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Keywords: ADAM10; breast cancer; migration; invasion; inhibitor; targeted therapy

ADAM10: a new player in breast cancer progression?

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Background: The ADAM proteases are best known for their role in shedding the extracellular domain of transmembrane proteins. Among the transmembrane proteins shed by ADAM10 are notch, HER2, E-cadherin, CD44, L1 and the EGFR ligands, EGF and betacellulin. As cleavage of several of these proteins has been implicated in cancer formation and progression, we hypothesised that ADAM10 is also involved in these processes.

Methods: ADAM10 expression was decreased by RNA interference and the effects of this on cell numbers, invasion and migration were determined. We also examined the effect of ADAM10 inhibition on breast cancer cell line invasion and migration.

Results: Using the triple-negative (TN) breast cancer cell lines, BT20, MDA-MB-231 and the non-TN cell line MDA-MB-453, knockdown of ADAM10 expression significantly decreased *in vitro* migration ($P < 0.01$; for each cell line). Similarly, treatment with the ADAM10-selective inhibitor GI254023X reduced migration in the three cell lines (for BT20, $P < 0.001$; for MDA-MB-231, $P = 0.005$; for MDA-MB-453, $P = 0.023$). In contrast, neither knockdown of ADAM10 nor treatment with the ADAM10-selective inhibitor GI254023X significantly affected cell numbers. Using extracts of primary breast cancers, higher levels of ADAM10 were found more frequently in high-grade vs low-grade tumours ($P < 0.001$) and in oestrogen receptor (ER)-negative compared with ER-positive tumours ($P = 0.005$). Analysis of pooled publicly available data sets found that high levels of ADAM10 mRNA were associated with adverse outcome in patients with the basal subtype of breast cancer.

Conclusions: Based on our combined cell line and breast cancer extract data, we conclude that ADAM10 is likely to be involved in breast cancer progression, especially in the basal subtype.

The ADAMs are a family of multifunctional proteins implicated in proteolysis and cell adhesion (Blobel, 2005; Murphy, 2008; Duffy *et al*, 2009). Unlike the matrix-degrading proteases such as the matrix metalloproteases and urokinase plasminogen activator, the main substrates for ADAM proteases are the extracellular domains of transmembrane proteins. Of the 21 human ADAMs, one of the most-detailed studied is ADAM17 (Sahin *et al*, 2004; Blobel, 2005; Murphy, 2008; Duffy *et al*, 2009). ADAM17 is involved in the release and activation of several growth factors and cytokine receptor ligands. Among the growth factors

activated by ADAM17 are TGF- α , amphiregulin, epiregulin and HB-EGF (Sahin and Blobel, 2007; McGowan *et al*, 2007). Because of its ability to activate these growth factors and thus stimulate EGFR signalling systems, ADAM17 have been implicated in cancer progression (Blobel, 2005; Murphy, 2008; Duffy *et al*, 2009). Indeed, in several different tumour model systems, ADAM17 has been shown to enhance cell proliferation, invasion and migration (Borrell-Pages *et al*, 2003; Franovic *et al*, 2006; Kenny and Bissell, 2007; McGowan *et al*, 2007). These effects of ADAM17 are likely to be at least partly mediated by release of the

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EGFR/HER ligands, which in turn activates EGFR signalling (Sahin and Blobel, 2007).

An ADAM structurally and functionally related to ADAM17 is ADAM10 (Duffy *et al*, 2003; Moss *et al*, 2008). Like ADAM17, ADAM10 has also been implicated in the activation of specific EGFR ligands, especially EGF and betacellulin (Sahin and Blobel, 2007). However, in addition to releasing these EGFR ligands, ADAM10 has also been shown to activate notch signalling (Hartmann *et al*, 2002; Bozhulak and Weinmaster, 2009; Weber *et al*, 2011) and cleave the extracellular domains of adhesion proteins, such as E-cadherin (Maretzky *et al*, 2005a,b; Solanas *et al*, 2011), N-cadherin, L1 and CD44 (Mechtersheimer *et al*, 2001; Hundhausen *et al*, 2003; Reiss *et al*, 2005; Maretzky *et al*, 2005a,b; Murai *et al*, 2006). The ability of ADAM10 to activate notch and EGFR signalling as well as cleave several adhesion proteins suggests that ADAM10, similar to ADAM17, may also have a role in cancer progression.

