ADAM-17 Expression in Breast Cancer Correlates with Variables of Tumor Progression

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Abstract The ADAMs are a family of membrane proteins possessing a disintegrin and metalloprotease domain. One of their main functions is shedding of membrane proteins. The aim of this study was to test the hypothesis that ADAM-17 (also known as tumor necrosis factor-α converting enzyme) is involved in breast cancer progression. Overexpression of ADAM-17 in MCF-7 breast cancer cells increased in vitro invasion and proliferation, whereas down-regulation of ADAM-17 expression in MDA-MB-435 cells decreased invasion and proliferation. At both mRNA and protein levels, ADAM-17 expression was significantly up-regulated in breast cancer compared with normal breast tissue. Using Western blotting, ADAM-17 protein in breast cancer was shown to exist in two forms migrating with approximate molecular masses of 100 and 120 kDa. Based on their known molecular mass, these bands were taken to represent the active and precursor forms of ADAM-17, respectively. The proportion of active to total ADAM-17 increased progressively from normal breast tissue to primary breast cancer to lymph node metastases (P = 0.017, Kruskal-Wallis test). In primary cancers, the active form was expressed more frequently in node-positive compared with node-negative tumors (P = 0.034, χ² test). Furthermore, in primary carcinomas, both forms of ADAM-17 correlated significantly (Spearman correlation analysis) with levels of urokinase plasminogen activator (precursor form: r = 0.246, P = 0.032, n = 83 and active form: r = 0.428, P = 0.0001, n = 83) and proliferating cell nuclear antigen (precursor form: r = 0.524, P < 0.0001, n = 73 and active form: r = 0.365, P = 0.002, n = 73). Our results support the hypothesis that ADAM-17 is involved in breast cancer progression.

The ADAM (a disintegrin and metalloprotease) proteins, which are also known as metalloprotease disintegrin cysteine rich, belong to the metzincin subfamily of metalloproteinases (for review, see refs. 1, 2). They are mostly transmembrane proteins and typically contain the following domains: a signal peptide, a propeptide, a metalloprotease domain, a disintegrin-like domain, a transmembrane sequence, and a cysteine-rich region, an epidermal growth factor (EGF)–like domain, a cytoplasmic tail. One of the most widely studied sheddases is ADAM-17, which is also known as tumor necrosis factor-α (TNF-α) converting enzyme (7). ADAM-17 was originally identified by its ability to release membrane-bound TNF-α from its precursor (8, 9). Subsequently, ADAM-17 has been shown to shed several membrane-bound proteins, including E-selectin, p75 TNF receptor, transforming growth factor-α, amphiregulin, heparin-binding EGF, and epiregulin (reviewed in refs. 1, 2).

The shedding of a number of these molecules is potentially important in cancer progression. For example, endogenous levels of TNF-α have been shown to promote tumor progression (for review, see ref. 10). Potential mechanisms by which TNF-α mediates progression include induction of matrix-degrading proteases and release of cytokines and chemokines (10). Direct evidence of a role for TNF-α in cancer was recently obtained by Suganuma et al. who showed that a deficiency of this cytokine rendered mice resistant to chemically induced skin carcinogenesis (11).

Similarly, because of its ability to shed ligands such as transforming growth factor-α, amphiregulin, heparin-binding EGF, and epiregulin (12), ADAM-17 is an important trans-activator of the HER family of receptors, especially EGF receptor...
(EGFR; refs. 13–16). EGFR plays a pivotal role in cell migration, mitogenesis, and angiogenesis and is currently undergoing intensive investigation as a target for anticancer therapies (17). Indeed, ADAM-17 – mediated release of specific EGFR ligands, following G-protein receptor stimulation, has been shown to transactivate EGFR and enhance mitogenesis and migration of different cell lines in vitro (13–16).

All of these findings, when taken together, suggest that ADAM-17 is likely to play a role in cancer progression. The aim of this study was therefore to test the hypothesis that ADAM-17 is involved in human breast cancer progression.

