Carcinoembryonic Antigen Cell Adhesion Molecule 6 Predicts Breast Cancer Recurrence following Adjuvant Tamoxifen

Loaie Maraqa, Michele Cummings, Mark B. Peter, Abeer M. Shaaban, Kieran Horgan, Andrew M. Hanby, and Valerie Speirs

Abstract

Purpose: Tamoxifen remains therapy of choice for premenopausal estrogen receptor α – positive breast cancer. However, resistance and recurrence are serious problems. Our previous work indicated that carcinoembryonic antigen cell adhesion molecule 6 (CEACAM6) was significantly up-regulated in tamoxifen-resistant (TAMr) MCF-7 derivatives. The aim of this study was to determine the functional role of CEACAM6 in endocrine-resistant breast cancer and to retrospectively test whether it was predictive of resistance in a large cohort of breast cancers with long-term follow-up.

Experimental Design: siRNA silencing of CEACAM6 was done in TAMr cells and effects on clonogenicity and anchorage independence were determined. CEACAM6 immunohistochemistry was done on a tissue microarray comprising 108 relapsed primary breast cancers and 243 tamoxifen-sensitive controls.

Results: siRNA-mediated silencing of CEACAM6 reduced both clonogenicity and anchorage-dependent and anchorage-independent growth of TAMr cells. Importantly, CEACAM6 silencing restored sensitivity of TAMr cells to 4-hydroxytamoxifen and proliferative response to 17β-estradiol. Immunohistochemistry showed significantly more CEACAM expression in the relapsed group compared with nonrelapsed controls [35 of 108 (33.3%) and 32 of 243 (13.2%), respectively; odds ratio, 3.16 (95% confidence interval, 1.83-5.47); P < 0.0001]. Additionally, we derived an outcome predictor model based on CEACAM expression that re-stratified patients in the Nottingham prognostic index intermediate-risk group into either higher-risk or lower-risk group.

Conclusions: Our data support an important role for CEACAM6 in endocrine resistance, which can serve as a powerful predictor of future recurrence.

Materials and Methods

Cell lines and culture. We developed a TAMr derivative of MCF-7, MML1 (4, 14). Briefly, MCF-7 cells were cultured in phenol red-free RPMI 1640 containing l-glutamine (Invitrogen) supplemented with 5% charcoal-stripped steroid-depleted FCS (Harlan SeraLab), 100 units/mL penicillin and 100 units/mL streptomycin, and 100 nmol/L 6 (CEACAM6) was significantly up-regulated by 20-fold in tamoxifen-resistant (TAMr) MCF-7 derivatives compared with sensitive parental controls (4). CEACAM6 is overexpressed in several human cancers including colorectal adenomas (5) and carcinomas (6), gastric cancers (7), and pancreatic cancers (8, 9). Moreover, CEACAM6 overexpression in colorectal cancer cells has been correlated with reduced overall survival and disease-free survival (6), and CEACAM6 overexpression in colon cancer cells prevented colonocyte differentiation and promoted tumorigenicity in nude mice (10). In pancreatic cancer cells, CEACAM6 overexpression was associated with anoikis resistance and in vivo metastasis (9). CEACAM6 is expressed in breast cancer (11, 12) and its expression in atypical ductal hyperplasia may be predictive of those cases that subsequently develop invasive breast cancer (13).

The aim of this study was to determine the functional role of CEACAM6 in endocrine-resistant breast cancer and to retrospectively test whether it was predictive of resistance in a large cohort of clinical breast cancer with long-term follow-up.
4-hydroxytamoxifen (4-HT; Sigma) for 12 months during which the 4-HT–resistant variant MCF-7MMU1 developed (4). Parental cells were cultured in the same media, but with ethanol vehicle. All experiments were conducted in phenol red–free RPMI 1640 supplemented with 5% charcoal-stripped steroid-depleted FCS. 4-HT or 17β-estradiol (E2). Mycoplasma checks were consistently negative.

