Dysregulated Expression of Adamalysin-Thrombospondin Genes in Human Breast Carcinoma

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ABSTRACT

The adamalysin-thrombospondin (ADAMTS) proteinases are a relatively newly described branch of the metzincin family that contain metalloproteinase, disintegrin, and thrombospondin motifs. They have been implicated in various cellular events, including cleavage of proteoglycans, extracellular matrix degradation, inhibition of angiogenesis, gonadal development, and organogenesis. However, in many cases, their normal physiological roles and their potential for dysregulation in malignancy remain to be established. The expression profile of *ADAMTS1–20* in human breast carcinoma was undertaken by real-time PCR using RNA isolated from malignant tumors, nonneoplastic mammary tissue, and breast cancer cell lines to identify altered regulation that may have potential pathogenetic and prognostic significance. Our studies show that seven of the *ADAMTS* genes (*ADAMTS1, 3, 5, 8, 9, 10,* and *18*) are consistently down-regulated in breast carcinomas with respect to nonneoplastic mammary tissue, irrespective of the heterogeneity of the samples and the tumor type or grade (Mann-Whitney *U* test, $P < 0.0001$ for each gene). Conversely, *ADAMTS4, 6, 14,* and *20* are consistently up-regulated in breast carcinomas ($P = 0.005$, $P < 0.0001$, $P = 0.003$, and $P = 0.001$, respectively). *ADAMTS2, 7, 12, 13, 15, 16, 17,* and *19* show no significant difference between the sample types. *ADAMTS1, 2, 7, 8, 10,* and *12* are expressed predominantly in stromal fibroblasts. *ADAMTS3, 4, 5, 6, 9,* and *13–20* inclusive are expressed predominantly in myoepithelial cells; all appear to be relatively poorly expressed in luminal epithelial cells. *ADAMTS15* has emerged as being an independent predictor of survival, with RNA expression levels significantly lower ($P = 0.007$) in grade 3 breast carcinoma compared with grade 1 and 2 breast carcinoma.

INTRODUCTION

The adamalysin-thrombospondin (ADAMTS) proteinases are a family of metalloproteinases with functions in extracellular matrix processing, organogenesis, and hemostasis (for review, see Ref. 1). To date, there are 19 published *ADAMTS* genes, numbered *1–10* and *12–20*. The proteins they encode all comprise an NH_2-terminal signal peptide, a prodomain, and metalloproteinase, disintegrin, cysteine-rich, and thrombospondin type 1 (TSP1) domains, followed by a spacer region and a variable number of TSP1-like motifs (for an overview of the domain organization, see Ref. 2); in some cases, there is a COOH-terminal protease and lacunin domain (3) or a complement subcomponent/embryonic sea urchin protein/bone morphogenic protein domain (4). Unlike the adamalysins, the ADAMTSs are not membrane-anchored but bind to extracellular matrix components such as heparin and heparan sulfate after their secretion (1, 5); this binding is mediated through their TSP1 motifs (6).

The prototype, ADAMTS-1, was first described by Kuno *et al.* (6) and isolated on the basis of elevated expression in adenocarcinomas that induce cachexia in mice. It is an effective inhibitor of angiogenesis (7) and has a role in follicular rupture at ovulation (8) and urogenital organogenesis, as evidenced by the phenotype of *ADAMTS1* knockout mice (9). ADAMTS-2, -3, and -14 are all procollagen N-proteinases, involved in the processing of procollagen I (ADAMTS-2 and -14) and procollagen II (ADAMTS-2 and -3) to collagen by cleavage of the NH_2 terminus propeptides (10–13). Deficiency in the *ADAMTS2* gene causes Ehlers-Danlos syndrome type VIIc in humans (14); *ADAMTS2* knockout mice develop severe skin fragility, and males are sterile, suggesting an additional role for ADAMTS-2 in spermatogenesis or fertility (15). Five of the ADAMTSs have been identified as aggrecanases, which are pivotal for cartilage degradation in osteoarthritis: ADAMTS-1 (16); ADAMTS-4 and ADAMTS-5/TS-11 (17, 18); ADAMTS-9 (19); and ADAMTS-15 (20). *ADAMTS6* and *7* have been described, but their protein function remains uncharacterized (21). ADAMTS-8, like ADAMTS-1, is a potent inhibitor of angiogenesis (7) and was also described independently by Georgiadis *et al.* (22). ADAMTS-9 is thought to function as an antiangiogenic agent (23), and both it and ADAMTS-20 may possibly have a role in gonadal development because of its high sequence homology to the *Caenorhabditis elegans ADAMTS* gene *gon-1* (19, 23). *ADAMTS12* transcripts have been isolated in gastric carcinoma and various tumor cell lines, suggesting a role in tumor processes (24). ADAMTS-13, which has been described very recently, has been characterized as the blood

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clotting protein von Willebrand factor cleaving protease (4, 25–27). Another recent paper describes seven novel *ADAMTS* genes, including *ADAMTS15–19* (2).

Like matrix metalloproteinases (MMPs), certain ADAMTSs have matrix-degrading activities and might therefore be involved in pathological tissue remodeling and cell invasion. At least two family members have antiangiogenic capabilities; others may do so via their TSP1 motifs. Therefore, it is important to explore possible links between ADAMTS genes and cancer, particularly because ADAMTSs are potential targets of synthetic metalloproteinase inhibitors that have entered clinical cancer trials (for review, see Ref. 28). In this study, real-time PCR was used to evaluate the expression profile of *ADAMTS1–20* in a series of human breast carcinomas, nonneoplastic mammary tissue, and purified populations of mammary tissue cell types to assess evidence of altered regulation with potential pathogenetic and prognostic significance.

MATERIALS AND METHODS

This study received the approval of the Norwich District Ethics Committee, the Norwich Research and Development Committee, and the Partners in Cancer Research Tissue Bank Committee.

Human Breast Tumor Samples. Forty-eight women ages 40–88 years (median age, 58 years) provided samples of 50 primary breast neoplasms after informed consent. Two patients who had bilateral disease gave two samples, one from each side. Forty-eight of the neoplasms were invasive carcinomas, and two were high-grade ductal carcinoma *in situ*, one of which showed foci of microinvasion. All samples were taken from fresh, unfixed carcinomas removed surgically. The specimens were sent immediately after removal to the laboratory, where, after inking the margins and slicing the specimen, a small part of the tumor was removed by a histopathologist for this study. Specimens were prepared and snap-frozen in the department's human tissue bank as described by Riddick *et al.* (29). The rest of the surgical specimen was fixed in 10% formal saline and processed for routine histopathological diagnosis and establishment of prognostic factors.

Nonneoplastic Human Mammary Tissue. Samples of nonneoplastic mammary tissue were obtained from 10 patients with informed consent at reduction mammoplasty. All samples were snap-frozen and stored as described for the cancers. An additional sample was obtained commercially as total RNA from normal human mammary tissue (Stratagene, La Jolla, CA).

