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Epithelial cell adhesion molecule (EpCAM) is overexpressed in breast cancer metastases

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Abstract

EpCAM (CD326) has diverse roles in cell adhesion and proliferation and is known to be overexpressed in primary breast carcinomas. While clinical and preclinical data suggest a role for EpCAM in metastases, the only prior study of EpCAM expression in breast cancer metastases suggested that EpCAM expression is *decreased* after first-line chemotherapy. This current study evaluates EpCAM expression in metastatic breast carcinoma (MBC) versus matched primary breast carcinoma (PBC). Rapid autopsies were performed on seventeen patients with widely metastatic breast cancer. Single patient tissue microarrays (TMAs) were constructed from archived PBC and post-mortem MBCs. In total, 169 spots from 17 PBCs and 895 spots from 195 MBCs were labeled for EpCAM by immunohistochemistry (IHC). Expression was scored as intensity (1–3) multiplied by percent membrane labeling (0–100%) and was subclassified as low (0–100), moderate (101–200), or high (201–300) labeling. PBCs exhibited exclusively low-moderate EpCAM labeling. EpCAM labeling was present in all metastases and was significantly increased in MBCs of 14 of 17 patients (p value range <0.05 to <0.0001, t test). In the remaining 3 patients, EpCAM labeling was nonsignificantly increased in 1 and unchanged in 2. High EpCAM labeling was verified using a different antibody for IHC, as well as in a separate series of surgically resected metastases compared to unmatched surgically resected primary breast cancers. In conclusion, EpCAM is highly expressed in MBCs compared to matched PBCs, verifying that it is a promising therapeutic target.

Keywords

Metastasis; EpCAM; Breast; Carcinoma

Introduction

The transmembrane protein epithelial cell adhesion molecule (EpCAM, CD326) is a 40kd glycoprotein which has a variety of roles in cell signaling, proliferation, adhesion, migration and tissue maintenance [1–2]. It is a unique glycoprotein that lacks the repeated subdomains that characterize the structure of other cell adhesion molecules, such as cadherins, integrins and selectins, but in contrast it shows frequent post-translational glycosylation changes [1,3]. In benign epithelium, EpCAM functions as an adhesion molecule.

EpCAM is expressed at low levels in luminal epithelial cells in benign breast tissue. However, EpCAM is overexpressed in many carcinomas relative to the corresponding normal epithelium, including breast carcinomas. The mechanism of overexpression is not clear, but a recent study has suggested that it may be a consequence of p53 dysfunction [4]. EpCAM overexpression in primary breast carcinomas correlates with diminished overall survival in patients with node-positive disease [5–6], suggesting that overexpression correlates with aggressive behavior. EpCAM has been shown to have a variety of activities which promote cancer progression and metastasis. While normally a pro-adhesive molecule, in cancer EpCAM can function as an antagonist to E-cadherin via disruption of the alpha-catenin/F-actin link [7], and actually loosen tight cell-cell adhesions. EpCAM forms complexes with CD44v6 and claudin 7 in highly metastatic colonic and pancreatic carcinomas [8], which correlates with resistance to apoptosis. Recently, intramembrane proteolysis of EpCAM has been shown to result in nuclear transport of the intracellular domain of EpCAM, resulting in association with β -catenin and gene transcription which promotes cell cycling [9]. Activation of the *wnt* signaling pathway is postulated to be a major role of EpCAM in normal and cancer stem cells [10]. Because of these activities and its overexpression in cancer, EpCAM has been considered a promising therapeutic target for breast cancer.

For EpCAM to be a viable therapeutic target for breast cancer, its expression and function in metastases must be verified. However, while the above clinical and preclinical data suggest a role for EpCAM in metastases, a study of breast cancer micrometastases to bone marrow suggested that EpCAM expression is *decreased* after first-line chemotherapy, which if true would limit the utility of EpCAM as a therapeutic target [11]. No prior studies have compared EpCAM expression in metastatic breast carcinoma (MBC) versus matched primary breast carcinoma (PBC). It should be noted that EpCAM expression has been shown in one study to be diminished in metastatic renal cell carcinomas compared to primaries [12], so underexpression in breast cancer metastases, as suggested by the prior study of Thurm et al., [11] would not be unprecedented.