Materials and Methods

**Patients and preparation of samples.** All samples were obtained with institutional board approval from St. Vincent’s University Hospital. Following surgical resection and pathologic evaluation, tissues were snap-frozen in liquid nitrogen and stored at −80°C. Tissue samples were homogenized using a Mikro-Dismembrator (Braun Biotech International, Melsungen, Germany). Table 1 summarizes the characteristics of the primary breast carcinomas analyzed. The normal breast tissues used for ADAM-17 mRNA were 22 samples adjacent to carcinoma, 8 samples adjacent to fibroadenoma, and 8 reduction mammoplasty specimens. It is important to point out that these “normal” breast tissues cannot be regarded as “healthy normal.” As similar levels of ADAM-17 were found in all of these types of “normal” breast tissue, they were combined into one group.

**Transfection of MCF-7 cells with ADAM-17 cDNA.** MCF-7 breast cancer cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in DMEM (Invitrogen/Life Technologies, Carlsbad, CA), supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer (Invitrogen, Carlsbad, CA), 10% fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin and maintained in a 37°C CO2-humidified incubator. The cDNA encoding ADAM-17 was kindly provided by Dr. Roy Black, and the pcDNA3.1 control vector was purchased from Invitrogen. Transfections were done using Nucleofection technology (Amaxa, Koeln, Germany) according to manufacturer's instructions. Briefly, 2 × 10⁶ mature MCF-7 cells were resuspended in Cell Line Nucleofector V solution (Amaxa), mixed with 2 μg cDNA, and pulsed as suggested by the manufacturer. Immediately after nucleofection, cells were transferred into wells containing culture medium (37°C). After 48 h, cells were selected using Geneticin (12.2 μg/mL; Invitrogen/Life Technologies).

**Transfection of MBA-MB-435 cells with ADAM-17 short hairpin RNA.** The human carcinoma cell line MBA-MB-435 (American Type Culture Collection) was grown in RPMI (Invitrogen), supplemented with 2 mmol/L L-glutamine, 10 mmol/L HEPES buffer (Invitrogen), 10% fetal bovine serum (Invitrogen), and 1% penicillin/streptomycin and maintained in a 37°C CO2-humidified incubator. Cells were successfully transfected with two pre-designed ADAM-17 short hairpin RNA (shRNA) vectors or scrambled vector (SureSilencing, SuperArray, Frederick, MD), according to the manufacturer’s instructions. To isolate neomycin-resistant colonies, 5 μg/mL Geneticin (Invitrogen) selection was applied. Silencing of ADAM-17 expression was confirmed by reverse transcription-PCR analysis and immunoblot.

**RNA Isolation.** For both conventional and real-time PCR, RNA from breast tissue samples was isolated using TRI reagent (Sigma-Aldrich, St. Louis, MO). RNA from cell lines was isolated using the RNeasy Mini kit (Qiagen, Hilden, Germany). All RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen).

**Conventional reverse transcription-PCR.** ADAM-17 mRNA was measured by both conventional and real-time reverse transcription-PCR. The primer sequences used for conventional PCR were as previously described (18). The PCR reaction mixture contained 1 μL cDNA, 50 ng of each primer (sense and antisense), 2.5 μL PCR Mg-free buffer, 2 mmol/L MgCl₂, 0.5 μL deoxynucleotide triphosphates, and 0.1 μL Taq polymerase (Promega, Madison, WI). The final volume for each reaction was 25 μL. The protocol used for amplification was as follows: 95°C for 5 min for initial denaturation followed by 30 cycles of 95°C for 1 min for denaturation, 60°C for 1 min for annealing, and 1 min at 72°C for extension. A final extension was carried out at 72°C for 10 min. PCR products were subjected to electrophoresis on a 2% agarose gel, and DNA was visualized by ethidium bromide staining. The intensity of the mRNA band observed was semiquantified by scanning densitometric analysis (EagleEye, Stratagene, United Kingdom) with normalization of ADAM-17 mRNA against the internal control glyceraldehyde-3-phosphate dehydrogenase. RNA isolated from BT-474 breast cancer cells was used as a positive control for ADAM-17.