Purified Samples of Nonneoplastic Human Mammary Cell Populations. Normal breast tissue was obtained from reduction mammoplasty procedures following consent. Primary myoepithelial, luminal epithelial, and fibroblast populations were isolated according to a modified version of the protocol described by Gomm *et al.* (30). Tissue was digested as described by Stampfer *et al.* (31) in 200 units/ml collagenase type IA and 125 units/ml hyaluronidase in DMEM containing 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone for 12–18 h. The resulting mixed organoid and single cell preparation was subjected to a single sedimentation step of 30 min at 4°C, and the supernatant, rich in fibroblasts, was removed and passed sequentially through 2 × 40-μm filters and then transferred to coverslips for characterization. Organoids were washed in Enriched Medium (1:1 DMEM:Ham's F-12 medium, 10% fetal bovine serum, 5 μg/ml insulin, 5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone; all from Sigma) and then digested in 20 ml of trypsin/EDTA containing 400 μl of DNase, incubated at 37°C for 10 min. After further filtration, the single cell suspension was resuspended in Enriched Medium for isolation of myoepithelial cells or Luminal Medium (Enriched Medium lacking epidermal growth factor) for isolation of the luminal population. Myoepithelial cells were selected using Pan-Mouse IgG Dynabeads (Dynal United Kingdom Ltd., Bromborough, United Kingdom) labeled with CALLA (CD10; Dako), as described previously (32). Luminal cells were isolated using the MiniMACS magnetic cell sorting system (Miltenyi Biotec Ltd.), according to the manufacturer's instructions. Briefly, cells were incubated with antibody to epithelial membrane antigen [EMA (clone E29; Dako)] for 10 min at 4°C with rotation. After washing in EDTA/PBS, the cells were incubated with MACS goat antimouse IgG microbeads in EDTA/PBS for 15 min at 4°C and then passed through a MiniMACS column. After several washes to elute the nonlabeled fraction, the column was removed from the magnet, and the labeled cell fraction was eluted.

Characterization of Isolated Cell Populations by Immunohistochemistry. Aliquots of isolated cells were plated onto poly-D-lysine-coated coverslips and grown for 48 h in appropriate media. The cells were then fixed in acetone for 10 min at 4°C, and immunohistochemistry was performed using antibodies to cytokeratin (CK) 14 and CK18 (LL001 and LE061, respectively; both received as gifts from Prof. E. B. Lane; Cancer Research UK Cell Structure Research Laboratories, Dundee, Scotland, United Kingdom), EMA, and vimentin (Dako) with a standard streptavidin/biotin detection system, as described previously (32). For analysis of RNA from isolated mammary cells to assess cell purity using primers for the desmosomal glycoproteins *Dsg2* and *Dsg3*, see Refs. 32 and 33.

Human Breast Cancer Cell Lines. The breast cancer cell lines MCF-7, T47D, MDA-MB-231, and BT549 were obtained from the American Type Culture Collection (Manassas, VA) and cultured as directed. All media and supplements were obtained from Life Technologies, Inc. (Paisley, United Kingdom).

RNA Extraction. Total RNA was isolated using a modification of the SV Total RNA Isolation System (Promega, Madison, WI; Ref. 34): 50–100 mg of tumor tissue were homogenized in 1 ml of RNazol B reagent (Biogenesis Ltd., Poole, United Kingdom) using an UltraTurrax T8 homogenizer (IKA). The homogenate was stored at −80°C pending the following step of the protocol: the samples were allowed to thaw completely at room temperature, then centrifuged at 14,000 rpm for 10 min and the clear supernatants were collected into 200 μl of chloroform. They were shaken vigorously for 15 s, incubated for 3 min at room temperature, and centrifuged for 15 min at 14,000 rpm. The upper phase was collected into 200 μl of 95% ethanol, mixed, and transferred into a spin basket assembly. The Promega protocol was then followed from step 7 to the end according to the manufacturer's instructions.

Reverse Transcription. One μg of total RNA was primed with 0.5 μg of random hexameric primers (Promega) and reverse transcribed into cDNA in a 20-μl reaction volume containing 200 units of SuperScript II reverse transcriptase, 5× first-strand buffer [final concentrations: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$], 10 mM DTT, 0.5 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Life Technologies, Inc.), and 40 units of RNasin RNase inhibitor (Promega). Each priming reaction was carried out at 70°C for 10 min and stopped by placing samples on ice; the reverse transcription was carried out at 42°C for 1 h followed by incubation at 70°C for 10 min.

PCR. The integrity of the RNA in each sample was checked by PCR amplification of the reverse-transcribed cDNA as described above, using primers for the *glyceraldehyde-3-phosphate dehydrogenase* gene. The sequence of the forward primer was 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ (nucleotides 78–101), and that of the reverse primer was 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ (nucleotides 384–361) to give a 307-bp amplicon (Marc Lafleur; University of East Anglia, Norwich, United Kingdom). The reactions contained 100 ng of cDNA, 20 pmol of each primer, 1× PCR buffer [final concentrations: 67 mM Tris-HCl (pH 8.8), 16 mM $(NH_4)_2SO_4$, and 0.01% Tween 20], 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Bioline, London, United Kingdom), and 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP; Life Technologies, Inc.) in a 50-μl reaction volume. The amplification reaction was carried out over 35 cycles with the following parameters: 94°C for 60 s; 60°C for 60 s; and 72°C for 90 s. The 72°C step was extended to 10 min for the final cycle to obtain full-length PCR products.

TaqMan Real-Time PCR. Forward and reverse primers and fluorescence-labeled oligonucleotide probes [using 6-carboxy-fluorescein (FAM) as the reporter dye and 6-carboxy-tetramethyl rhodamine (TAMRA) as the quencher dye] were designed for each of the human *ADAMTS* genes and the c-*erb*B-2 gene using Primer Express 1.0 software (PE Applied Biosystems, Foster City, CA) and synthesized by PE Applied Biosystems (Warrington, United Kingdom). The primer and probe sequences for *MMP-9* and *-11*, designed in our laboratory, are now the intellectual property of Applied Biosystems. The primer and probe set for *cyclin D1* was obtained directly from Applied Biosystems (as before). BLASTN searches were undertaken on all our designed primer and probe sequences to confirm gene specificity. To ensure against genomic DNA amplification, the forward and reverse primers were placed in separate adjacent exons. Since the genomic sequences of the human *ADAMTS* genes were not published at the time of primer design, the mouse *Adamts1* genomic sequence (35) was used to predict their intron-exon boundaries using the CLUSTALW program (Biology Workbench 3.2).[6] The correct identity of the PCR products obtained was confirmed by direct sequencing of the amplicons. Details of the primers and probes are given in Table 1. For each human *ADAMTS* gene, a standard curve with concentrations ranging from 25 ng to 1 ng was produced using human placental cDNA dissected from fetal villi as the template. An XY scatter plot was for each gene was produced using Microsoft Excel Chart Wizard software (Microsoft Office 2001), and values for the equation $y = mx + b$ (where m = the slope of the standard curve and b = the y intercept of that line) and R^2 were obtained. The *18S* rRNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. The *cyclin D1* gene was also used as a further control to confirm that differences in gene expression between sample types were not due merely to the proliferation rate of the tumors. PCR reactions for all samples were performed in duplicate in 96-well optical plates with 5 ng of cDNA (1 ng of cDNA for the *18S* gene), 100 nM probe, 200 nM each primer, and 12.5 μl of TaqMan Universal 2× PCR Master Mix (PE Applied Biosystems, Warrington, United Kingdom) in a 25-μl reaction volume. The amplification reaction was carried out over 40 cycles with the following parameters: an initial holding stage of 2 min at 50°C and then 10 min at 95°C, followed by a two-step cycling program of 15 s at 95°C and 1 min at 60°C.

