Expression of membrane type-1 matrix metalloproteinase, MT1-MMP in human breast cancer and its impact on invasiveness of breast cancer cells

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Abstract. MT1-MMP (membrane type-1 matrix metalloproteinase), otherwise known as MMP14 is a proteolytic enzyme known to be involved in degradating extracellular matrix and assist progression of cancer invasion and progression. We investigated the impact of targeting the expression of MT1-MMP in breast cancer and its clinical relevance. Human breast cancer cell line MDA-MB-231 was used. Expression of MT1-MMP in the breast cancer cell line was manipulated by way of retroviral ribozyme transgene. The *in vitro* invasion, growth and cell migration were determined on cell lines transfected with either the transgene or control plasmid. Protein and message levels of MMP14 was also assessed using immunohistochemistry and real-time quantitative analysis, and correlated with clinical and pathological information of the patients. Retroviral ribozyme transgene to human MT1-MMP successfully knocked down the levels of MT1-MMP mRNA from MDA-MB-231 cells. Reduction of MT1-MMP from the breast cancer cells resulted in significant reduction of *in vitro* invasiveness and loss of response to an invasion stimulus, HGF, compared with control and wild-type cells. The invasion index for MT1-MMP knockdown cells were 13±3.1 (without HGF) and 16.4±2.3 (with HGF, p=0.14), and the index for transfection control cells 25.3±4.3 (without HGF) and 40.4±4.1 (with HGF, p=0.0049). Transfection with the transgenes did not change the rate of cell growth. In clinical breast cancer, MT1-MMP staining was both membranous and cytoplasmic. Tumour cells displayed stronger staining compared with normal mammary epithelial cells. Tumour tissues had a marginally higher levels of the MMP14 transcript (8.6±1.9), compared with normal tissues (4.7±1.4), p=0.13. No significant difference was observed between node positive and node negative tumours (9.0±2.2 vs 8.7±3.1, p=0.24). Marginally higher levels of the MMP14 transcript were seen in tumours which developed metastasis and local recurrence. However, tumours from patients who died of breast cancer related causes had significantly higher levels of the transcript, compared with tumours from patients who remained disease-free 10 years after initial surgery (12.2±2.5 vs 6.3±1.2, p=0.0091). MT1-MMP is a proteolytic enzyme that is pivotal in controlling the invasiveness of breast cancer cells. It is highly expressed in aggressive breast tumours and is associated with clinical outcome. The enzyme is a potential therapeutic target in breast cancer.

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Introduction

Membrane type-1 metalloproteinase, also known as MMP14, is a member of the matrix metalloproteinases (MMPs), which are Zn(2+)-binding endopeptidases that degrade various components of the extracellular matrix (ECM) (1,2). Metalloproteinases have been implicated in a number of physiological and pathological processes, including tissue remodeling processes, wound repair, angiogenesis, cellular migration, and invasion and metastasis of tumour cells. Extracellular matrix substrate for MT1-MMP includes fibronectin, vitronectin, laminin-1, fibrin, and collagens type-I, -II and -III, CD44 and tissue transglutaminase. MT1-MMP is delivered to the leading edge of the migratory cells and assembles other molecules including MMP2 allowing degradation of different ECM components in the basement membrane and stroma. MT1-MMP also degrade other molecules including CD44 and tissue transglutaminase, to facilitate cell migration (3,4).

A number of *in vitro* and *in vivo* model have suggested the potential role of MT1-MMP in cancer. In xenograft breast tumour model, breast tumours induced MT1-MMP production from stromal cells (5). Extracellular matrix metalloprotease inducer (EMMPRIN) which can induce the expression of MT1-MMP has been found to be highly positive in metastatic breast tumour cells and related to the clinical outcome of patients in breast cancer and with survival of the patients (6). Human breast carcinoma cell surface-associated MT1-MMP,

via activating proMMP2, stimulates TCIPA (tumour cell-induced platelet aggregation), which may contribute to the deposit and aggregation of breast cancer cells (7). It has been shown that myofibroblasts from normal mammary tissue can reduce the invasion of breast cancer cells including MDA-MB-231 and MCF-7. This was partly associated with a reduction of MMPs including MT1-MMP (8). Vice versa, breast cancer cells are able to induce the expression of MT1-MMP in fibroblasts (9).