In contrast to ADAM17, little detailed work has been carried out on ADAM10 in cancer (Arima *et al*, 2007; Lee *et al*, 2010; Gaida *et al*, 2010), especially in breast cancer. In one of the few reports on ADAM10 in breast cancer, Feldinger *et al* (2014) recently reported that elevated expression of this ADAM predicted resistance to trastuzumab (Herceptin). ADAM10 may thus have a clinical role in breast cancer.

The aim of this study was therefore to perform a detailed investigation on ADAM10 in breast cancer. Here we show that inhibition of ADAM10 expression using RNA interference or blockage of its activity using a selective low molecular weight inhibitor significantly decreased breast cancer cell line invasion and migration. We also show that ADAM10 protein levels were significantly elevated in high-grade compared with low-grade tumours and in oestrogen receptor (ER)-negative compared with ER-positive tumours. In addition, we found that high expression of ADAM10 predicted poor outcome in patients with basal-type breast cancer. Our preclinical results, when combined with those from extracts of human cancers, suggest that ADAM10 is involved in the progression of breast cancer.

MATERIALS AND METHODS

Cell lines and reagents. The breast cancer cell lines BT20, MDA-MB-231 and MDA-MB-453 were obtained from the American Tissue Culture Collection (Manassas, VA, USA). Cell lines were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (Invitrogen Life Sciences, Carlsbad, CA, USA) and maintained in a 37 °C CO₂-humidified incubator. Cell line identity was confirmed by analysis of Short Tandem Repeat Loci (IdentiCell, Aarhus, Denmark). Cells were routinely tested for mycoplasma infection. The ADAM10 selective inhibitor GI254023X (Hundhausen *et al*, 2003; Ludwig *et al*, 2005) was obtained from GlaxoSmithKline (GlaxoSmithKline UK Ltd, Stevenage, UK) via material transfer agreement. A ten millimolar stock solution was prepared and stored at 4 °C.

RNA interference. All cell lines were transiently transfected using the reverse transfection method with commercially available ADAM10 siRNA pooled sequences (Dharmacon, Thermo Fisher Scientific, Lafayette, CO, USA; Thermo Scientific, Rockford, IL, USA). Briefly, a 20- μ M stock solution of ADAM10 siRNA was prepared in siRNA buffer according to the manufacturer's instructions. ADAM10 siRNA solution (50–75 nM) or corresponding scrambled control siRNA were incubated in Opti-MEM (Invitrogen Life Sciences) for 10 min. The transfection reagent (DharmaFECT) was also incubated in Opti-MEM for 10 min at room temperature. Following incubations, both solutions were combined and incubated for an additional 30 min at room temperature. The transfection solution was added to 500 μ l of cell solution and incubated overnight at 37 °C. Fresh RPMI was added to the cells,

24 h post-transfection. ADAM10 silencing was confirmed using reverse transcription reaction (PCR).

Cell viability assays. Cell viability was measured using direct cell counting. Following reverse transfection with 5×10^4 cells per well as described above, cells were trypsinised and cell suspensions were counted using the Countess Automated Cell Counter (Invitrogen Life Technologies). For assays examining the effect of GI254023X on cell numbers, 5×10^4 cells per well were incubated for 24 h and treated with either GI254023X or corresponding vehicle control for 5 days. Cells were then washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 following the addition of trypsin. Cell suspensions were also counted using the Countess Automated Cell Counter (Invitrogen Life Technologies). Results are shown as cell numbers (%) compared with vehicle/scrambled control-treated cells. Results are shown as mean of independent experiments carried out in triplicate \pm s.e.m.