Statistical Analysis. The data were found not to be normally distributed (Kolmogorov-Smirnov test; data not shown). Significant differences in the normalized input values, representing RNA expression levels, of invasive carcinoma and normal mammary tissue for each *ADAMTS* gene were determined using the Mann-Whitney *U* test. Correlations between the normalized input values of the invasive carcinoma samples and various clinical characteristics were undertaken using Spearman's rank correlation. Kaplan-Meier survival functions were plotted to evaluate the expression levels of each *ADAMTS* gene from the normalized input values of the invasive carcinoma samples against event-free survival for those patients. P values < 0.05 were deemed statistically significant in all analyses. All data were analyzed using SPSS 11.0; the normalized input values are in arbitrary units.

RESULTS

Expression levels for *ADAMTS* RNA were analyzed in invasive breast carcinoma ($n = 50$), nonneoplastic mammary tissue ($n = 11$), and breast cancer cell lines ($n = 4$). Normalized input values are presented graphically in Fig. 1, and the grouped median values of the three sample groups for each gene are given in Table 2. Comparison of RNA expression levels in invasive breast carcinomas with those of nonneoplastic mammary tissue showed that gene expression levels of *ADAMTS1, 3, 5, 8, 9, 10,* and *18* were significantly lower in the malignant samples ($P < 0.0001$ for each gene). By contrast, the same evaluation showed that expression of *ADAMTS4, 6, 14,* and *20* was significantly higher in the malignant samples as compared with normal mammary tissue ($P = 0.005$, $P < 0.0001$, $P = 0.003$, and $P = 0.001$, respectively). No significant difference was seen between the two sample types for *ADAMTS2, 7, 12, 13, 15, 16, 17,* or *19.*

To define the cellular origins of the *ADAMTS* genes expressed in normal mammary gland, we isolated fibroblasts, epithelial and myoepithelial cells using a combination of collagenase digestion and affinity separation procedures. Immunohistochemistry demonstrated that CALLA-extracted cells were

[6] http://www.workbench.sdsc.edu/.

Table 1 TaqMan primer and probe sets for the human ADAMTS[a] and c-*erb*B-2 genes

Target gene	Forward primer sequence Reverse primer sequence Probe sequence (all 5′–3′)	Target accession no.	Position	Product size (bp)	Annealing temperature (°C)	PCR cycle no.
ADAMTS1	GGACAGGTGCAAGCTCATCTG	NM_006988.2	2207–2227	72	59	40
	TCTACAACCTTGGGCTGCAAA		2278–2258		59	
	CAAGCCAAAGGCATTGGCTACTTCTTCG		2229–2256		69	
ADAMTS2	CTGGCAAGCATTGTTTTAAAGGA	NM_014244.1	1622–1644	95	59	40
	GGAGCCAAACGGACTCCAA		1716–1698		60	
	ATCTGGCTGACACCTGACATCCTCAAACG		1651–1679		70	
ADAMTS3	GCAGCATTCCATCGTTACCA	NM_014243.1	1328–1347	145	58	40
	CCATAGAATAATTGATTCCAGGAAGTT		1472–1446		58	
	CCATTCCTATGACTGTCTCCTTGATGACCC		1387–1416		68	
ADAMTS4	CAAGGTCCCATGTGCAACGT	NM_005099.2	974–993	115	60	40
	CATCTGCCACCACCAGTGTCT		1088–1068		60	
	CCGAAGAGCCAAGCGCTTTGCTTC		1027–1050		70	
ADAMTS5	TGTCCTGCCAGCGGATGT	NM_007038.1	2096–2113	117	60	40
	ACGGAATTACTGTACGGCCTACA		2212–2190		59	
	TTCTCCAAAGGTGACCGATGGCACTG		2162–2187		70	
ADAMTS6	GGCTGAATGACACATCCACTGTT	NM_014273.2	680–702	96	60	40
	CAAACCGTTCAATGCTCACTGA		775–754		60	
	CACTACCAATTAACAACACACATATCCACCACAGACAG		710–747		70	
ADAMTS7	CAGCCTACGCCCAAATACAAA	NM_014272.1	1711–1731	143	59	40
	CCCTTGTAGAGCATAGCGTCAAA		1853–1831		59	
	AAGCGCTTCCGCCTCTGCAACC		1756–1777		69	
ADAMTS8	CCGCCACCCAGAGCACTA	NM_007037.2	1625–1642	138	59	40
	TCGATCACGGAGCAGCTTTT		1762–1743		60	
	CCATCCTGCTCACCAGACAGAACTTCTGTG		1651–1680		70	
ADAMTS9	TTAGTGAAGATAGTGGATTGAGTACAGCTT	AF488803.1	1291–1320	137	59	40
	TGTTGGAGCCATGACATGCT		1427–1408		59	
	ATCGCCCATGAGCTGGGCCA		1326–1345		69	
ADAMTS10	AGAGAACGGTGTGGCTAACCA	NM_030957.1	1241–1261	127	58	40
	TCTCTCGCGCTCACACATTC		1367–1348		59	
	CAGTGCTCATCACACGCTATGACATCTGC		1270–1298		69	
ADAMTS12	CACGACGTGGCTGTCCTTCT	NM_030955.1	1341–1360	133	60	40
	CCGAATCTTCATTGATGTTACAACTG		1473–1448		60	
	AGGACATCTGTGCTGGTTTCAATCGCC		1369–1395		70	
ADAMTS13	CAGAGCGAGAGAATATGTCACATTTC	AJ305314.1	1722–1747	96	59	40
	ACCGCCAAGTGTGTGAAGAGA		1817–1797		59	
	CCAACCTGACCAGTGTCTACATTGCCAAC		1760–1788		69	
ADAMTS14	CGCTGGATGGGACTGAGTGT	AF366351.1	1495–1514	130	60	40
	CGCGAACATGACCCAAACTT		1624–1605		60	
	CCCGGCAAGTGGTGCTTCAAAGGT		1518–1541		70	
ADAMTS15	ATGTGCTGGCACCCAAGGT	NM_139055.1	1886–1904	91	60	40
	CAGCCAGCCTTGATGCACTT		1976–1957		60	
	CCTGACTCCACCTCCGTCTGTGTCCA		1927–1952		70	
ADAMTS16	GCCCATGAGTCTGGACACAA	NM_139056.1	1294–1313	129	58	40
	GCAGGGTGACCAGGAGAAGA		1422–1403		59	
	TGCAAAAAGTCCGAGGGCAACATCAT		1348–1373		69	
ADAMTS17	GGTCTCAATTTGGCCTTTACCAT	NM_139057.1	1161–1183	92	59	40
	GACCTGCCAGCGCAAGAT		1252–1235		59	
	CCACAACTTGGGCATGAACCACGA		1199–1222		69	
ADAMTS18	CTCATTGGAAAGAATGGCAAGAG	NM_139054.1	1202–1224	141	59	40
	GGTACAACTTCGGTACTTAGAGCACAT		1342–1316		59	
	TGTGACACTCTAGGGTTTGCCCCCAC		1283–1308		69	
ADAMTS19	GGTGTAAGGCTGGAGAATGTACCA	AJ311904.1	1856–1879	120	60	40
	TGCGCTCTCGACTGCTGAT		1975–1957		59	
	CCTCAGCACCTGAACATCTGGCCG		1886–1909		70	
ADAMTS20	ATTTCTGCTTTTACTATAGCCCATGA	AF488804.1	1186–1211	109	58	40
	GGGCCATTACATGATACTTTGTAACT		1294–1269		58	
	CTTGGGCACACACTTGGTGTTCAACA		1213–1238		68	
c-*erb*B-2	GGCTCTCACACTGATAGACACCAA	M11730.1	687–710	114	59	40
	CGCGTCAGGCTCTGACAAT		800–782		59	
	CTCTCGGGCCTGCCACCCCT		714–733		69	