**Quantitative real-time PCR.** For real-time PCR, the primer sequences were as follows: sense, CCCGCTGTGTGCCCTATGT and antisense, CCAGGACAGACCCAA (designed using Genefisher software). Each reaction mixture contained 200 ng cDNA, 2.0 mmol/L MgCl₂, 80 ng of each sense and antisense primer, and 2 μL of LightCycler FastStart reaction master mix containing FastStart Taq DNA polymerase and DNA double-stranded–specific SYBR Green 1 dye (Roche Diagnostics GmbH, Mannheim, Germany). The optimized amplification protocol consisted of an initial denaturation step of 95°C for 10 min followed by 36 amplification cycles at 93°C for 10 s, annealing at 58°C for 5 s, and elongation at 72°C for 10 s. A constant temperature ramp of 20°C/s was used throughout each of the steps. Measurements of fluorescence were taken at the end of the annealing phase for each cycle. The PCR

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<table>
<thead>
<tr>
<th>Tumor Characteristics</th>
<th>ADAM-17 mRNA, n (%)</th>
<th>ADAM-17 protein, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size (cm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤2</td>
<td>29 (20.1)</td>
<td>16 (19.3)</td>
</tr>
<tr>
<td>&gt;2</td>
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<td>9 (10.8)</td>
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<td></td>
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<td>1 and 2</td>
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<td>3</td>
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<td>12 (14.5)</td>
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<td>18 (21.7)</td>
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<td>7 (8.4)</td>
</tr>
<tr>
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<td>10 (6.9)</td>
<td>8 (9.7)</td>
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products were melted by increasing the temperature to 95°C (0.1°C/s). Finally, the samples were cooled to 40°C. As a positive control, RNA extracted from HeLa cells was used. For the negative control, CDNA was replaced with deionized water. To quantify gene expression, the internal control transcript glyceraldehyde-3-phosphate dehydrogenase was used as a reference.

**Immunoblot analysis.** Protein was extracted from tissue samples using 50 mmol/L Tris-HCl (pH 7.4) containing protease inhibitors [i.e., 1 mmol/L benzamidine-HCl, 1 μmol/L trans-epoxyisuccinyl-l-leucylalanyl-(4-guanidino) butane (E-64), and 10 mmol/L EDTA; Sigma-Aldrich; 2 ml per 100 mg sample (19)] and Triton X-100 (1% (w/v)) agitation at 4°C for 1 h. A Bicinechonic Acid assay (Pierce, Rockford, IL) was used to determine total protein concentration. Equal amounts of protein were separated on SDS-PAGE and transferred to nitrocellulose membranes (Sigma, St. Louis, MO). Membranes were blocked in 5% low-fat dry milk (Marvel instant dried skimmed milk) in TBS-T for 1 h at room temperature and then stained with polyclonal rabbit anti-ADAM-17 antibody (5 μg/ml; ProSci, Inc., Poway, CA). Following three washes for 10 min in TBS-T, the membrane was incubated with 1:1,000 horseradish peroxidase–conjugated anti-rabbit Ig secondary antibody (Sigma) for 1 h at room temperature before incubation with 6 mL of chemiluminescence reagent (Luminol, Santa Cruz Biotechnology, Santa Cruz, CA) for 1 min. Membranes were exposed to X-ray film (Fujifilm with deionized water and solubilized with 0.2% Triton X-100 (Sigma). The lower chamber was filled with fibroblast-conditioned medium. After 48 h of incubation, nonmigrated cells in the lower chamber were removed from the upper surface of the filters with a PBS-soaked cotton swab. This was followed by fixation using 1% glutaraldehyde and staining with 0.1% crystal violet (Pro-LaB Diagnostics, Cheshire, United Kingdom). All experiments were carried out in triplicate using three independent assays. Cells fixed on the lower face of the Matrigel chambers were counted under a light microscope at a magnification of ×10. The same procedure was followed for parental, vector-transfected, and ADAM-17 shRNA–transfected MDA-MB-435 cells.