**siRNA vector construction and stable transfection.** Double-stranded oligonucleotides containing an inverted repeat of the CEACAM6 siRNA sequence (5¶-CCGGACAGTTCCATGTATA-3¶; ref. 9) were designed using the pSilencer insert design tool and cloned into pSilencer 3.1-H1 neo (Ambion). pSilencer-CEACAM6 was transfected into MMU1 cells using LipofectAMINE 2000 (Invitrogen). Control cells were transfected with pSilencer-3.1-H1 neo-control (Ambion), encoding a nontargeting shRNA. Stable clones were selected and maintained in G418-containing medium (250 µg/mL).

**Real-time PCR.** Total RNA was extracted using Trizol (Invitrogen) and treated with TURBO-DNase (Ambion). RNA (0.5 µg) was reverse transcribed with 250 pg of random hexamers using Superscript II reverse transcriptase (Invitrogen). Real-time PCR for CEACAM6 mRNA was carried out using SYBR green master mix (Applied Biosystems) on the ABI Prism 7700, and expression was normalized to RPLP0 mRNA (4). Primer sequences were CEACAM6, 5¶-CACCGTCCGGCATCAGCA-3¶ (forward) and 5¶-AGGAGTTGAGCGCTGTCAGC-3¶ (reverse); RPLP0, 5¶-GAACTCTGCATTCTCGCTTCC-3¶ (forward) and 5¶-GATGCAAGTGTTGAGCCA-3¶ (reverse).

**Western blotting.** Cells were washed with PBS and scraped into chilled lysis buffer [50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA, 1% NP40, 0.25% Na deoxycholate] containing protease inhibitor cocktail (Roche) and 1 mmol/L sodium orthovanadate. Lysates were assayed for protein using Bradford reagent (Pierce), resolved by denaturing SDS-PAGE, then electroblotted onto polyvinylidene difluoride membranes (Millipore). Proteins were detected with anti-CEACAM6 (Abcam) and anti–β-actin (clone AC-15; Sigma) and visualized with SuperSignal West Pico enhanced chemiluminescence reagent (Pierce).

**Clonogenic and anchorage-independent growth assays.** MMU1 cells were transfected with pSilencer-CEACAM6 or pSilencer-control, then selected with G418-containing medium for 20 days at 37°C. Colonies were fixed with 70% ethanol, stained with 0.1% crystal violet, and counted. Anchorage-independent growth assays were established by seeding 5 × 10⁵ cells per well (in triplicate) in six-well plates in growth medium containing 0.4% noble agar (U.S. Biologicals) onto a bottom layer of 0.6% noble agar in growth medium. Following 16 days of incubation at 37°C, colonies >200 µm in diameter were counted.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays.** Cells were seeded in 96-well plates. The next day, 4-HT (0.1-100 nmol/L), E2 (0.01-10 nmol/L; both Sigma), or ethanol vehicle was added. At 96 h posttreatment, cells were incubated in 1 mmol/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide for 4 h. The formazan product was solubilized in propan-1-ol and read at 570 nm (Opsys MR 15, Dynex Technologies).

**Clinical material.** Ethical approval was obtained from the Leeds (East) Local Research Ethics Committee at St. James’s University Hospital, Leeds, United Kingdom. A series of 351 cases of primary operable invasive breast carcinoma from patients presenting from 1987 to 2005 was used, all of whom had received tamoxifen and comprised 243 cases that did not experience a relapse and 108 cases that did. Mean follow-up time for the former was 77 months (range, 11-229; SD, 49.7)
and for the latter, 84 months (range, 1-142; SD, 38.8). Histopathologic details are presented in Supplementary Table S1.

**Tissue microarray construction and immunohistochemistry.** Tissue microarrays were constructed from the above cases using 0.6-mm cores selected from the most representative tumor area. Immunohistochemical analysis of CEACAM6 expression was done with a mouse monoclonal antibody (9A6; Abcam). Following dewaxing and antigen retrieval, CEACAM6 antibody was applied to tissue microarray sections at 1:200 dilution for 60 min at room temperature. Negative controls, in which the primary antibody was omitted, and three positive controls of colorectal carcinoma of varying staining intensities were included in each batch of immunohistochemistry. Staining was scored based on intensity where 0 indicates no staining; 1, minimal; 2, moderate; and 3, strong. Scoring was overseen by two specialized breast consultant histopathologists (A.M.S. and A.M.H.).