Migration assay. Cells were seeded at a density of 5×10^4 cells in the upper compartment of migration inserts (Becton Dickinson, Franklin Lakes, NJ, USA). The lower chamber was filled with media containing fibroblast-conditioned media. Cells were incubated for 48 (MDA-MB-231) or 72 h (BT20 and MDA-MB-453). Following incubation, non-migrated cells were removed using a PBS-soaked cotton swab. In all, 1% glutaraldehyde (Sigma, St Louis, MO, USA) was used to fix the migrated cells, followed by staining with 0.1% crystal violet (Pro-Lab Diagnostics, Chesire, UK). Migrated cells were counted using a light microscope at a magnification of $\times 20$. Five fields were counted in each well and

Table 1. Pathological features and hormone receptor status of the cohort of primary breast carcinomas analysed during the ADAM10 investigation

Tumour characteristic	N	%
Age (years)		
≤ 50	40	34.2
> 50	77	65.8
Unknown	0	0.0
Tumour size (cm)		
≤ 2	32	27.3
> 2	76	65.0
Unknown	9	7.7
Grade		
1 and 2	46	39.3
3	60	51.3
Unknown	11	9.4
Nodal status		
Negative	56	47.9
Positive	56	47.9
Unknown	5	4.2
ER status		
Negative	38	32.5
Positive	79	67.5
Unknown	0	0.0
HER2 status		
Negative	100	85.5
Positive	6	5.1
Unknown	11	9.4
Histological type^a		
Ductal (D)	92	78.6
Lobular (L)	16	13.7
D and L	5	4.3
Other	0	0.0
Unknown	4	3.4

Abbreviation: ER, oestrogen receptor.

^aAll tumours were infiltrating carcinomas. Ductal carcinoma *in situ* and lobular carcinoma *in situ* were excluded.

averaged. The percentage of migrated cells was calculated compared with scrambled or vehicle control cells. Results are shown as mean of independent experiments carried out in triplicate \pm s.e.m.

Matrigel invasion assay. Similar to the migration assay described above, cells were seeded at a density of 5×10^4 cells per well in the upper compartment of Matrigel-coated inserts ($8 \mu\text{m}$ pore size, BioCoat, BD Biosciences, Erembodegem-Dorp, Belgium). Cells were incubated for 48 or 72 h at 37°C depending on the cell line. Chambers were stained and counted in a similar manner to the migration assay.

Studies on breast tumours. Human breast tumour samples were obtained following ethical approval from the institutional board at St. Vincent's University Hospital. Following pathological evaluation, tumours were snap frozen and stored at -80°C . Tissue homogenisation was carried out using a Mikro-Dismembrator (Braun Biotech International, Melsungen, Germany). Protein extraction was performed as previously described (McGowan *et al.*, 2007). Briefly, tissues were lysed with $200 \mu\text{l}$ of 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (Complete mini EDTA-free protease inhibitor cocktail, Roche Applied Science, West Sussex, UK), phosphatase inhibitors (PhosStop phosphatase

inhibitor cocktail, Roche Applied Science) and Triton X-100 at a final volume of 1%. This was then passed through a 19-gauge needle, vortexed and agitated for 20 min at 4°C . Supernatants were subsequently collected and transferred to fresh tubes. Total protein concentrations in the resulting supernatants were determined using the Nanodrop 1000 A_{280} program (Thermo Scientific). Clinical and pathological characteristics of the human breast cancers investigated are shown in Table 1. ADAM10 protein levels in extracts of primary breast carcinomas were measured by ELISA (USCN Life Science Inc., Wuhan, P.R. China), according to the manufacturer's instructions. For each sample, 1.5 mg of protein was used. The relationship between ADAM10 mRNA expression and survival was investigated using the publicly available data set, BreastMark (Madden *et al.*, 2013), as previously described (McGowan *et al.*, 2013).

Statistical analysis. The Student's paired *t*-test was employed when determining the possible effects of either ADAM10 knock-down or ADAM10 inhibition on cell migration and invasion compared with control cells. Mann-Whitney *U*-tests were used when relating ADAM10 levels to tumour characteristics. All data were analysed using PASW Statistics Version 18 (SPSS Inc., Chicago, IL, USA) and Prism version 5.0b software (GraphPad Software, San Diego, CA, USA).

