Gene Ther* 1999;6:1469–74.
18. Osta WA, Chen Y, Mikhitarian K, et al. EpCAM is overexpressed in breast cancer and is a potential target for breast cancer gene therapy. *Cancer Res* 2004;64:5818–24.
19. Schmidt M, Scheulen ME, Dittrich C, et al. An open-label, randomized phase II study of adecatumumab, a fully human anti-EpCAM antibody, as monotherapy in patients with metastatic breast cancer. *Ann Oncol* 2010;21:275–82.
20. Balzar M, Briaire-De Bruijn IH, Rees-Bakker H, et al. Epidermal growth factor-like repeats mediate lateral and reciprocal interactions of Ep-CAM molecules in homophilic adhesions. *Mol Cell Biol* 2001;21:2570–80.
21. McShane LM, Altman DG, Sauerbrei W, Taube SE, Gion M, Clark GM. Statistics subcommitee of the NCI-EORTC working group on cancer diagnostics. Reporting recommendations for tumour marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005;97:1180–4.
22. Spizzo G, Obrist P, Ensinger C, et al. Prognostic significance of Ep-CAM and Her-2/neu over-expression in invasive breast cancer. *Int J Cancer* 2002;98:883–8.
23. Spizzo G, Went P, Dirnhofer S, et al. High Ep-CAM expression is associated with poor prognosis in node-positive breast cancer. *Breast Cancer Res Treat* 2004;86:207–13.
24. R Development Core Team. *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing, 2011, ISBN 3–900051–07–0, URL. http://www.R-project.org/.
25. Greenacre M. *Correspondence Analysis in Practice*, 2nd edn. London: Chapman & Hall/CRC, 2007.
26. Schoenfeld D. Partial residuals for the proportional hazard regression model. *Biometrica* 1982;69:239–41.
27. Schemper M, Smith TL. A note on quantifying follow-up in studies of failure time. *Clin Trials* 1996;17:343–6.
28. Litvinov SV, Balzar M, Winter MJ, et al. Epithelial cell adhesion molecule (Ep-CAM) modulated cell-cell interactions mediated by classic cadherins. *J Cell Biol* 1997;139:1337–48.
29. Munz M, Kieu C, Mack B, Schmitt B, Zeidler R, Gires O. The carcinoma-associated antigen EpCAM upregulates c-myc and induces cell proliferation. *Oncogene* 2004;23:5748–58.
30. Tandon AK, Clark GM, Chamness GC, McGuire WL. Association of the 323/A3 surface glycoprotein with tumour characteristics and behaviour in human breast cancer. *Cancer Res* 1990;50:3317–21.
31. Winter MJ, Nagelkerken B, Mertens AE, Rees-Bakker HA, Briaire-de Bruijn IH, Litvinov SV. Expression of Ep-CAM shifts the state of cadherin-mediated adhesions from strong to weak. *Exp Cell Res* 2003;285:50–8.

32. Winter MJ, Cirulli V, Briaire-de Bruijn IH, Litvinov SV. Cadherins are regulated by Ep-CAM via phosphaditylinositol-3 kinase. *Mol Cell Biochem* 2007;302:19–26.
33. Bilder D, Perrimon N. Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* 2000;403:676–80.
34. Albertson R, Chabu C, Sheehan A, Doe CQ. Scribble protein domain mapping reveals a multistep localization mechanism and domains necessary for establishing cortical polarity. *J Cell Sci* 2005;117:6061–70.
35. Grifoni D, Garoia F, Bellosta P, et al. aPKC cortical loading is associated with Lgl cytoplasmic release and tumor growth in Drosophila and human epithelia. *Oncogene* 2007;26:5960–5.
36. Gardiol D, Zacchi A, Petrera F, Stanta G, Banks L. Human discs large and scrib are localized at the same regions in colon mucosa and changes in their expression patterns are correlated with loss of tissue architecture during malignant progression. *Int J Cancer* 2006;119:1285–90.
37. Zhan L, Rosenberg A, Bergami KC, et al. Deregulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* 2008;135:865–78.
38. Lisovsky M, Dresser K, Baker S, et al. Cell polarity protein Lgl2 is lost or aberrantly localized in gastric dysplasia and adenocarcinoma: an immunohistochemical study. *Mod Pathol* 2009;22:977–84.