**Statistical analysis.** Pearson χ² and Spearman correlation were used using SPSS 14.0 statistical software. Survival curves were calculated by the Kaplan-Meier method. Mann-Whitney U test was used to determine statistical significance for real-time reverse transcription-PCR and Student's t test for clonogenic assays and growth curves. All P values were two sided; P < 0.05 was considered significant. Appropriate CEACAM6 scoring thresholds were determined by receiver operating characteristic analysis.

**Results**

CEACAM6 is up-regulated at the mRNA and protein levels in TAMr MMU1 cells and can be silenced by siRNA. We confirmed our original Affymetrix data (4) by quantitative reverse transcription-PCR, showing that CEACAM6 mRNA was significantly up-regulated in TAMr MMU1 cells compared with tamoxifen-sensitive MCF-7 controls (Fig. 1A). Similarly, by Western blotting, CEACAM6 protein was undetectable in

![Fig. 2](image-url)
control MCF-7 cells but was strongly expressed in TAMr cells (Fig. 1B). Stable transfection of TAMr MMU1 cells with a siRNA vector targeted to CEACAM6 showed knockdown of CEACAM6 mRNA (Fig. 1C) and protein (Fig. 1D) in two independent clones compared with control MMU1 cells stably transfected with a nontargeting siRNA vector.

Stable silencing of CEACAM6 increases sensitivity of TAMr MMU1 cells to E2 and 4-HT and reduces clonogenicity and anchorage-independent growth of MMU1 cells.

The effect of siRNA silencing of CEACAM6 on hormone sensitivity was next investigated. In the presence of 4-HT (1-100 nmol/L), MMU1 cells stably transfected with a nontargeting siRNA vector were not growth inhibited. However, CEACAM6-knockdown clones showed partial reversal of sensitivity to 4-HT with significant inhibition of growth at doses above 10 nmol/L (Fig. 2A). CEACAM6-silenced clones had partially restored sensitivity to E2 compared with control tamoxifen-sensitive MCF-7 cells, whereas both parental MMU1 cells and control MMU1 stable transfectants were insensitive to E2 at all concentrations tested (Fig. 2B). Different levels of hormone sensitivity in the two CEACAM6-silenced clones may be reflective of nonequivalent starting levels of expression of endogenous CEACAM6 (Fig. 1C), which may subsequently affect the level of gene silencing.

While generating the stable CEACAM6 knockdown MMU1 clones, we observed that fewer colonies were generated from MMU1 cells transfected with the CEACAM6 siRNA vector compared with those transfected with the control vector. Evaluation of anchorage-dependent growth showed a reduction in MMU1 cells where CEACAM6 was silenced (Fig. 2C) and that this was reproducible in clonogenic assays, where silencing of CEACAM6 resulted in a ~3-fold reduction in clonogenicity (Fig. 2D). Soft-agar colony-forming assays showed that tamoxifen-sensitive MCF-7 cells formed no colonies in soft agar, but TAMr MMU1 cells did form colonies and hence have acquired the capacity for anchorage-independent growth.

Silencing of CEACAM6 reduced the number of colonies formed by TAMr MMU1 cells in excess of 10-fold (Fig. 2E), showing the importance of CEACAM6 in the anchorage-independent phenotype of these cells. In summary, stable knockdown of CEACAM6 partially restores both the proliferative response to E2 and sensitivity to 4-HT and reduces the tumorigenic potential of TAMr-MMU1 cells.