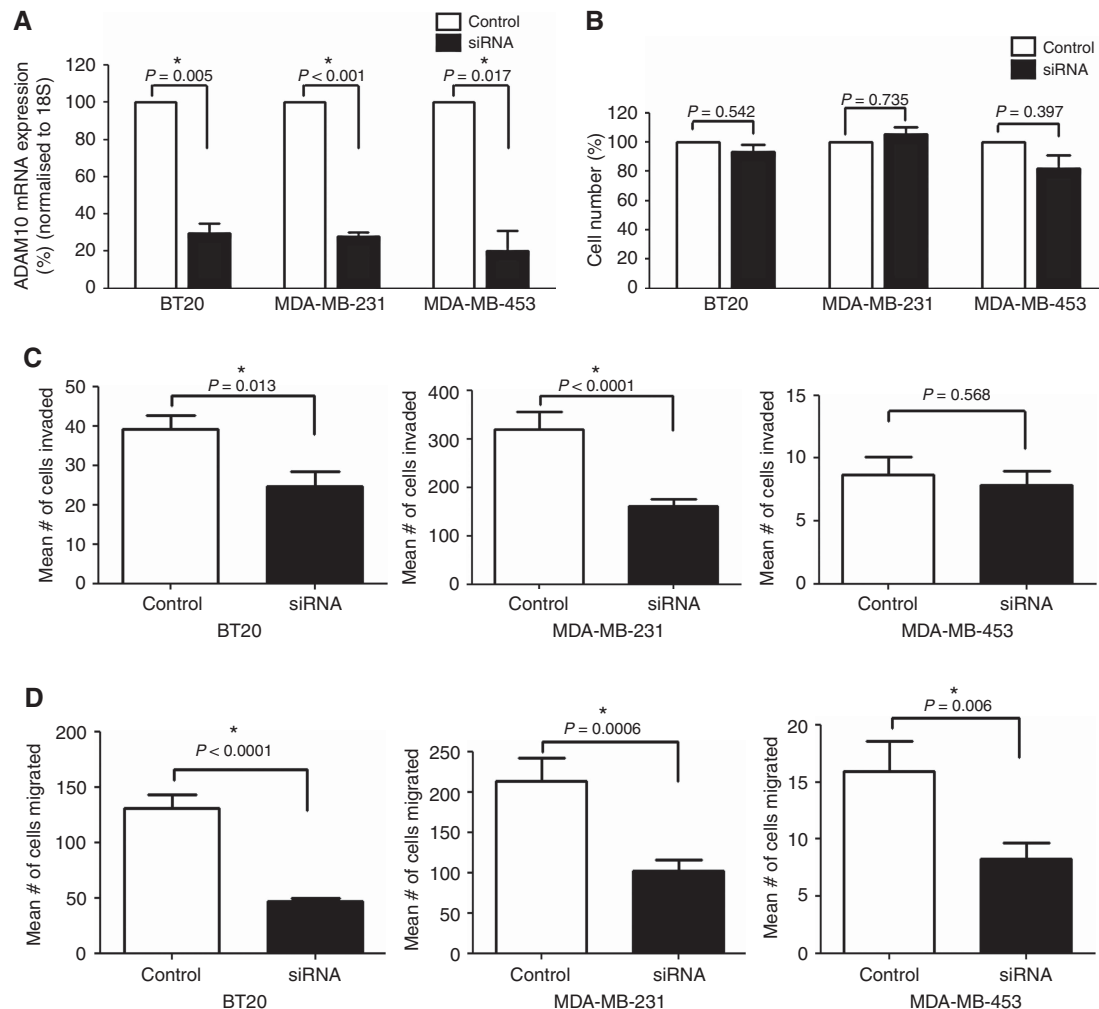


Figure 1. Effect of ADAM10 RNA interference on breast cancer cell line numbers, invasion and migration. Barchart showing the effects of decreased ADAM10 expression on BT20, MDA-MB-231 and MDA-MB-453 (A) mRNA expression, (B) cell line numbers, (C) invasion and (D) migration compared with scrambled control-treated cells. The white bars represent the control cells and the black bars represent the ADAM10 siRNA-treated cells. Data were analysed using Student's paired *t*-test. All experiments were carried out in triplicate. Results are means \pm s.e.m.