[a] ADAMTS, adamalysin-thrombospondin.

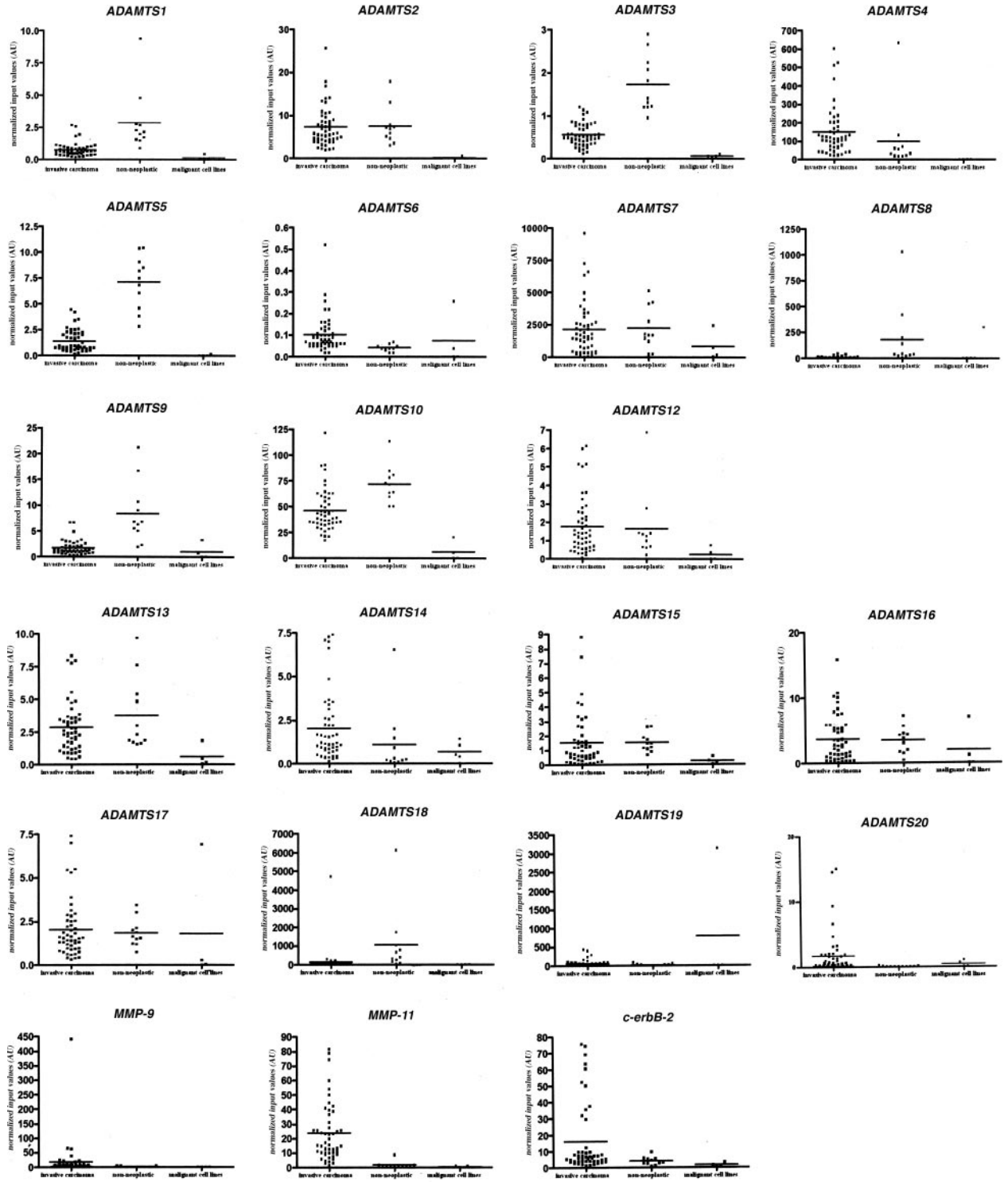

Fig. 1 Graphs to show RNA expression levels in invasive carcinoma, nonneoplastic mammary tissue, and breast cancer cell lines.

uniformly positive for CK14 and vimentin but negative for CK18 and EMA, in keeping with a myoepithelial phenotype. EMA-isolated cells were positive for CK18 and EMA but negative for CK14 and vimentin, consistent with a luminal epithelial phenotype, and fibroblasts displayed uniform positivity for vimentin but were negative with the other markers. The reverse transcription-PCR analysis on isolated populations showed that EMA-isolated cells expressed *DSg2* but not *DSg3*, whereas

Table 2 Real-time PCR (TaqMan) ADAMTS[a] profiling data showing standardized median values