At our institution, we have performed a series of “rapid autopsies” on patients who have died of metastatic breast cancer [13]. For most of these cases, the primary tumor is available in our files. This provides us with the unique opportunity to compare protein expression in metastatic breast carcinoma sites to the matched primary cancers from the same patient. Using this resource, we evaluate EpCAM expression in metastases relative to matched primary breast carcinomas.

Materials and Methods

Rapid autopsies

Rapid autopsies (< 4 hours post-mortem interval on all but one case) were performed on seventeen patients with terminal, widely metastatic breast carcinoma (Table 1). Consent was obtained at the time of death from the patient’s designated next of kin. The protocol was reviewed and approved by the Institutional Review Board of Johns Hopkins Hospital and Department of Defense. At autopsy, all organs were grossly examined for metastases, and metastases were snap frozen and fixed in formalin. Formalin-fixed tissue was processed similar to surgical breast specimens at The Johns Hopkins Hospital and was examined microscopically.

Tissue microarray construction

Seventeen single patient tissue microarrays (TMAs) were constructed from paraffin tissue blocks of the patient’s archived PBC and from both normal tissue and multiple MBCs

sampled at autopsy. These TMAs consisted of 99 spots, each measuring 1.4 mm in diameter. The structure of the typical TMA is shown in Fig. 1. Four to five spots (mean, 4.5) per tumor sample were placed on each TMA to minimize sampling error. The seventeen TMAs contained 169 spots of PBCs and 895 spots derived from, in total, 195 different MBCs.

In addition, a separate breast cancer metastasis TMA was constructed, composed of 16 metastases sampled not at autopsy but rather in routine surgical pathology. This TMA consisted of 99 spots, each measuring 1.4 mm in diameter. The distribution of the sixteen metastatic sites were as follows: one liver, one pleura, one bone, six lung, and seven brain. Each metastasis was represented by 4 spots (14 cases) or 2 spots (2 cases). In addition, the TMA contained two unrelated primary breast cancers (4 spots per case) for comparison.

Immunohistochemistry and expression scoring

The TMAs were immunohistochemically labeled for EpCAM expression using standard methods. Briefly, unstained 5- μ m sections were cut from paraffin TMA blocks; slides were deparaffinized by routine techniques, steamed for 30 min at 90 C in 1X sodium citrate buffer, cooled for 5 min, then incubated with the mouse monoclonal primary antibody C-10 (Santa Cruz, sc-25308), which recognizes an epitope between amino acids 24–93 in the extracellular domain of EpCAM. EpCAM expression was scored as labeling intensity (1–3) multiplied by percent membrane labeling (0–100%). Each patient's average PBC and MBC labeling score was subclassified as low (score 0–100), moderate (score 101–200), or high (score 201–300). To confirm the results, selected TMAs were also labeled with the Dako antibody Ber-Ep4 (M0804), which recognizes a different epitope of EpCAM's extracellular domain. Both antibodies were detected using the same detection system (iView from Ventana Medical System, Tucson, AZ).

Quantitative gene expression analysis

Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) was performed on microdissected unmatched frozen PBCs and MBCs to quantify EpCAM mRNA expression. We identified 11 highly cellular primary invasive ductal carcinomas on which frozen tissue was available in our surgical pathology tumor bank. A frozen section was made from each case, and the OCT-embedded tissue block was trimmed to remove contaminating normal breast ducts and yield tumor cellularity of 60–90% (mean, 78%). Frozen sections were also prepared from liver metastases from 10 cases in this study. We chose liver metastases because of their availability and the fact that contaminating normal hepatocytes are nonreactive to EpCAM, in contrast to the positive staining in mammary stroma. These blocks were similarly trimmed to remove contaminating normal tissue and yield tumor cellularity of 40–90% (mean 77%).