RESULTS

Effect of ADAM10 knockdown on cell numbers, migration and invasion. In order to select suitable cells for ADAM10 knockdown, we first measured the endogenous expression of ADAM10 in a broad panel of breast cancer cell lines (Supplementary Figure S1). Based on this data, two TN cell lines, that is, BT20 and MDA-MB-231 and one non-TN control cell line, that is, MDA-MB-453 that expressed relatively high levels of ADAM10 were selected. Following transfection of these breast cancer cell lines, expression of mRNA for the ADAM10 was reduced by 70% in the BT20 cells, 72% in the MDA-MB-231 cells and 80% in the MDA-MB-453 cells (Figure 1A).

Despite this decrease in mRNA for ADAM10, no significant effect was observed on breast cancer cell numbers (Figure 1B) ($P > 0.05$, for all three cell lines examined). In contrast, suppression of ADAM10 expression resulted in a significant decrease in the ability of the TN breast cancer cell lines BT20 ($P = 0.013$) and MDA-MB-231 ($P < 0.0001$) to invade through Matrigel chambers. No effect however, was observed on the ability of the non-TN cell line MDA-MB-453 ($P = 0.568$) to invade Matrigel chambers (Figure 1C). We also examined the effect of ADAM10 suppression on tumour cell Transwell migration. Downregulation of ADAM10 significantly decreased the ability of the three breast cancer cell lines to migrate through the Transwell chamber (for BT20, $P < 0.0001$; for MDA-MB-231, $P = 0.0006$; and for MDA-MB-453, $P = 0.006$) (Figure 1D).

Effect of GI254023X on cell numbers, migration and invasion.

We next investigated the effects of the selective ADAM10 inhibitor GI254023X ($5 \mu\text{M}$) on cell numbers, migration and invasion. Similar to the effects observed with RNA interference, addition of GI254023X did not affect MDA-MB-231 ($P = 0.963$) and MDA-MB-453 ($P = 0.483$) cell numbers. In contrast to siRNA-treated cells, a statistically significant but modest reduction in cell numbers was observed following treatment of the TN BT20 cell line with GI254023X ($19.04 \pm 6.62\%$, $P < 0.01$; Figure 2A). As with RNA interference, treatment with GI254023X significantly decreased invasion of both BT20 ($P = 0.02$) and MDA-MB-231 ($P = 0.02$) cell lines. Unlike RNA interference however, GI254023X significantly decreased invasion of the MDA-MB-453 cell line ($P = 0.007$; Figure 2B). GI254023X also significantly decreased migration of these three cell lines (for BT20, $P < 0.0001$; for MDA-MB-231, $P = 0.005$; and for MDA-MB-453, $P = 0.023$) (Figure 2C).

ADAM10 levels in breast cancers. ADAM10 protein levels were measured by ELISA in 117 primary breast tumour extracts. Levels varied from undetectable to 19.2 ng mg^{-1} , the median value being 1.16 ng mg^{-1} protein. Levels of ADAM10 protein expression were related to established variables of tumour progression, including patient age at diagnosis, tumour size, tumour grade, lymph node status, ER status, HER2 status and histological type. Increased levels ($>$ median) were found more frequently in higher-grade vs lower-grade tumours ($P < 0.001$) and in ER-negative than in ER-positive tumours ($P = 0.005$). Significantly elevated levels of ADAM10 were detected in tumours from patients who were