Gene[b]	Nonneoplastic mammary tissue (n = 11)	Invasive breast carcinoma[c] (n = 48)	P	Ct value median (range)		Normalized input median (range)	
				Nonneoplastic mammary tissue	Invasive breast carcinoma	Nonneoplastic mammary tissue	Invasive breast carcinoma
ADAMTS1	1	0.29	<0.0001	23.10 (21.26–25.00)	24.70 (22.13–26.75)	2.14 (0.86–9.39)	0.63 (0.17–2.66)
ADAMTS2	1	0.85	0.796	26.23 (24.53–29.32)	26.04 (23.41–28.67)	6.88 (3.03–17.99)	5.85 (2.04–25.72)
ADAMTS3	1	0.35	<0.0001	29.65 (28.75–31.48)	31.28 (29.30–34.98)	1.41 (0.96–2.90)	0.49 (0.12–1.21)
ADAMTS4	1	3.56	0.005	27.37 (23.81–28.73)	25.47 (23.10–28.26)	33.89 (18.69–635.75)	120.74 (22.75–603.24)
ADAMTS5	1	0.11	<0.0001	23.48 (23.12–26.43)	26.27 (24.11–29.55)	7.54 (2.83–10.46)	0.83 (0.16–4.46)
ADAMTS6	1	1.82	<0.0001	29.31 (28.14–32.68)	27.96 (24.76–31.47)	0.04 (0.02–0.07)	0.08 (0.02–0.52)
ADAMTS7	1	0.94	0.599	30.07 (28.33–34.43)	30.05 (27.57–36.57)	1727.50 (237.10–5164.04)	1625.50 (21.89–9647.94)
ADAMTS8	1	0.094	<0.0001	32.15 (27.92–34.48)	34.63 (31.26–38.64)	39.74 (7.16–1033.80)	3.73 (0.13–46.72)
ADAMTS9	1	0.17	<0.0001	25.93 (24.66–29.36)	28.04 (25.43–30.80)	6.73 (1.94–21.27)	1.14 (0.30–6.65)
ADAMTS10	1	0.59	<0.0001	27.02 (25.54–29.67)	27.29 (25.41–29.68)	71.57 (50.26–113.56)	41.96 (17.17–121.41)
ADAMTS12	1	1.05	0.724	27.27 (24.62–31.90)	27.35 (25.11–33.13)	1.23 (0.20–6.89)	1.29 (0.03–6.15)
ADAMTS13	1	1.05	0.457	31.93 (29.38–33.67)	31.04 (28.94–34.87)	2.32 (1.57–9.74)	2.43 (0.46–8.35)
ADAMTS14	1	5.39	0.003	34.66 (29.35–40.00)	31.34 (28.34–35.41)	0.22 (0.03–6.53)	1.19 (0.28–7.41)
ADAMTS15	1	0.58	0.102	25.05 (23.53–27.40)	25.49 (21.65–30.41)	1.48 (0.67–2.68)	0.85 (0.04–8.86)
ADAMTS16	1	0.73	0.759	28.31 (26.61–32.84)	28.10 (25.78–33.17)	3.84 (0.56–7.28)	2.82 (0.11–15.85)
ADAMTS17	1	0.94	0.684	28.64 (27.56–30.46)	28.46 (25.58–31.80)	1.63 (0.73–3.42)	1.53 (0.30–7.41)
ADAMTS18	1	0.10	<0.0001	26.88 (24.16–31.26)	29.11 (24.16–37.27)	380.12 (26.47–6126.86)	39.45 (0.02–4724.45)
ADAMTS19	1	1.35	0.684	29.55 (27.28–31.56)	29.10 (25.82–33.40)	15.16 (3.39–84.50)	20.43 (0.40–432.49)
ADAMTS20	1	22.00	0.001	38.71 (35.79–40.00)	34.03 (29.21–40.00)	0.02 (0.01–0.24)	0.44 (0.00–15.07)

[a] ADAMTS, adamalysin-thrombospondin; Ct, cycle threshold.
[b] Normalized input values between different genes are not directly comparable.
[c] Values for invasive breast carcinoma are represented as a percentage of the normalized input values obtained for nonneoplastic mammary tissue for each given gene.

CALLA-isolated cells expressed both genes, consistent with the myoepithelial cell-restricted expression of *DSg3* reported previously (33). Fibroblasts did not express either desmosomal gene. Together, these results indicate substantially pure populations of different breast cell types.

Gene expression localization studies using the sorted cell populations from pooled nonneoplastic mammary tissue showed that expression levels of *ADAMTS1, 2, 7, 8, 10,* and *12* were highest in stromal fibroblasts, whereas expression levels of *ADAMTS3, 4, 5, 6, 9,* and *13–20* inclusive were highest in myoepithelial cells. All of the *ADAMTS* genes appeared to be relatively poorly expressed in luminal epithelial cells (Fig. 2).

Whereas the data in Fig. 1 provide a quantitative comparison between samples for each *ADAMTS* gene normalized to *18S* RNA levels, the analyses did not indicate the relative expression levels of each gene. To resolve this, cycle threshold results and their ranges are also presented in Table 2. These data indicate that, of the gene family, *ADAMTS1, 5,* and *15* show the highest levels of expression.

To characterize further the tissues that were analyzed in this study, expression analysis of several genes that have previously been reported to be linked with malignancy in mammary neoplasms was undertaken. The results obtained for *MMP-9, MMP-11,* and c-*erb*B-2 (Fig. 1) were consistent with previously published expression patterns for these genes in both invasive breast carcinoma and nonneoplastic mammary tissue (36–40). All three genes showed significant overexpression at the RNA level in carcinomas compared with nonneoplastic mammary tissue. A control analysis using *cyclin D1* as a proliferation marker of mammary neoplasia showed that the altered regulation seen with the *ADAMTS* genes was independent of tumor cell proliferation (data not shown).

Table 3 summarizes the clinical and pathological features of patients with invasive breast carcinoma, and the clinical features of the nonneoplastic breast tissue patients are given in Table 4. Possible correlations between RNA expression levels in invasive carcinoma and tumor grade, estrogen receptor status (Spearman's rank correlation), and differences with respect to axillary lymph node involvement (Mann-Whitney U test) were evaluated for each *ADAMTS* gene. *ADAMTS15* expression levels were significantly lower in patients with grade 3 tumors compared with grade 1 or grade 2 tumors ($P = 0.007$). Analysis of event-free survival of the invasive breast carcinoma patient set was undertaken for each *ADAMTS* gene using Kaplan-Meier survival functions. The median normalized input value for each gene was used to divide the patients into two groups: those with values at or above the median *versus* those with values below the median. This showed that only *ADAMTS15* expression in the tumors was an independent predictor of survival ($P = 0.0024$; shown graphically in Fig. 3). When combined with Nottingham Prognostic Index values, prediction of those patients who have died or relapsed increased from 17% to 75% (data not shown). Survival data of the patients with breast carcinoma are presented in Table 5.