**Cell proliferation assays.** Parental, ADAM-17–transfected, and vector-transfected MCF-7 cells (2.5 × 10^4 per well) were seeded in the upper compartment of Matrigel-coated inserts (8-μm pore size; BioCoat, BD Biosciences, Erembodegem-Dorp, Belgium). The lower chamber was filled with fibroblast-conditioned medium. After 48 h of incubation, nonmigrated cells in the upper chamber were removed from the upper surface of the filters with a PBS-soaked cotton swab. This was followed by fixation using 1% glutaraldehyde and staining with 0.1% crystal violet (Pro-LaB Diagnostics, Cheshire, United Kingdom). All experiments were carried out in triplicate using three independent assays. Cells fixed on the lower face of the Matrigel chambers were counted under a light microscope at a magnification of ×10. The same procedure was followed for parental, vector-transfected, and ADAM-17 shRNA–transfected MDA-MB-435 cells.

**Other assays.** Estrogen receptor (ER) and progesterone receptor (PR) levels were measured by ELISA (Abbott Diagnostics, North Chicago, IL; ref. 21). The cutoff point for ER was 200 fmol/g wet weight tissue, whereas the cutoff for progesterone receptor was 1,000 fmol/g wet weight tissue. Proliferation rates were measured using an ELISA for proliferating cell nuclear antigen (PCNA; Merck, Darmstadt, Germany; ref. 22). Urokinase plasminogen activator (uPA) was measured by ELISA using kits obtained from American Diagnostica, Inc. (Stamford, CT).

**Statistical analysis.** The Spearman rank correlation (for continuous variables), the Mann-Whitney U test, and Kruskal-Wallis test (for continuous/nominal variables) and χ² test (for nominal data) were used to determine relationships between various variables. The Student’s paired t test was used to compare ADAM-17 expression in paired tissue from the same patient. All statistics were calculated using StatView for Windows, version 5.0.1 (SAS Institute, Inc., Cary, NC). A two-sided P < 0.05 was considered statistically significant.

**Results**

**Effect of overexpressing ADAM-17 on Matrigel invasion and cellular proliferation of MCF-7 cells in vitro.** Overexpression of ADAM-17 protein was confirmed by immunoblot (Fig. 1A). To evaluate the effect of ADAM-17 overexpression on cellular invasion, we carried out Matrigel invasion assays using parental, vector-transfected, and ADAM-17–transfected MCF-7 cells (Fig. 1B). MCF-7 cells were chosen for transfection as they express low levels of ADAM-17. When compared with vector-transfected or parental cells, ADAM-17–transfected cells showed a significant increase in invasive ability, when expressed as a ratio of cells invading the Matrigel compared with number of cells migrating through control inserts (Student’s paired t test: P = 0.0005 and P = 0.0078, respectively; n = 9; Fig. 1C).

Cellular proliferation rates were also compared in parental, vector-transfected, and ADAM-17–transfected MCF-7 cells. As shown in Fig. 1D, the total number of adherent cells was significantly higher in the ADAM-17–transfected cells compared with both vector-transfected and untransfected MCF-7 cells (Student’s paired t test: P < 0.0001 and P < 0.0001, respectively; n = 9).

**Effect of knockdown of ADAM-17 expression on Matrigel invasion and cell proliferation of MDA-MB-435 cells in vitro.** To further investigate the effects of ADAM-17 on cancer cell migration and proliferation, we blocked the endogenous expression of ADAM-17 by RNA interference. MDA-MB-435 cells were used for these experiments as they express high levels of ADAM-17. Suppression of ADAM-17 expression was confirmed by both reverse transcription-PCR and immunoblot analysis of total cell lysates following shRNA transfection (Fig. 2A and B). Two transfected clones (designated clone 1 and clone 2) were obtained with 60% and 95% ADAM-17 silencing, respectively (Fig. 2A). As shown in Fig. 2C and D, both clone 1 and clone 2 showed a significant reduction in invasive capacity compared with parental cells (Student’s paired t test: P = 0.0001 and P < 0.0001, respectively). Cell proliferation rates were also compared in parental, vector-transfected, and ADAM-17 shRNA–transfected MDA-MB-435 cells. As shown in Fig. 2E, the total number of adherent cells was significantly reduced in the ADAM-17 shRNA–transfected clones compared with parental MDA-MB-435 cells (Student’s paired t test: P < 0.0001, n = 9).