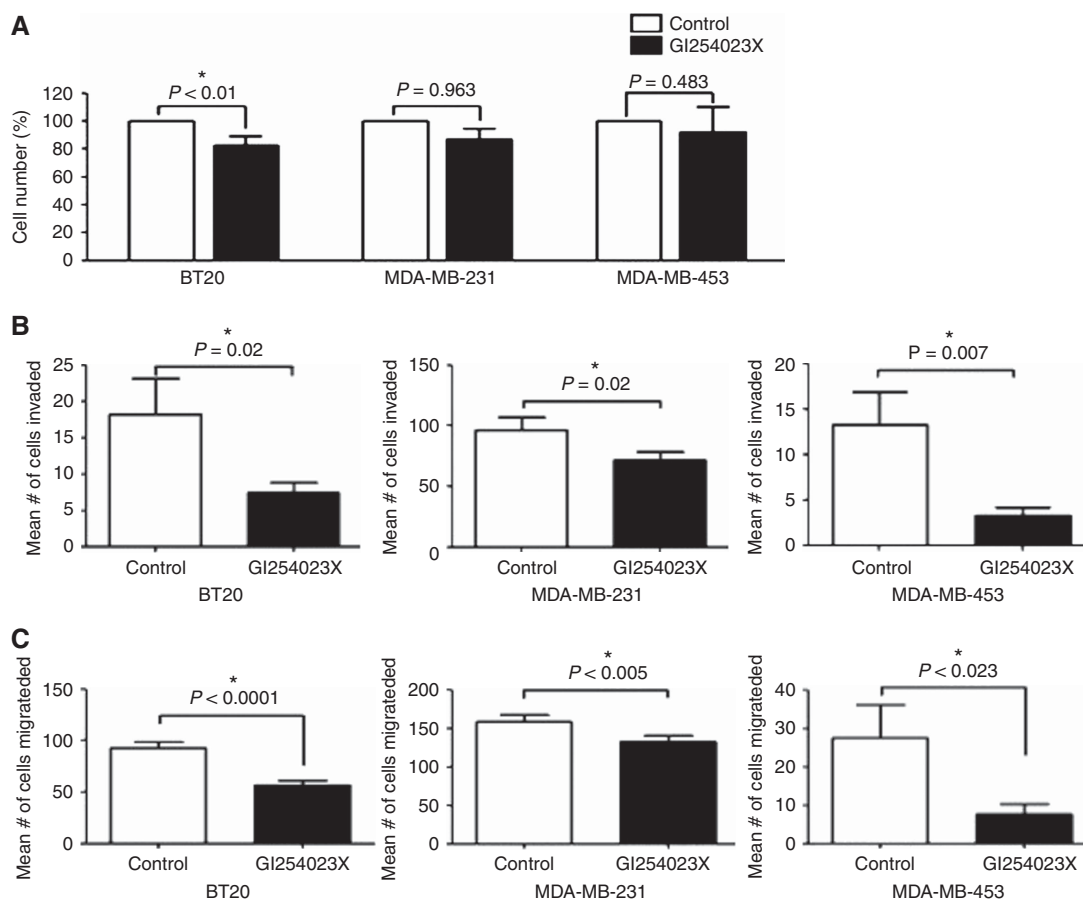


Figure 2. Effect of the ADAM10 inhibitor GI254023X on breast cancer cell line numbers, invasion and migration. Bar chart showing the effects of GI254023X on BT20, MDA-MB-231 and MDA-MB-453 (A) cell line numbers, (B) invasion and (C) migration compared with vehicle control-treated cells. The white bars represent the control cells and the black bars represent GI254023X-treated cells. Data were analysed using Student's paired *t*-test. All experiments were carried out in triplicate. Results are means \pm s.e.m.

younger than 50 years at diagnosis compared with those who were older ($P = 0.018$). In contrast, no significant associations were found between ADAM10 protein levels and tumour size, lymph node status, HER2 status or histological subtype (Table 2).

ADAM10 levels and patient outcome. In order to establish whether ADAM10 levels predicted patient outcome, we analysed its expression using cutoffs at the median expression level in a cohort of pooled publicly available data sets ($n = 2592$; Madden *et al*, 2013). ADAM10 was categorised as the expression

greater than or less than the median ADAM10 expression level. Although, no significant relationship was found between ADAM10 mRNA levels (median cutoff) and disease-free survival in the total population of patients (hazard ratio = 1.01; $P = 0.365$; Figure 3A), increased ADAM10 expression predicted disease-free survival in the basal subgroup of patients (hazard ratio = 1.57 (1.18–2.09); $P = 1.83 \times 10^{-4}$; Figure 3B). In contrast to this finding in the basal subtype, ADAM10 expression was not associated with outcome in either the luminal or HER2-positive subtypes (data not shown).