It has also been shown that MT1-MMP-controls cross-talk between αvß3 and α2ß1 integrins, which in turn supports binding of aggressive, MT1-MMP-, and αvß3 integrin-expressing malignant cells on type-I collagen (10). This has contributed to the increased migration of breast cancer cells (11,12). Furthermore, VEGF and MT1-MMP interplay and jointly contribute to the growth of tumours (13). The key mechanism of MT1-MMP in its induction of tumour growth is the activation of MMP2 (14,15) and possibly MMP13 (16).

In clinical cancer, staining of MT1-MMP was significantly associated with the presence of lymph node metastasis (17). The location of MT1-MMP protein in mammary tumours remains controversial, with some studies showing MT1-MMP in stromal cells (18) and other in cancer cells (14). Inhibition of MT1-MMP has been indicated to have a potential role in cancer. Ro-28-2653, an inhibitor with high selectivity for MMP2, MMP9, and MT1-MMP have been shown to inhibit the *in vitro* invasion of breast cancer cell (19). The same compound also reduces the growth of breast tumours that was driven by fibroblasts.

Despite these studies to demonstrate the role of MT1-MMP in cancer cells including breast cancer cells, there has been no information on the relationship between the expression of MT1-MMP with clinical outcome, such as long-term survival in human breast cancer. The current study aimed at investigation of MT1-MMP at protein and mRNA level in clinical breast cancer and establishing a relationship between MT1-MMP and clinical outcomes and patient survival. We further developed retroviral ribozyme transgenes which allowed elimination of expression of MT1-MMP in breast cancer cells.

Materials and methods

Human mammary cancer cells, MDA-MB-231 and an immortalised non-tumourigenic human fibroblast cell line MRC5 were from ECACC (the European Collection of Animal Cell Culture, Salisbury, UK) and were routinely maintained in DMEM F12 with 10% foetal calf serum. Recombinant human hepatocyte growth factor/scatter factor (HGF/SF) was a gift from Dr T. Nakamura, Osaka University Medical School, Osaka, Japan. Matrigel (reconstituted basement membrane) was purchased from Collaborative Research Products (Bedford, MA, USA). A rabbit anti-human MT1-MMP, anti-CK19, anti-Ki67 and peroxidase conjugated anti-IgG were from Santa Cruz Biotechnologies (Santa Cruz, CA, USA) and Sigma (Poole, Dorset, UK), respectively. A chemiluminescence detection kit for Western blotting and protein A/G conjugate were from Santa Cruz Biotechnologies. A transwell plate equipped with a porous insert (pore size 8 μm) was from Becton-Dickinson Labware (Oxford, UK). DNA restriction enzymes and T4 DNA ligase were obtained from New England Biological Laboratories (Hertfordshire, UK). DNA gel extraction and plasmid extraction kits were from Qiagen (Crawley, UK).

Table I. Clinical and pathological information.

	n
Node status	
Negative	65
Positive	55
Grade	
1	23
2	41
3	56
Histology	
Ductal	94
Lobular	14
Medularry	2
Tubular	2
Mucinous	4
Others	4
TNM staging	
TNM 1	69
TNM 2	40
TNM 3	7
TNM 4	4
Clinical outcome	
Disease-free	81
With metastasis	7
With local recurrence	5
Died of breast cancer	20
Died of unrelated diseases	7

Both primary breast cancer (n=120) and normal tissues which were away from tumour tissues and free from cancer cells (n=32) were collected immediately after surgery and stored frozen until use. Patients were routinely followed clinically after surgery. The median follow-up period was 120 months (June 2004). The presence of tumour cells in the collected tissues was verified by examination of frozen sections using H&E staining. Details of the samples are given in Table I.