**Effect of ADAM-17 silencing on sensitivity to gefitinib.** ADAM proteases have been implicated in the shedding of a number of EGFR ligands (23–25). To test whether the ADAM-17 regulation of proliferation in MDA-MB-435 cells was mediated via EGFR, we investigated the effect of ADAM-17 knockdown on response to the EGFR inhibitor gefitinib (AstraZeneca, Macclesfield, Cheshire, United Kingdom; ref. 26). ADAM-17 silencing resulted in a decrease in the IC₅₀ of gefitinib from 8.34 to 6.96 μmol/L and 5.98 μmol/L for clone 1 and clone 2, respectively (Student’s paired t test: P = 0.0041 and P = 0.0022,
respectively) when compared with the IC50 for nontreated parental cells. This finding suggests that EGFR may be involved in mediating the effects of ADAM-17 on proliferation in MDA-MB-435 cells.

ADAM-17 mRNA expression in normal, benign, and malignant breast tissue. ADAM-17 mRNA was measured by both conventional and real-time reverse transcription-PCR in human breast tissue. Figure 3A illustrates a representative ethidium bromide–stained agarose gel of ADAM-17 mRNA expression, as measured by conventional PCR, in normal, benign, and malignant breast tissue. Although ADAM-17 tended to be expressed more frequently in the axillary node metastases (9 of 14, 64.3%) and primary breast carcinomas (77 of 144, 53.5%) than in either the normal breast specimens (15 of 38, 39.4%) or fibroadenomas (8 of 23, 34.8%), this difference was not statistically significant. For 17 patients, however, matching normal and malignant breast tissue was available. For these matching samples, ADAM-17 was detected more frequently in the cancers compared with the surrounding normal tissue (* Student’s paired t test; P = 0.04, n = 15 pairs).

**Immunoblotting of ADAM-17 protein in normal, benign, and malignant breast tissue.** Figure 3B illustrates a representative immunoblot of ADAM-17 protein expression in normal breast tissues, fibroadenomas, primary breast carcinomas, and axillary node metastases, following electrophoresis under reducing conditions. Two specific bands were detected, migrating with molecular masses of 120 and 100 kDa. The intensity of both these bands was reduced in the presence of excess blocking peptide (Fig. 3C). Based on their molecular mass, these bands were regarded as the precursor (120 kDa) and active (100 kDa) forms of ADAM-17 protein.

Using the Spearman rank correlation test, a significant correlation was found between the two forms of ADAM-17 in primary breast carcinomas (r = 0.633, P < 0.0001, n = 83). Of the 83 samples investigated, 58 (69.8%) were positive for both proteins, whereas 8 (9.6%) were negative for both forms. Seventeen samples (20.5%) expressed the 100-kDa form in the absence of the 120-kDa form. None of the samples studied expressed the 120-kDa form in the absence of the 100-kDa band.

Table 2 summarizes the frequency of expression and relative levels of the two forms of ADAM-17 protein in the different breast tissue types investigated. The 120-kDa form of ADAM-17 was expressed more frequently (χ² test: P = 0.019) and at higher levels (Mann-Whitney U test: P = 0.0056) in primary

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**Fig. 1.** Effect of overexpressing ADAM-17 on Matrigel invasion and cellular proliferation of MCF-7 cells. **A.** ADAM-17 protein expression, following transfection of vector and ADAM-17 cDNA, was examined by immunoblot analysis. **B.** Comparison of the in vitro invasive potentials of MCF-7 cells transfected with vector or ADAM-17 cDNA. Invasion assays were done in 24-well Transwell chambers containing polyethylene terephthalate membranes, with 8-μm diameter pores, coated with Matrigel. Cells that migrated to the undersurface of the membranes were stained using crystal violet, and photographs were taken under the microscope (visual field at ×10). C. Invasive indices of parental, vector-transfected, and ADAM-17–transfected MCF-7 cells. Boxes, 75th percentile with the median indicated; bars, 10th and 90th percentiles. Analyzed using the Student’s paired t test (n = 9) * P < 0.01, for ADAM-17 transfection compared with parental and vector-transfected controls. **D.** Cell proliferation of parental, vector-transfected, and ADAM-17–transfected MCF-7 cells. Absorbance at 590 nm was expressed as a percentage of the control absorbance (deemed as 100%). Analyzed using the Student’s paired t test (n = 9). ** P < 0.0001, for ADAM-17 transfection compared with parental and vector-transfected controls. Columns, mean; bars, SE.
breast carcinomas compared with normal breast tissue. Although median levels of this form of ADAM-17 were ~3-fold higher in primary breast carcinomas compared with fibroadenomas, this difference was not statistically significant. Similar levels of the 120-kDa form were found in primary breast carcinomas and axillary node metastases.