The data were also assessed by coexpression analysis to look for potential patterns of coregulation of the genes in mammary tissues. The mRNA expression of each *ADAMTS* gene in the entire tissue collection (*i.e.*, with malignant and nonneoplastic cases grouped, but excluding cell lines) was compared with each of the other *ADAMTSs* and with *MMP-9, MMP-11,* and c-*erb*B-2. All statistically significant correlations are shown in Fig. 4. This analysis revealed particular *ADAMTS* gene pairs that showed strong association of their expression. These pairs can be seen to form two principal groupings that show positive

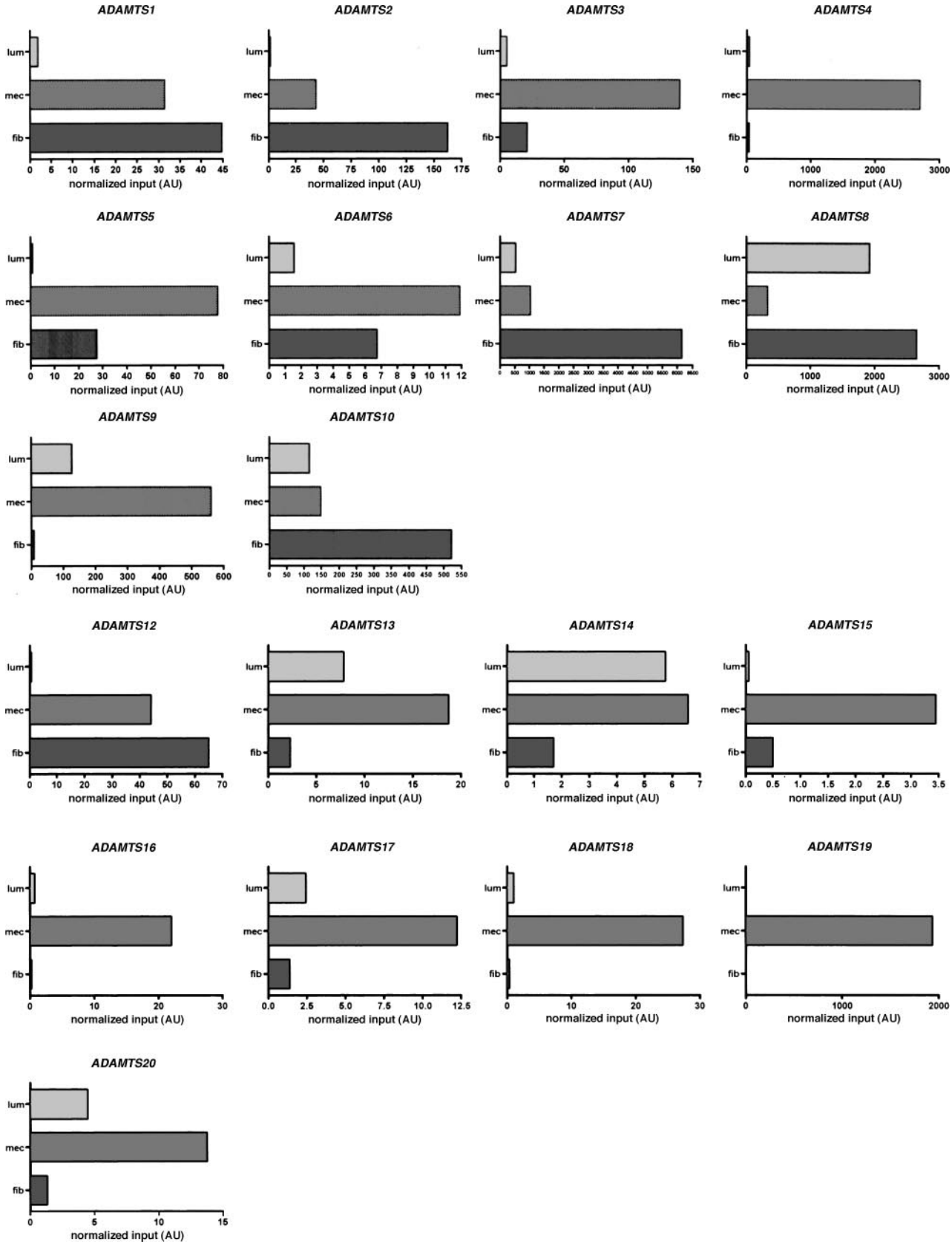

Fig. 2 Graphs to show RNA expression localization within nonneoplastic mammary tissue.

Table 3 Clinical and pathological features of patients with invasive breast carcinoma

	No. of patients
Histological classification	
Ductal carcinoma, NOS[a]	29
Ductal carcinoma, mucinous	2
Ductal carcinoma, tubular	1
Ductal carcinoma, cribriform	1
Metaplastic carcinoma	1
Lobular carcinoma	10
Mixed ductal (all types) and lobular	6
Tumor size (cm)	
<2	19
2–4	25
>4	4
No size	2
Tumor grade	
Grade 1	9
Grade 2[b]	22
Grade 3	16
No grade	3
NPI	
Good (<3.4)	13
Intermediate (3.4–5.4)	28
Poor (>5.4)	4
No NPI[c]	5
No. of axillary LN metastases	
0	25
1–3	15
>3	6
Axilla not sampled	4
ER status	
Negative	6
Positive	24
Strongly positive	12
Unknown	8
Age median (range) (yrs)	58 (40–88)
Total	50

[a] NOS, not otherwise specified; NPI, Nottingham Prognostic Index; LN, lymph node; ER, estrogen receptor.
[b] Includes all lobular carcinomas and four mixed carcinomas.
[c] Includes simple mastectomies only and one ungraded case of metaplastic carcinoma.

pairwise correlations of mRNA expression at significance levels of $P < 0.0001$: the first set includes *ADAMTS1, 3, 5, 8, 9, 10,* and *18*; and the second set comprises *ADAMTS2, 4, 6, 7, 12,* and *14*. Expression of c-*erb*B-2 did not correlate strongly (*i.e.*, $P < 0.0001$) with any of the other genes assessed, but *MMP-11* expression showed strong negative correlation with *ADAMTS1, 5, 8,* and *9*.

DISCUSSION

This study is the first detailed investigation of the *ADAMTS* genes in human breast cancer. The most striking observation is that 11 of the 19 *ADAMTS* genes studied show altered expression in malignant compared with nonneoplastic tissue. *ADAMTS1, 3, 5, 8, 9, 10,* and *18* are down-regulated in human breast carcinoma; *ADAMTS4, 6, 14,* and *20* are up-regulated; and *ADAMTS2, 7, 12, 13, 15, 16, 17,* and *19* show no significant difference in mRNA expression levels between malignant and nonneoplastic tissue. Whereas all of the ADAMTSs that are up-regulated in breast carcinoma are expressed predominantly in myoepithelial cells in nonneoplastic mammary tissue, those that are down-regulated are not confined to one cell type in nonneoplastic mammary tissue; some are expressed predominantly in myoepithelial cells, and some are expressed predominantly in stromal fibroblasts. It could be argued that genes that show a myoepithelial-restricted expression pattern in nonneoplastic tissue could be subject to down-regulation in tumors due to the absence of a myoepithelial population in invasive carcinomas. Whereas we cannot rule this out for genes such as *ADAMTS3* and *18* (and possibly *ADAMTS9*), it is clearly not the case for the other largely myoepithelial-restricted genes, including *ADAMTS4*, which is profoundly up-regulated in tumors. Thus, it is clear that both the level and location of expression of several members of the *ADAMTS* gene family become dysregulated during mammary tumorigenesis.