Both normal and tumor RNA were extracted using the Trizol method, and all cDNAs were prepared with 500ng of RNA in SuperScript II (Invitrogen, Carlsbad, CA) reactions according to the instructions of the manufacture. TaqMan PCR reactions were conducted using pre-made kits from Applied Biosystems, and 1 μ l of cDNA was used in each 25 μ l of reaction volume. The PCR reactions were run in an Applied Biosystems Sequence Detection System 7500. Relative expression levels were determined using the C_t method using *GAPDH* as endogenous control. The TaqMan gene expression assays were assessed with Taqman probes: Hs00901888_g1 for *EpCAM*, 4326317E for *GAPDH*.

Results

Clinicopathologic Features of Patients

Clinicopathologic characteristics of patients are seen in Table 1. To summarize, of the seventeen patients studied, one had an invasive lobular carcinoma primary (Case 1) and the remaining sixteen had invasive ductal carcinoma. The ages at diagnosis ranged from 28–71 years, while the ages at death ranged from 36–79 years. The number of metastatic sites detected per patient at autopsy ranged from 3–18. All patients were ultimately refractory to all chemotherapy and hormonal therapy. Of the seventeen primary carcinomas, eleven were ER positive (10 HER2 negative, 1 HER2 positive) and six were ER negative (5 HER2 negative, 1 HER2 positive).

Immunohistochemistry

Membranous EpCAM labeling was detected in all PBCs. The PBCs exhibited low (score 0–100) to moderate (score 101–200) average EpCAM labeling, and no high labeling (Table 2). The intensity of membranous EpCAM labeling in the invasive lobular carcinoma, Case 1, was uniformly faint (intensity score of 1); however, the percentage membrane labeling was high.

Membranous EpCAM labeling was also detected in all MBCs. EpCAM labeling did not decrease from the primary cancer to the metastatic sites in any patient. In fact, in 82% of the patients (14/17), EpCAM labeling was statistically significantly increased in the metastatic sites compared to the matched primary cancer (p values ranging from <0.05 to <0.0001, t test) (Table 2). In four of these patients (Cases 5, 6, 9, 12), the membranous EpCAM labeling was strikingly increased from low in the PBCs to high in the MBCs (p values from <0.001 to <0.0001, t test), as is illustrated in Case 9 (Fig. 2).

Of the remaining 3 patients, EpCAM labeling was unchanged in two and nonsignificantly increased in one. Case 10 showed moderate EpCAM labeling in both the PBC and MBCs ($p>0.1$), and Case 11 showed low EpCAM labeling in both the PBC and MBCs ($p>0.1$). Case 3 showed a nonsignificant increase from low to medium expression from the PBC to the MBCs ($p>0.1$).

EpCAM labeling was consistent in virtually all metastases within a patient, including a wide variety of sites including the liver, lymph nodes, spleen, brain, lung, pleura, ovary and adrenal glands (Supplemental Table 1). The only site where a slight decrease in expression was seen was in bone metastases, which were extensively decalcified and therefore the diminished labeling likely represents artifact. There was no association between ER, PR or Her2 status of the primary carcinoma and the EpCAM expression in either the primary or the metastatic sites.

However, in three cases, increased labeling was noted in nests of metastatic carcinoma cells as compared to the lesser labeling in more dispersed, infiltrative metastatic carcinoma cells. In Case 15, marked heterogeneity was noted in the splenic metastases. Rounded, nested metastases were more strongly positive than irregularly infiltrative metastases which invaded the splenic parenchyma (score 101–200) (Fig. 3a,b). Of note, in this case, nests of carcinoma cells located within lymphatic spaces in the primary breast cancer specimen similarly showed stronger EpCAM expression than the surrounding primary breast cancer cells irregularly invading stroma. In another patient (Case 16), rounded intravascular nodules of carcinoma in the pleura were more strongly positive than invasive small nests of carcinoma which invaded the fibrous pleural tissue. In the third patient (Case 4), rounded nests of metastatic carcinoma cells in lymphovascular spaces in the pericardium were more strongly positive than infiltrative cells in the surrounding fat (Fig. 3c,d).