Table 2. Relationship between ADAM10 protein expression and variables of tumour progression

Tumour characteristic	N	Median ADAM10 protein levels (ng mg ⁻¹)	P-value ^a
Age (years)			
≤50	40	1.73	0.018
>50	77	1.08	
Tumour size (cm)			
≤2	32	1.07	0.315
>2	76	1.20	
Grade			
1 and 2	46	0.84	<0.001
3	60	1.74	
Nodal status			
0	56	1.64	0.146 ^b
1–3	30	0.84	
4–9	15	1.08	
>10	9	1.13	
ER status			
Negative	38	1.79	0.005
Positive	79	1.08	
HER2 status			
Negative	100	1.08	0.452
Positive	6	1.54	
Histological type			
Ductal (D)	92	1.18	0.496 ^b
Lobular (L)	16	1.02	
D and L	5	1.03	

Abbreviation: ER, oestrogen receptor.
^aData were analysed using the Mann–Whitney *U*-test.
^bData were analysed using Kruskal–Wallis analysis.

DISCUSSION

To our knowledge, this is one of the most comprehensive reports to date on ADAM10 in any malignancy. Using three different breast cancer cell lines, we show using both RNAi knockdown and a selective low molecular inhibitor that ADAM10 is involved in cell migration. In contrast, our results suggest that ADAM10 does not reduce cell numbers, at least in two of the three cell lines investigated in our study. With the BT20 cell line however, although ADAM10 knockdown failed to affect cell counts, treatment with GI254023X had a modest impact in reducing cell numbers, that is, by 19%, a finding that was not observed in the MDA-MB-231 cell line. The biological relevance of such a modest reduction in cell numbers is unclear. The most likely explanation for the different effects of ADAM10 knockdown and GI254023X in the BT20 cell is that the low molecular weight inhibitor is not only absolutely specific for ADAM10 but may also be marginally impacting on a pathway having a role in cell proliferation or viability.

Previously, Arima *et al* (2007) reported that decreased ADAM10 expression in a prostate cancer cell line blocked growth on only 1 of the 4 days in which this end point was determined. However, following stimulation with dihydrotestosterone, decreased growth was found on 2 of the 4 days. In another *in vitro* study, Lee *et al* (2010) showed that downregulation of ADAM10 expression in melanoma cells resulted in both suppression of anchorage-independent cell growth and reduced migration. Similar to our findings with breast cancer cell lines, Gaida *et al* (2010), using pancreatic cancer cells, reported that downregulation of ADAM10 failed to decrease cell proliferation but led to reduced invasion and migration. Thus the effects of ADAM10 may be variable, depending on the cell type.

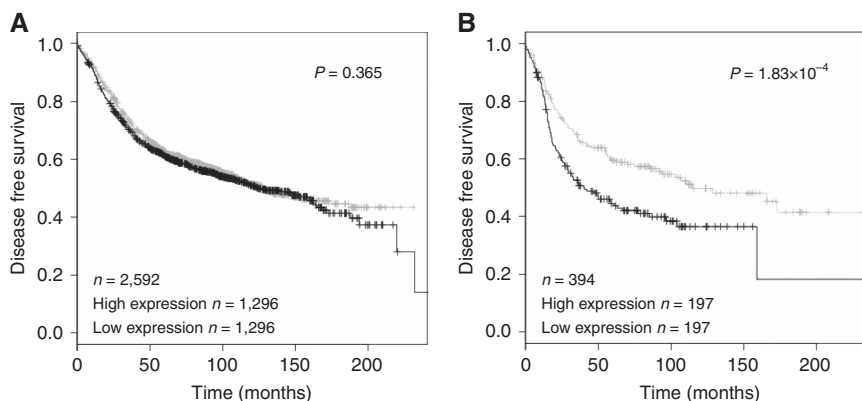


Figure 3. Relationship between ADAM10 mRNA expression and disease-free survival in the basal-like breast cancer subgroup. Kaplan–Meier curves showing the association between ADAM10 mRNA expression and disease-free survival using the default settings of BreastMark (Madden *et al*, 2013) (A) across all molecular subtypes of breast cancer and (B) in the basal-like breast cancer patient subtype. High expression is shown in black and low expression is shown in grey. All survival analysis was carried out using the publicly available data set BreastMark (Madden *et al*, 2013). Data were analysed using the Log Rank test and Kaplan–Meier analysis.