Although members of the ADAMTS family show on average only between 20% and 40% homology to each other (1), two groups, *ADAMTS2, 3,* and *14* and *ADAMTS4* and *5*, share very similar functions and substrates (13, 18). It was anticipated that these groups of genes might display the same overall trend with respect to relative gene expression levels and distribution in breast tissue, but this was shown not to be the case. Instead, pairwise analysis of expression levels provided evidence for potential coregulation of *ADAMTS1, 3, 5, 8, 9, 10,* and *18*, all of which were down-regulated in cancer specimens. There was also evidence for coexpression of *ADAMTS2, 4, 6, 7, 12,* and *14*. None of the *ADAMTS* genes showed coexpression with c-*erb*B-2, which was overexpressed in 22% of carcinoma samples, consistent with previous reports (36, 37). However, several *ADAMTS* genes that are down-regulated in malignancy showed significant negative correlation of expression with that of *MMP-11*, a protease that is up-regulated in stromal fibroblasts in mammary carcinomas (38). Thus, there may be some common mechanisms that achieve reciprocal regulation of the *ADAMTS* genes and *MMP-11*.

The predominance of myoepithelial cell expression of the ADAMTSs suggests a possible role for some of these genes in the control of basement membrane remodeling in the normal breast. Furthermore, it has been reported that myoepithelial cells exhibit an antiangiogenic phenotype (41), and the expression of ADAMTSs may contribute to this. Because ADAMTS proteins have catalytically intact metalloproteinase domains, disintegrin domains (that may interact with integrins), and regions resembling the antiangiogenic thrombospondins, their contributions to tumorigenesis may be complex. Indeed, the best-characterized

Table 4 Clinical features of the nonneoplastic breast tissue patients

	No. of patients
Indication for surgery	
Reduction mammoplasty	6
Prophylactic mastectomy: positive FH[a]	2
Prophylactic mastectomy: contralateral BC	2
Menopausal status	
Premenopausal	8
Postmenopausal	2
Age median (range) (yrs)	32 (21–67)
Total	10

[a] FH, family history; BC, breast carcinoma.

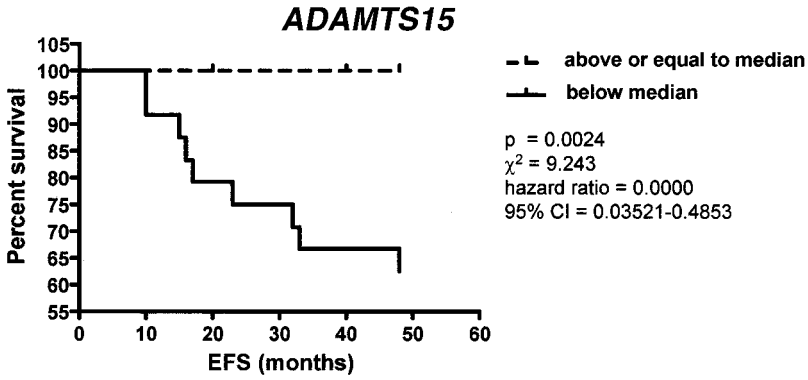

Fig. 3 Graph to show event-free survival based on *ADAMTS15* normalized input values. Cases have been censored at the minimum follow-up period of 48 months.

family member, ADAMTS-1, is an aggrecanase (16) that has also been shown to inhibit endothelial cell proliferation (7). In the corneal micropocket and chorioallantoic membrane assays, both ADAMTS-1 and ADAMTS-8 display antiangiogenic activities that are more potent than that of thrombospondin-1 at the same molar concentration (7).

As with the situation in breast cancer, the expression of *ADAMTS1* has also been found to be lower in pancreatic cancer compared with noncancerous tissue (42). However, among the pancreatic cancer cases, those with higher levels of *ADAMTS1* showed poorer prognosis, with evidence of increased local invasion and lymph node metastasis, suggesting that *ADAMTS1* may play different roles in the normal organ compared with its involvement in tumor progression. There was no association

Table 5 Survival data of patients with breast carcinoma

	No. of patients							
Overall survival[a]								
Dead	10 (20%)							
Relapsed	2 (4%)							
Alive & event-free:	34 (68%)							
Lost to follow-up:	4 (8%)							
Total	50							
Tumor grade								
1	Dead	0	Relapsed	0	AEF[b]	8	LFU	1
2	Dead	3	Relapsed	1	AEF	15	LFU	3
3	Dead	6	Relapsed	1	AEF	9	LFU	0
No grade	Dead	1	Relapsed	0	AEF	2	LFU	0
Tumor size (cm)								
<2	Dead	0	Relapsed	0	AEF	15	LFU	4
2–4	Dead	9	Relapsed	0	AEF	16	LFU	0
>4	Dead	0	Relapsed	2	AEF	2	LFU	0
No size	Dead	1	Relapsed	0	AEF	1	LFU	0
Tumor type								
IDC	Dead	9	Relapsed	1	AEF	20	LFU	2
ILC	Dead	0	Relapsed	1	AEF	8	LFU	1
Mixed	Dead	0	Relapsed	0	AEF	5	LFU	1
DCIS	Dead	1	Relapsed	0	AEF	1	LFU	0
Axillary lymph node metastases								
0	Dead	5	Relapsed	0	AEF	17	LFU	3
1–3	Dead	1	Relapsed	1	AEF	12	LFU	1
>3	Dead	1	Relapsed	1	AEF	4	LFU	0
No data	Dead	3	Relapsed	0	AEF	1	LFU	0
NPI								
Good (<3.4)	Dead	0	Relapsed	0	AEF	9	LFU	4
Intermed. (3.4–5.4)	Dead	4	Relapsed	1	AEF	23	LFU	0
Poor (>5.4)	Dead	1	Relapsed	1	AEF	2	LFU	0
No NPI	Dead	5	Relapsed	0	AEF	0	LFU	0
Estrogen receptor status								
Negative	Dead	4	Relapsed	0	AEF	2	LFU	0
Positive	Dead	5	Relapsed	2	AEF	15	LFU	2
Strongly positive	Dead	1	Relapsed	0	AEF	10	LFU	1
Unknown	Dead	0	Relapsed	0	AEF	7	LFU	1

[a] Median follow-up (range) in months: 61 (48–)64.

[b] AEF, alive and event-free; LFU, lost to follow-up; IDC, invasive ductal carcinoma; ILC, invasive lobular carcinoma; DCIS, ductal carcinoma *in situ*; NPI, Nottingham Prognostic Index; Intermed., intermediate.