Levels of the 100-kDa form of ADAM-17 were significantly higher in primary breast cancers compared with both normal breast tissue (Mann-Whitney U test: \( P = 0.0003 \)) and fibroadenomas (Mann-Whitney U test: \( P = 0.018 \)). In contrast to the 120-kDa form, levels of the 100-kDa form were also significantly higher in lymph node metastases than in primary breast carcinomas (Mann-Whitney U test: \( P = 0.031 \)). Using the Kruskal-Wallis test, levels of both the 120-kDa and 100-kDa forms of ADAM-17 protein increased progressively from normal breast tissues to primary breast carcinomas to nodal metastases (Kruskal-Wallis statistical test: \( P = 0.019 \) and \( P < 0.0001 \), respectively; Fig. 3D).

Relative levels of active and precursor forms of ADAM-17 in the different types of breast tissue. The ratio of active to total ADAM-17 was 64.4% in both normal breast tissue and fibroadenomas, 73.6% in the primary carcinomas, and 81.9% in the lymph node metastases (data acquired from Table 2). Using the Kruskal-Wallis test, the ratio of the active form to the precursor form increased progressively from the normal breast specimens to the primary carcinomas to the axillary node metastases (i.e., 2.5) and axillary node metastases (i.e., 4.7) than in normal breast tissue (i.e. 1.3; Mann-Whitney U test: \( P = 0.009 \) and \( P = 0.028 \), respectively).

Relationship between ADAM-17 and characteristics of the primary breast carcinomas. ADAM-17 protein levels, as determined by immunoblotting, were related to established prognostic factors for breast cancer (i.e., tumor size, presence, or absence of axillary node metastasis, tumor grade, histology type, ER status, and progesterone receptor status). No significant correlation was found between ADAM-17 protein expression and hormone receptor status, tumor size, or patient age at diagnosis. However, the 100-kDa form was expressed more frequently in node-positive than in node-negative primary breast carcinomas (\( \chi^2 \) test: \( P = 0.034 \)). The ratio of

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**Fig. 2.** Effect of knockdown of ADAM-17 expression on Matrigel invasion and cell proliferation of MDA-MB-435 cells. ADAM-17 mRNA and protein expression, following transfection with ADAM-17 shRNA (two clones) or neomycin-resistant negative control vector, was examined by (A) reverse transcription-PCR and (B) immunoblot analysis. C, comparison of the *in vitro* invasive potentials of MDA-MB-435 cells transfected with vector or ADAM-17 shRNA – transfected MDA-MB-435 cells. Boxes, 75th percentile with the median indicated; bars, 10th and 90th percentiles. Analyzed using the Student’s paired *t* test \( (n = 9) \). \({ }^* * \), \( P < 0.0001 \), for ADAM-17 shRNA – transfected clones compared with parental and vector-transfected controls. E, cell proliferation of parental, vector-transfected, and ADAM-17 shRNA – transfected MDA-MB-435 cells (see Fig. 3D). Analyzed using the Student’s paired *t* test \( (n = 9) \). \({ }^{**} \), \( P < 0.0001 \), for ADAM-17 shRNA – transfected clones compared with parental and vector-transfected controls. Columns, mean; bars, SE.
active to precursor ADAM-17 tended to be higher in tumors with a diameter of ≥2 cm compared with tumors with diameter of ≤2 cm (Mann-Whitney U test: \( P = 0.056 \)) and in ER-negative than in ER-positive tumors (Mann-Whitney U test, \( P = 0.055 \)).