CEACAM6 is overexpressed in TAMr breast tumors. As our in vitro studies confirmed that CEACAM6 was a valid target for endocrine resistance, we conducted a retrospective immunohistochemical study of its expression in clinical breast cancer. CEACAM6 was mainly seen in primary tumors with subsequent relapse (Fig. 3). CEACAM6 expression was dichotomized using receiver operating characteristic curves with moderate to strong expression corresponding to a cutoff value of 2 (Supplementary Fig. S1). As illustrated in Table 1, CEACAM6 was significantly associated with relapsed cases. Although there were multiple factors correlating with recurrence using multivariate Cox regression analysis only Nottingham prognostic index (NPI),

**Table 1.** Total, membrane, and cytoplasmic CEACAM6 expression in tamoxifen relapsed and tamoxifen-sensitive breast tumors

<table>
<thead>
<tr>
<th>CEACAM6</th>
<th>Study group</th>
<th>Control group</th>
<th>Odds ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 108 [n (%)]</td>
<td>n = 243 [n (%)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>35 (33.3)</td>
<td>32 (13.2)</td>
<td>3.16 (1.83-5.47)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Negative</td>
<td>73 (66.7)</td>
<td>211 (86.8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** Cox multivariate analysis of factors associated with breast cancer recurrence

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimated coefficient (B)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate NPI</td>
<td>-1.332</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High NPI</td>
<td>-0.940</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Progesterone receptor</td>
<td>0.559</td>
<td>0.041</td>
</tr>
<tr>
<td>CEACAM6</td>
<td>-0.735</td>
<td>0.005</td>
</tr>
</tbody>
</table>

**Fig. 3.** Immunohistochemical expression of CEACAM6 in breast and colorectal control tissue microarrays. CEACAM6 was expressed in colorectal cancer—positive (A), but not colorectal cancer—negative (B), controls. Breast carcinomas scoring 0 (C), 1 (D), 2 (E), and 3 (F) are also shown. Original magnification, ×10.
CEACAM6 membrane staining and progesterone receptor expression remained significant predictors of recurrence (Table 2). A prediction equation was initially calculated: \( \text{NPI} + \text{CEACAM6} - \text{progesterone receptor} \), where for NPI low risk = 1, moderate risk = 2, and high risk = 3. For CEACAM6 and progesterone receptor, no staining = 0 and positive staining = 1. However, progesterone receptor expression did not differ significantly from CEACAM6 expression (odds ratio, 0.77; 95% confidence interval, 0.41-1.44; \( P = 0.42 \)) and was therefore dropped from the analysis. The final prediction equation was \( \text{NPI} + \text{CEACAM6} \).

To test the above equation, cases were plotted against disease-free and overall survival. The robustness of the data was first analyzed using NPI as a separate predictor followed by CEACAM6 and then finally using the combined equation. Both NPI and CEACAM6 were able to identify groups at risk of recurrence and death from breast cancer (Fig. 4A-D). However, progesterone receptor expression did not differ significantly from CEACAM6 expression (odds ratio, 0.77; 95% confidence interval, 0.41-1.44; \( P = 0.42 \)) and was therefore dropped from the analysis. The final prediction equation was \( \text{NPI} + \text{CEACAM6} \).

Discussion

There have been previous observational reports of CEACAM6 expression in breast cancer (11, 12), but relatively little is known about its functional role in this disease. Global microarray analysis of atypical ductal hyperplasia, a preinvasive breast lesion with a high likelihood to progress to invasive
breast cancer, showed that CEACAM6 expression was higher in cases that progressed to breast cancer (15). This was later substantiated by immunohistochemistry (13).

Initial clues on the potential importance of CEACAM in endocrine resistance came from Affymetrix microarray analysis of TAMr MCF-7 cells, where CEACAM6 was shown to be up-regulated by 20-fold over tamoxifen-sensitive cells (4). We have investigated the functional consequences and showed that, unlike tamoxifen-sensitive MCF-7 cells, TAMr MMU1 cells are capable of anchorage-independent growth and that anchorage independence is reversed by siRNA silencing of CEACAM6. Thus, overexpression of CEACAM6 conveys tumorigenic properties to these cells. It has previously been shown that CEACAM6 expression increases anoikis resistance and promotes cell survival under anchorage-independent conditions, a characteristic associated with tumorigenesis and metastasis (16, 17).