To exclude the possibility that our results are artifactual due to the clone of the antibody used, we labeled serial sections of three TMAs (Cases 12, 16 and 17) with the BerEP4 antibody which also recognizes EpCAM. Although the membranous staining of EpCAM was uniformly fainter with the BerEP4 antibody than the C-10 antibody, the results were similar in that in all three cases, metastases showed greater labeling than the matched primary. In Cases 12 and 16, the average membranous EpCAM labeling was significantly increased from low to moderate ($p < 0.0001$). In Case 17, the membranous EpCAM labeling was nonsignificantly increased from low to moderate ($p > 0.1$); in this case, the BerEP4 antibody failed to or only lightly labeled metastases to the bone.

To help exclude the possibility that the increased labeling for EpCAM seen in the metastases was due to autopsy-related fixation artifacts, we labeled the TMA containing 16 different breast cancer metastases and 2 primary breast cancers harvested in surgical pathology with the C-10 antibody to EpCAM. Similar moderate to high expression in these metastases was seen, with an average expression score across all sites of 153. The two primary breast cancers showed only low expression, with an average expression score of 69.

RNA Expression

To test the mRNA expression levels of *EpCAM*, real time RT-PCR was performed by using Taqman gene expression assay probes. cDNAs from 6 organoids isolated from normal mammary gland reduction tissues, 11 primary breast carcinomas, and 10 breast cancers metastatic to the liver were used for the assay. QRT-PCR revealed that while there was marked variation in expression of EpCAM mRNA in metastases, there was not a significant upregulation of EpCAM mRNA transcripts in metastases versus unmatched primary tumors (Fig. 4).

Discussion

EpCAM is unique among the family of cell adhesion molecules. It can function as an antagonist to E-cadherin and actually loosen tight cell-cell adhesions [7]. The interaction of EpCAM between the metastasis-promoting cell adhesion molecule CD44 and the tight junction molecule claudin 7 forms a complex that influences cell-cell and cell-matrix adhesion [8]. Furthermore, EpCAM has recently been shown to act as a mitogenic signal transducer via nuclear translocation of the intracellular domain, in association with FHL2, β -catenin, and Lef-1, after intramembrane proteolysis of the EpCAM protein [9]. The cleaved intracellular domain of EpCAM and its associated nuclear complex induces transcription of *c-myc*, as well as *cyclin A* and *E* [14]. These findings further support EpCAM's role in regulating cell proliferation and cell cycling.

No prior study has evaluated EpCAM expression in breast cancer metastases relative to matched primaries. The one study of EpCAM in breast cancer metastases in the literature is a study of breast cancer micrometastases to bone marrow, and it suggested that EpCAM expression is decreased after first-line chemotherapy [11]. If true, this would limit the utility of EpCAM as a therapeutic target. However, the authors of this study note that "it is extremely difficult to get sets of autologous primary tumors and metastases from the same patient." Our rapid autopsy cohort is an ideal resource to use to address this issue. Our findings indicate that EpCAM labeling is consistently higher in the metastases harvested at autopsy compared to their matched primary cancers of patients who died of widely metastatic breast cancer. This corroborates experimental data which suggests that EpCAM promotes metastasis, and validates EpCAM as a therapeutic target in metastatic breast cancer. Indeed, an EpCAM-targeting monoclonal antibody, edrecolomab, was shown to reduce and even eliminate EpCAM+ breast cancer cells in the bone marrow of patients with metastatic or locally recurrent disease [15].

Given the increased immunohistochemical labeling for EpCAM in metastases versus matched primaries, we were somewhat surprised to not find increased EpCAM mRNA expression via QRT-PCR in breast cancer metastases versus the unmatched primaries. Several possible explanations for this result exist. The first is that EpCAM overexpression results from post-translational mechanisms. Indeed, the frequent glycosylation known to occur to the EpCAM protein may contribute to protein overexpression via differential stabilization of the protein. Another possibility is that the fact that the unmatched nature of primary tumors used to compare to the metastases for mRNA expression (which was necessary given the requirement for frozen tissue), masked mRNA overexpression in the metastases.