Relationship between ADAM-17 protein and both uPA protein and PCNA protein. uPA is a serine protease that is causally involved in cancer invasion and metastasis and is one of the most powerful prognostic factors thus far described for breast cancer (27–29). We therefore compared levels of both the 120-kDa and 100-kDa forms of ADAM-17 with uPA levels. Levels of uPA were determined in all primary breast cancers that had been investigated for ADAM-17 by immunoblotting.

Table 2. Expression of the 120- and 100-kDa forms of ADAM-17 protein in the different types of breast tissue examined

<table>
<thead>
<tr>
<th>ADAM-17 protein</th>
<th>Tissue type</th>
<th>n</th>
<th>No. positive (%)</th>
<th>Median (range)</th>
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<tbody>
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<td>120 kDa</td>
<td>Normal breast tissue</td>
<td>33</td>
<td>15 (45.5)</td>
<td>0 (0-0.760)</td>
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<td>Fibroadenoma</td>
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<td>Primary breast carcinoma</td>
<td>83</td>
<td>58 (69.9)</td>
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<td>Nodal metastases</td>
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<td>100 kDa</td>
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<td>33</td>
<td>24 (72.7)</td>
<td>0.150 (0-1.740)</td>
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<tr>
<td></td>
<td>Fibroadenoma</td>
<td>22</td>
<td>15 (68.2)</td>
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<td>Primary breast carcinoma</td>
<td>83</td>
<td>75 (90.4)</td>
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<td></td>
<td>Nodal metastases</td>
<td>18</td>
<td>17 (94.4)</td>
<td>1.016 (0-6.218)</td>
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</table>

NOTE: Levels are expressed as arbitrary units of ADAM-17/\( \beta \)-actin.
correlated significantly with uPA levels (120-kDa form: \( r = 0.246, P = 0.032, n = 83 \); 100-kDa form: \( r = 0.428, P = 0.0001, n = 83 \)).

PCNA is a nuclear protein that is required for the activity of DNA polymerase delta and is widely used as a marker of cell proliferation (30). Seventy-three representative primary breast cancers that were analyzed by immunoblotting were also investigated for PCNA expression. Using Spearman rank analysis, a positive correlation was found between relative levels of both the 120-kDa and 100-kDa protein and PCNA protein levels (120-kDa form: \( r = 0.524, P < 0.0001, n = 73 \); 100-kDa form: \( r = 0.365, P = 0.002, n = 73 \); Fig. 4C and D).

**Discussion**

ADAM-17 is one of the most widely investigated ADAMs and one of the most important sheddases identified to date (1, 2). Because of its ability to release biologically important ligands such as TNF-\(\alpha\), transforming growth factor-\(\alpha\), amphiregulin, heparin-binding EGF, and epiregulin (9–16), it might be expected to play an important role in cancer progression. Our results with both cell lines and human breast tumors are consistent with this hypothesis.

Using the breast cancer cell line MCF-7, we showed that overexpression of ADAM-17 enhanced both invasion and proliferation in vitro. Conversely, decreased ADAM-17 expression in MDA-MB-435 cells reduced both invasion and proliferation. These findings are in agreement with other reports that showed that ADAM-17–mediated release of specific EGFR ligands, as a result of G-protein–coupled stimulation, also stimulated cell mitogenesis and migration in vitro (13–16). Evidence implicating EGFR in ADAM-17–mediated control of cell proliferation in the present study comes from the observation that ADAM-17 knockdown resulted in a significant decrease in the IC\(_{50}\) of gefitinib when compared with the IC\(_{50}\) for nontreated parental cells.

Using real-time PCR to measure ADAM-17 mRNA and Western blotting to determine ADAM-17 protein, we showed significant up-regulation in human breast cancer. Our results thus confirm and extend the findings of Lendeckel et al. (31) who reported higher levels of ADAM-17 mRNA in 24 breast cancers compared with corresponding normal breast tissue.