Importantly, our in vitro data were substantiated in clinical breast cancer where we showed that CEACAM6 was overexpressed in primary breast tumors that subsequently relapsed, further highlighting its importance in this process. Furthermore, in a multivariate analysis, only CEACAM6 remained a significant predictor of recurrence.

Currently, NPI is an important predictor of tumor behavior, based on tumor size, grade, and lymph node status (19). NPI compares favorably with many other predictor models (20) and provides information about suitability for chemotherapy. Patients in the low-risk group would normally be candidates for endocrine therapy only, provided that the tumor is estrogen receptor (ER)/progesterone receptor (PR) positive. Those in the high-risk group are candidates for chemotherapy, in addition to endocrine therapy. On the other hand, the intermediate-risk group can have either option. The absence of a unified treatment plan for those patients can therefore create discrepancy in treatment.

Fig. 4 Continued. Those at intermediate risk (using NPI) were analyzed separately and CEACAM6-positive cases behaved similarly to high-risk patients (G and H). In the presence of estrogen receptor (ER)/progesterone receptor (PR), this separation was still evident, with positive CEACAM6 immunohistochemistry associated with higher risk of recurrence and death (I and J). Similar divergences were observed with respect to grade 3 tumors (K and L).
From our cohort of 351 patients, we derived an outcome predictor model using NPI and CEACAM6. This resulted in patients being stratified into either low-risk or high-risk group, with no intermediate-risk patients. As patients in the intermediate NPI were reclassified using our model, we carried out subgroup analyses of each group and grade type. Patients in the high-risk group remained unchanged because of the high weighting of NPI in the upper scale of our model, whereas those in the low-risk group also remained unchanged as the majority did not express CEACAM6. However, the most clinically relevant finding was the subcategorization of intermediate-risk patients into lower-risk and higher-risk groups. For example, consider a 60-year-old patient with average health diagnosed with a stage 2 lymph node–negative and estrogen receptor–positive 25-mm invasive carcinoma (NPI = 3.5). Treated with tamoxifen, Adjuvant! Online (21) would predict a 23.5% 10-year recurrence. Adding CMF-like regimen (the majority of chemotherapy in this cohort) would add a further 3.2% benefit. However, our outcome predictor model reclassified this group. CEACAM6-negative patients were still correctly placed in a similar 10-year risk (30% risk in our analysis) but crucially highlighted a further cohort of patients with a 10-year recurrence risk of 68% if CEACAM6 positive. This subgroup might therefore benefit from aggressive therapies. We suggest that our model could provide a clearer rationale for therapy selection but requires further validation before implementation in the clinic.

In summary, we have presented in vitro and in vivo evidence showing the importance of CEACAM6 in endocrine-resistant breast cancer. Not only can this serve as a powerful predictor of future recurrence, modulating CEACAM6 expression has marked effects on endocrine sensitivity in vitro, but it may also represent a promising new therapeutic target for breast cancer.

Acknowledgments

We thank Dr. S. Ko for help with soft-agar cloning assays.

References

Correction: Article on CEACAM6 and Breast Cancer Recurrence

In the article on CEACAM6 and breast cancer recurrence in the January 15, 2008, issue of *Clinical Cancer Research*, there was an error in Fig. 4. The correct figure is shown below.

Fig. 4. Kaplan-Meier survival curves showing the relationship between NPI and CEACAM6 expression on disease-free and overall survival. Both NPI (A, B) and CEACAM6 (c, d) identified to identify groups at risk of recurrence (A, C) and death (B, D) from breast cancer. Using a combined predictor equation 4 groups were observed. Cases scoring 1 or 2 fell into the low risk group those scoring 3 or 4 fell into the higher risk category (E, F). Those at intermediate risk (using NPI) were analysed separately and CEACAM6-positive cases behaved similarly to high risk patients (G, H). In the presence of ER/PR, this separation was still evident, with positive CEACAM6 immunohistochemistry associated with higher risk of recurrence and death (I, J). Similarly divergences were observed with respect to grade 3 tumours (K, L).
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