A third possibility to exclude is that the expression of EpCAM protein is no different in primary tumors compared to metastases, but that different processing of the autopsy tissues versus the matched primaries from surgical pathology accounted for the increased immunohistochemical labeling of the metastases. One could envision that the post-mortem interval of the autopsy, in which metastases rest in the patient's still warm body, enhances autolysis relative to a surgical specimen, which cools more rapidly at room temperature, and that this promotes detection of EpCAM by IHC. Arguing against this are the results on the splenic (Case 15), pleural (Case 16) and pericardial (Case 4) metastases we noted in Results, where in the same tissue block the nested tumor cells labeled more strongly for EpCAM than the infiltrative ones, whose architecture simulated that of a primary tumor. If the increased labeling we saw in metastases was artifactual, one would expect both of these patterns of metastases to label similarly. In addition, the increased labeling of the surgically-resected metastases relative to the surgically-resected primaries on the additional TMA studied argues against this possibility. A fourth possibility to consider is that an artifact specific to the C-10 antibody to EpCAM is responsible for the increased labeling of metastases. The fact that we saw similar patterns of labeling in the MBC TMAs using the BerEp4 antibody argues against this possibility.

Our results fit a model in which membranous EpCAM expression may be relatively diminished in small groups of carcinoma cells actively invading stroma at the primary site or in transit to metastases (such as dispersed cells found in the bone marrow), but is increased in nests of intravascular tumor emboli or established metastases, which largely have a nested growth pattern to allow for expansile tumor growth. This would explain the low expression seen in the prior bone marrow aspirate study, and is consistent with several other observations in the literature. For example, Jojovic et al., found lower expression in smaller (<15 cell) compared to larger colon cancer metastases in a mouse model [16]. In addition, Gosens et al., found loss of EpCAM expression at the invasive edge of colorectal carcinomas [17]. These studies and ours highlight the concept that metastasis is a multistep process, and that expression of key proteins involved in the process (such as EpCAM) may not be the same in all steps as the cancer cells adapt to different environmental pressures [18–20] (Fig. 5). The functional role of EpCAM in metastases remains to be determined. For example, high membrane expression could potentially overwhelm inhibitors and lead to increased nuclear signaling by EpCAM intracellular domain [10]. Given that currently available EpCAM antibodies do not detect the intracellular domain of EpCAM, we could not address this possibility in our study. Further functional studies are needed to address this question.

Finally, given that EpCAM is implicated in stem cell signaling [10], one might postulate that the increase in EpCAM expression in breast cancer metastases that we found implies an increase in the stem cell population of these metastases. However, we urge caution regarding this conclusion. The literature is conflicting regarding the presence of an increased stem cell population in breast cancer metastases. In a study of 50 bone marrow specimens containing

disseminated breast cancer tumor cells, Balic et al., found that the majority of disseminated tumor cells had the CD44+CD24– putative stem cell phenotype, while this phenotype comprises only a minority of cells in primary breast cancers [21]. In contrast, Shipitson et al. found an increase in CD24+ cells in established metastatic cancers sampled at autopsy [22]. One way to reconcile these data is to postulate that cancer cells with the self-renewal capability of stem cells are required for transit and initiation of metastatic growth, while a transition to a more differentiated phenotype occurs with permanent establishment and growth of a metastatic lesion. However, we believe that further studies, including direct assays of breast cancer stem cell functional properties of metastatic tumor cells *in vivo*, are likely required to properly address the prevalence of cancer stem cells in metastatic breast cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Structure of a typical single patient tissue microarray

Each tissue microarray consisted of 99 spots with 9 spots of control tissue and 4–5 spots per tumor. (Abbreviations: PBC, primary breast cancer; CTL, control; MET, metastasis; R, right; L, left; NL, normal; ADR, adrenal; LN, lymph node; DIAPHR, diaphragm)

Fig. 2. Striking increase of EPCAM labeling in metastatic sites compared to matched primary cancer in Case 9