**Fig. 4.** Relationship among ADAM-17 protein, uPA, and PCNA. Bivariate scattergrams illustrating the positive correlations between uPA levels and (A) the 100-kDa form and (B) the 120-kDa form of ADAM-17 (\( n = 83 \)). Bivariate scattergrams illustrating the positive correlations between PCNA levels and (C) the 120-kDa form and (D) the 100-kDa form of ADAM-17 (\( n = 73 \)). Analyzed using the Spearman rank statistical test.
Similarly, ADAM-17 was shown to be up-regulated in hepatocellular (32), ovarian (33), and colorectal carcinoma (34). Up-regulation of ADAM-17 may thus be a common event in human malignancy.

Using Western blotting, ADAM-17 protein was found to exist in two forms, migrating with approximate molecular masses of 120 and 100 kDa. Based on their known molecular masses, these proteins are thought to represent the precursor and active forms of ADAM-17, respectively (35). A novel finding in this study was that the proportion of active to precursor ADAM-17 in human breast tissue increased with increasing malignancy (i.e., the ratio increased from nonmalignant breast tissues to the primary breast carcinomas to the lymph node metastases). Indeed, levels of the active, but not the precursor form of ADAM-17, were significantly increased in the lymph node metastases vis-a-vis primary breast cancers. These findings suggest increased processing of the ADAM-17 precursor protein occurs with increasing malignancy. Furthermore, the increased expression of the active form in nodal metastases suggests that this protein may play a role in dissemination of primary breast cancers to axillary nodes.

In agreement with our findings across different types of breast tissue, we also showed that within primary cancers the expression of ADAM-17 correlated with variables of progressive disease. Thus, the ratio of active to precursor form of ADAM-17 tended to increase with increasing size of the tumor and with ER negativity. Furthermore, consistent with our in vitro findings, levels of ADAM-17 in the primary breast tumors correlated with cell proliferation as measured by PCNA. The correlation between ADAM-17 levels and proliferation in the primary breast cancers might relate to the release of endogenous mitogenic ligands (12–16).

As well as correlating with proliferation rates, ADAM-17 protein levels were also found to be significantly associated with those of uPA. uPA is a serine protease causally involved in invasion and metastasis and is one of the most potent biological prognostic factors thus far described for breast cancer (27–29). Recently, its prognostic effect in lymph node–negative breast cancer patients was validated in both a prospective randomized trial (28) and a pooled analysis (29). The correlation between ADAM-17 levels and uPA in the breast cancers could result from ADAM-17–mediated shedding of ligands that induce expression of uPA such as amphiregulin (14, 36) and TNF-α (37).

Although our data suggest that ADAM-17 plays a role in human breast cancer progression, Peduto et al. (38) recently reported that that another ADAM (i.e., ADAM-9) was involved in the pathogenesis of prostate cancer in a mouse model. In this system, a deficiency of ADAM-9 delayed or prevented tumor progression after the well-differentiated stage. A potential mechanism by which ADAM-9 potentiated progression in this model system was by releasing EGF and/or fibroblast growth factor receptor 2iih (38). In another mouse model system, a deficiency of ADAM-15 resulted in decreased pathologic neo-vascularization and reduced tumor growth (39).

In summary, our cell line and human tumor results when taken together support the hypothesis that ADAM-17 is involved in human breast cancer progression. Further work with appropriate animal models, however, will be necessary this hypothesis. If our results are confirmed using in vivo experiments, ADAM-17 could be a new target for the treatment of breast cancer (i.e., selective inhibition of ADAM-17 could prevent the release of multiple ligands potentially important in promoting tumor growth). In this context, it is worth noting that etanercept, a recombinant human soluble p75 TNF receptor that inhibits TNF-α biological activity, has recently been evaluated in clinical trials for the treatment of advanced breast (40) and ovarian cancer (41). Blockage of ADAM-17 protease activity might be expected to inactivate not only the paracrine actions of TNF-α but also those of the EGF ligands, transforming growth factor-α, heparin-binding EGF, amphiregulin, and epiregulin (13–16). In recent years, a number of selective inhibitors of ADAM-17 have been described (26, 42–47), some of which have been shown to reduce tumor growth in model systems. Whether selective ADAM inhibitors will be more effective or exhibit fewer side effects than the broad-spectrum matrix metalloproteinase inhibitors previously investigated remain to be shown.

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References

ADAM-17 Expression in Breast Cancer Correlates with Variables of Tumor Progression


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