Construction of retroviral hammerhead ribozyme transgenes targeting human MT1-MMP and generation of active viral hammerhead ribozymes. The procedure has been previously reported (20,21). Briefly, the secondary structure of human MT1-MMP was generated using Zucker's RNA mFold software (22). A hammerhead ribozyme that specifically targets two *GUC* sites of human MT1-MMP, was generated using touch-down PCR with respective primers (Table II).

Table II. Sequence of primers.

	Sense primer (5'-3')	Antisense primer (5'-3')
MT1-MMP Q-PCR	cttggactgtcaggaatgag	actgaacctgaccgtacacaggtaaaagggtgccatgat
MT1-MMP	aggccgacatcatgatcttcttt	tggtatccatccatcacttggtta
MT1-MMP	atgtctcccgccccaag	tcagaccttgtccagcag
MT1-MMP ribozyme1	ctgactgcagattatgttgccatttgactga tgagtccgtgaggacgaaaccctgctgat gatgccgtgaggacgaaa	ccatactagtcgaaggaagcgtacgccatttcgtcctcacggac tcatcaggtccctttcgtcctcacggactcat
Viral vector	cccttgaacctcctcgttcgacc	cccttgaacctcctcgttcgacc
UBAM primers	ggatccgccaaccgaaagt	gtacgattaacaactaaga
ß-actin	gctgatttgatggagttgga	tcagctacttgttcttgagtgaa
CK19	caggtccgaggttactgac	actgaacctgaccgtacacactttctgccagtgtgtcttc

PCR products generated antisense-hammerhead ribozyme, flanked by PstI and SpeI restriction sites, were first T-A cloned into a pCR2.1 TOPO cloning vector (Invitrogen, Paisley, Scotland, UK), which was amplified in the OneShot™ *E. coli* (Invitrogen). Clones with correct oriented insert were verified using PCR. Plasmid was subsequently purified from the bacterial preparation and dually digested using PstI and SpeI. The digest was separated on a 2% agarose gel, followed by purification of the ribozyme insert from the gel. Gel purified ribozymes (with PstI and SpeI overhangs) were then ligated into either pLXSN (RetroX, K1060) or pRevTRE292 vector (RevTet-On, K1267, Clontech Laboratories, Palo Alto, CA, USA), which were modified in our laboratory by inserting a U1 promoter in front of the multicloning site (MCS) from our previous work (generous gift from Dr J. Laterra) (23,24). Ligated products were used to transform JM109 *E. coli*. Clones with correctly ligated anti-MT1-MMP ribozyme, termed here as pRevTRE-MT1MMP, and control plasmid (termed pRevTRE-cont) was subsequently identified. pRevTRE carried a tetracycline responsive gene and were used throughout the *in vitro* and *in vivo* studies. pLXSN ribozymes were not used in the subsequent studies. The direction and sequence were verified using a plasmid specific primer LXSNF and U1 specific primers (UBAMHF and UBAMHR, Table II).

Plasmid, extracted and purified using a plasmid extraction kit (Qiafilter, Qiagen, Crawley, UK), was introduced to a retroviral packaging cell line, PT67, using electroporation as previously described (25), followed by selection with G418 containing-medium for over 3 weeks. Viral titres from stably transfected PT67 cells were tested using NIH3T3 cells, and were found to be on average $8x10^5$ cfu/ml. Active viral stocks (a combination of pRevTRE-MT1-MMP or control stock and the pRevTet-On viral stock which was separately generated from PT67) were used to transduce MDA-MB-231 mammary cancer cells in the presence of polybrene (8 μg/ml final concentration). Each transduction lasted 24 h and 3 consecutive transductions were carried out. Transduced cells were subject to dual selection with G418 (for pRevTRE-MT1MMP) and hygromycin (for pRevTet-On) (Calbiochem, Nottingham, UK), each at 100 μg/ml for over 3 weeks in order to obtain stably transduced strains, that carried both the inducing vector pRevTet-On and expression vector pRevTRE-MT1MMP (or control). These stably transduced and subsequently verified cells were designated the following names and are used throughout the text: MDA-MB-231^{WT}- MDA-MB-231 wild-type; MDA-MB-231^{pRevTRE} MDA-MB-231 tranduced with pRevTRE empty vector; MDA-MB-231$^{\Delta MT1MMP}$: MDA-MB-231 transduced with pRevTRE-MT1MMP transgene.