Immunohistochemical labeling with the C10 antibody for membranous EpCAM expression via tissue microarray analysis

Fig. 3. Heterogeneity in EPCAM expression in metastases

The rounded nests of metastatic carcinoma cells involving the splenic parenchyma in case 15 are more strongly positive than the irregularly infiltrative tumor cells (a,b, 40X). The rounded nests of metastatic carcinoma cells involving pleura in Case 16 are more strongly positive than the irregularly infiltrative tumor cells c,d, 40X)

Fig. 4. EpCAM mRNA expression in unmatched breast cancer primaries and metastases

EpCAM mRNA expression was assessed by Taqman gene expression assays. The relative expression levels of *EpCAM* were normalized with average expression in organoids isolated from normal mammary gland reduction tissues and plotted with Graphpad Prism. *Columns*, Mean; *bars*, SD

Fig. 5. Schema of Metastases Stages

The stages of metastases progress through metastasis *initiation*, *progression* and *establishment*. To *initiate* metastatic spread, the tumor cells must first express proteins that enable detachment from the basement membrane and allow them to travel through the extracellular matrix into the capillary vasculature via intravasation. To *progress* from the primary site to the distant site, the tumor cells must then survive in the blood stream and activate factors that allow for extravasation. Finally, to *establish* a metastatic focus, the tumor cells must adhere, recruit vessels and evade host immune surveillance

Table 1

Clinicopathologic Information for Cases 1–17

(ILC, invasive lobular cancer. IDC, invasive ductal cancer)

Case	Age at Diagnosis	Stage at Diagnosis	Primary Tumor Type and Grade	Age at Death	Post-Mortem Interval	Number of Metastatic Sites at Autopsy
1	54	T2N0M0	ILC, Grade 2	65	3 hours, 45 min	10
2	33	T3N1M0	IDC, Grade 3	37	4 hours	18
3	40	T1N1M0	IDC, Grade 2	48	2 hours	13
4	59	T1N0M0	IDC, Grade 2	68	4 hours	15
5	56	T2N1M0	IDC, Grade 2	61	3 hours, 30 min	14
6	51	T2N0M0	IDC, Grade 3	53	1 hour, 15 min	15
7	48	T2N3M0	IDC, Grade 3	54	3 hours	6
8	38	T2NXMX	IDC, Grade 3	43	3 hours	13
9	71	T2N0MX	IDC, Grade 3	79	3 hours	18
10	35	T2N1MX	IDC, Grade 3	36	2 hours, 30 min	11
11	57	T4N1M1	IDC, Grade 2	58	2 hours, 45 min	10
12	28	T2N1MX	IDC, Grade 2	38	11 hours	15
13	33	T1N1MX	IDC, Grade 3	37	2 hours	5
14	47	T2N1MX	IDC, Grade 3	48	3 hours	9
15	42	T3N1M1	IDC, Grade 2	47	2 hours	8
16	44	T1N1MX	IDC, Grade 2	57	2.5 hours	11
17	43	T2N1MX	IDC, Grade 2	60	4 hours	2

Table 2
Membrane-bound EpCAM protein expression by immunohistochemistry with the C10 antibody in metastatic breast carcinomas (MBCs) with matched primary breast carcinomas (PBCs)

(Expression was quantified as labeling intensity (1–3) multiplied by percent membrane labeling (0–100%) and subclassified as low (score 20–100), moderate (score 101–200), or high (score 201–300))

Case	PBC Average EpCAM Expression	MBC Average EpCAM Expression	p value
1	44	110	p<0.001
2	160	242	p<0.01
3	63	154	p>0.1
4	125	218	p<0.05
5	96	221	p<0.001
6	88	205	p<0.001
7	19	97	p<0.0001
8	169	243	p<0.001
9	76	257	p<0.0001
10	146	170	p>0.1
11	25	48	p>0.1
12	85	258	p<0.0001
13	63	190	p<0.0001
14	180	226	p<0.01
15	187	255	p<0.001
16	86	165	p<0.01
17	91	163	p<0.01