How ADAM10 promotes *in vitro* breast cancer cell invasion or migration in our study is not clear. However, as mentioned in the Introduction above, ADAM10 is known to cleave E-cadherin (Maretzky *et al*, 2005a; Solanas *et al*, 2011). Using fibroblasts and keratinocytes, Maretzky *et al* (2005b) showed that ADAM10-mediated cleavage of E-cadherin resulted in altered cell–cell adhesion and enhanced invasion. This effect on E-cadherin, however, may also be cell-type dependent, as ADAM10 siRNA-treated pancreatic cancer cell lines displayed no alteration in E-cadherin protein levels (Gaida *et al*, 2010). Other potential mechanisms by which ADAM10 could promote migration and invasion include cleavage of membrane proteins such as N-cadherin (Reiss *et al*, 2005), L1 (Mechtersheimer *et al*, 2001; Maretzky *et al*, 2005b) or CD44 (Murai *et al*, 2006). Alternatively, ADAM10 could promote invasion and migration via activation of the EGFR ligands, betacellulin and EGF.

Consistent with a role in invasion/migration, we found that high levels of ADAM10 in the extracts of breast cancer correlated with indices of aggressive disease, such as ER-negativity and high tumour grade. Perhaps, more importantly, we showed that high ADAM10 mRNA levels were associated with adverse outcome in patients with the basal subform of breast cancer. Seventy to 80% of basal breast cancer lack ER, progesterone receptors and HER2 and are thus referred to as TN breast cancer (TNBC) (Duffy *et al*, 2012). Consequently, most patients with basal breast cancer are unable to receive a targeted therapy. This inability to be treated with a targeted therapy when combined with their intrinsic aggressiveness and lack of a validated prognostic marker means that patients with TNBC tend to have a poor outcome (Duffy *et al*, 2012). Unfortunately, it was not possible to evaluate a potential prognostic impact for ADAM10 in TNBC in our pooled collection of databases as too few of the patients had data on HER2 gene amplification/overexpression.

Our results suggest that ADAM10 mRNA may be a new prognostic biomarker for this subgroup of patients with breast cancer. Indeed, as ADAM10 correlates with outcome in basal-type breast cancer, it may be involved in their progression and thus function as a therapeutic target for these patients. If confirmed, the selective ADAM10 inhibitor used in the present investigation, that is, GI254023X, could be a potential treatment for basal carcinoma. GI254023X should thus now be investigated for efficacy and toxicity in an animal model of basal breast cancer.

Although the main focus of our work was on ADAM10 in TN and basal breast cancer, as mentioned in the Introduction above, Feldinger *et al* (2014) recently reported that increased expression of this ADAM was associated with resistance to Herceptin. Furthermore, high levels of ADAM10 predicted adverse patient outcome (Feldinger *et al*, 2014). In our study however, ADAM10 was not associated with outcome in HER2-positive patients. These different findings with ADAM10 may relate to different treatments administered in the two studies.

Before concluding, it is important to point out limitations in this report. First, although substantial knockdown of ADAM10 was observed at the mRNA levels, we did not confirm this at the protein level. Furthermore, we used a pooled mixture of four ADAM10 siRNA sequences to target ADAM10. Although the use of such a pooled mixture is likely to result in greater knockdown of ADAM10 expression than use of a single species, it could potentially increase the likelihood of off-target effects.

In addition, although we showed that high ADAM10 mRNA levels predicted a shortened disease-free interval in patients with basal-type breast cancer, we have not confirmed this at a protein level. As basal breast cancer constitute only approximately 15–20% of all breast cancer, a large total population of patients with breast cancer is necessary to obtain an adequate number of the basal-type samples for performing outcome studies. To address this problem, we used a publicly available database derived from

12 independent studies and containing 2718 patients (Madden *et al*, 2013). To our knowledge, a similar database for protein expression is currently not available. A further limitation of our study is that we did not investigate the effects of ADAM10 knockdown on cell numbers, migration and invasion at multiple time points.

In conclusion, our results suggest that ADAM10 may be a new player in breast cancer progression, especially in mediating invasion and migration. Inhibition of ADAM10 might thus be used to block these processes and thus prevent breast cancer progression. Indeed, as ADAM10 was particularly associated with adverse outcome in patients with basal breast cancer, it may be a new therapeutic target for the treatment of this form of the disease.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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