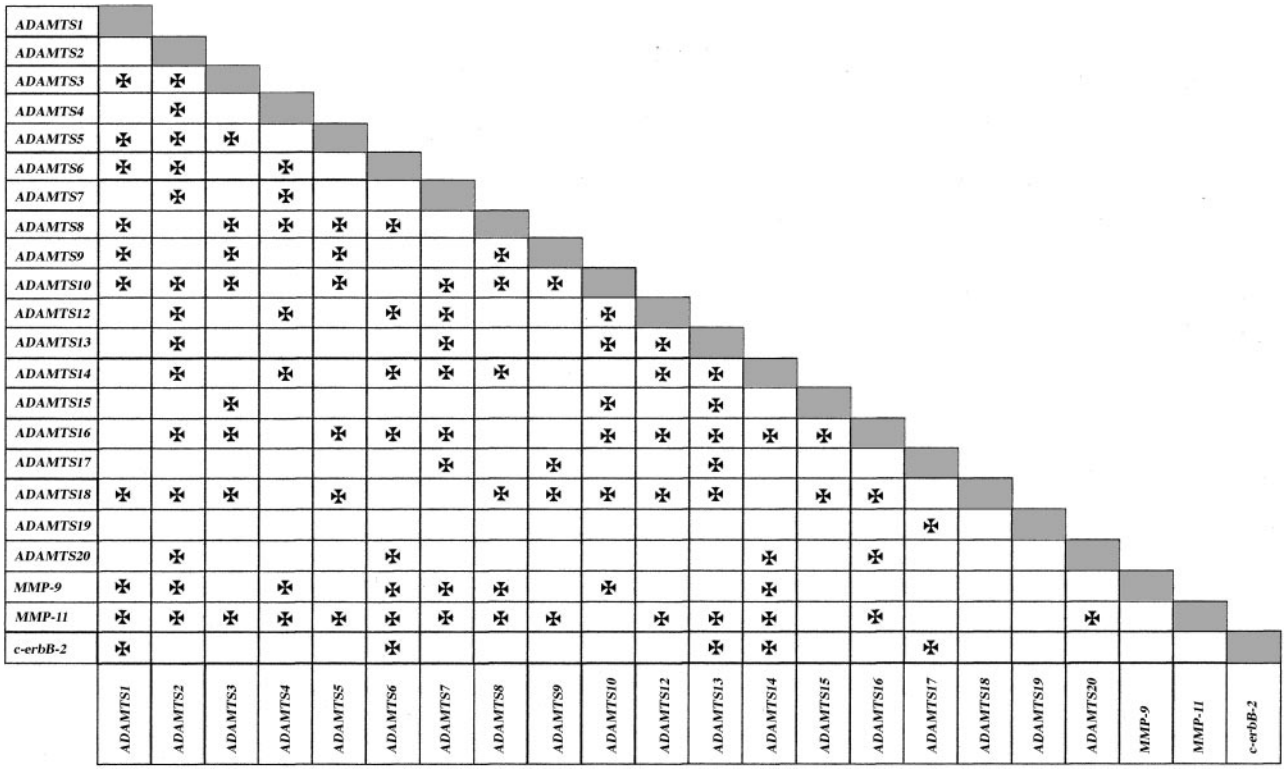

Fig. 4 Association of cDNA expression between *ADAMTS* genes. ✠, statistically significant association.

between *ADAMTS1* and microvessel density in the pancreatic cancer cases, and *ADAMTS8* was expressed at very low levels in both normal and cancer tissue, indicating that neither gene is closely linked with regulation of angiogenesis at this site. Analysis of clinical data revealed no strong links of *ADAMTS1* expression with clinicopathological features of the breast cancer cases in our study set; however, interestingly, *ADAMTS15* expression showed correlations with tumor grade and also correlated with event-free survival: normalized input values below 0.5 identify a subset of patients with a poorer prognosis; and values above 1.0 suggest a better prognosis. When evaluated together with two of the standard prognosticators, tumor grade and size, 75% of those patients who have died or relapsed were identified as falling into a poor risk category, compared with the Nottingham Prognostic Index, which (where available) identified only 17% of cases who have died or relapsed as poor risk.

The regulation of the *ADAMTS* genes in breast is not known. However, it might be reasonable on present evidence to speculate that progesterone may have some regulatory effect. One study has shown that *ADAMTS1* is a direct target of progesterone receptor-mediated activation in murine ovarian granulosa cells in follicles destined to ovulate, suggesting a possible role for *ADAMTS1* in follicular rupture (8). TSP1 itself has been shown to be regulated by progesterone in the human endometrium (43). It is possible, therefore, that certain *ADAMTS* genes expressed in mammary tissue may also be progesterone-regulated.

A further speculation is that some *ADAMTS* genes that are down-regulated in breast cancer may have been silenced by methylation, although this remains to be confirmed. Although two *ADAMTS* genes that are down-regulated in breast carcinoma, *ADAMTS8* and *ADAMTS9*, map to two regions known to be frequently deleted in breast cancer and other malignancies [11q25 and 3p14.2, respectively (44, 45)], we tested one gene, *ADAMTS8*, and found no evidence of loss of heterozygosity in several breast carcinoma cell lines assayed by Southern blotting and no evidence of a gross deletion of the gene locus by cytogenetic G-banded analysis.[7] The cytogenetic evaluation is also relevant with regard to *ADAMTS15*, approximately 20 kb distal to *ADAMTS8* at 11q25. Several authors postulate the existence of a hitherto undiscovered tumor suppressor gene at 11q25 in breast carcinoma (44, 46, 47). These observations, coupled with our findings of marked down-regulation of *ADAMTS8* in human breast carcinoma, indicate the value of further investigations to demonstrate whether or not *ADAMTS8* or *15* functions as a tumor suppressor gene in mammary tissue.

In summary, our data demonstrate for the first time that there is down-regulation at the level of RNA expression of several members of the *ADAMTS* family in breast cancer compared with nonneoplastic mammary tissue. This is of potential interest in the context of breast carcinomas because they are frequently vascular tumors, and their angiogenic index, or ratio of microvessels to tumor volume or tumor cell number, has emerged as a reliable indicator of early metastasis and thereby

[7] S. Porter, A. Hogan, and M. Weber, unpublished observations.

prognosis, especially in small tumors (48, 49). Thus, disease progression in breast cancer likely involves suppression or abrogation of antiangiogenic molecules, and the down-regulation of *ADAMTS1* and *8* in breast carcinoma may be significant. We note that, at the end of their paper on endogenous regulators of angiogenesis, Carpizo and Iruela-Arispe (50) mention their unpublished work on *ADAMTS1* and breast carcinoma. Human T47D breast carcinoma cells, stably transfected with *ADAMTS1* cDNA and injected s.c. into nude mice, show marked inhibition of tumor growth compared with tumors derived from control T47D cells transfected with an empty vector and concurrently injected. We await their further observations with interest, especially because they correspond closely with our ongoing studies of *ADAMTS1* and *8* in human breast carcinomas.

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