RNA preparation and RT-PCR. RNA from cells and tissues was extracted using an RNA extraction kit (AbGene Ltd., Surrey, UK) and quantified using a spectrophotometer (Wolf Laboratories). cDNA was synthesised using a first strand synthesis with an oligo dt primer (AbGene). PCR primers are given in Table II. The polymerase chain reaction (PCR) was performed using sets of primers with the following conditions: 5 min at 95˚C, and then 20 sec at 94˚C, 25 sec at 56˚C, 50 sec at 72˚C for 36 cycles, and finally 72˚C for 7 min. *ß-actin* was amplified and used as a housekeeping control. PCR products were then separated on a 0.8% agarose gel, visualised under UV light, photographed using a Unisave camera (Wolf Laboratories, York, UK) and documented with Photoshop software.

Quantitative analysis of MT1-MMP. The level of MT1-MMP transcripts from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor™ technology, modified from previous reported (26,27). Briefly, pairs of PCR primers were designed using the Beacon Designer software (version 2, CA, USA), but to one of the primers, an additional sequence was added, known as the Z sequence (5'-actgaacctgaccgtaca-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK). A Taqman detection kit for ß-actin was purchased from Perkin-Elmer. The reaction was carried out using: Hot-start Q-master mix (AbGene), 10 pmol of specific forward primer, 1 pmol reverse primer which has the Z sequence (Table II, underlined), 10 pmol of FAM-tagged probe (Intergen Inc.), and cDNA

from approximately 50 ng RNA. The reaction was carried out using IcyclerIQ™ (Bio-Rad) equipped with an optic unit that allows real-time detection of 96 reactions, using the following condition: 94˚C for 12 min, 50 cycles of 94˚C for 15 sec, 55˚C for 40 sec and 72˚C for 20 sec. Cytokeratin-19 (CK19) was used for comparison of cellularity during the analysis and primers for CK19 were 5'-caggtccgaggttactgac-3' and 5'-actgaacctgaccgtacacactttctgccagtgtgtcttc-3' respectively (28). The levels of the transcripts were generated from an internal standard (20,28) that was simultaneously amplified with the samples, and are shown here in two ways: levels of transcripts based on equal amounts of RNA, or as a target/CK19 ratio.

In vitro invasion analysis and cell growth assay. This was performed as previously reported and modified in our laboratory (29,30). Briefly, transwell inserts (upper chamber) with 8 μm pore size were coated with 50 μg/insert of Matrigel and air-dried, before being rehydrated. Cells (20,000) were added to each well with, or without HGF/SF. After 72 h, cells that had migrated through the matrix and adhered to the other side of the insert were fixed and, stained with 0.5% (w/v) crystal violet. Cells that have invaded and stained with crystal violet were extracted with 10% (v/v) of acetic acid and absorbance obtained using a multiplate reader.

For cell growth assay, MDA-MB-231^{WT}, MDA-MB-231^{pRevTRE}, or MDA-MB-231$^{\Delta MT1\text{-}MMP}$ cells were plated into 96-well plate at 2,500 cells/well. Cells were fixed in 10% formaldehyde at the day of plating, day 1, 2, 3, 4, 5, and 6 after plating, and then stained with 0.5% (w/v) crystal violet. Following washing, stained crystal was extracted with 10% (v/v) acetic acid and absorbance determined using a multiplate reader. The growth of cells are shown here as absorbance (mean ± SD).

Immunohistochemical staining of MT1-MMP. Frozen sections of tissues (32 paired normal and tumour tissues, as well as dissected tumour tissues) were cut at a thickness of 6 μm using a cryostat (28,31). The sections were mounted on super frost plus microscope slides, air dried and then fixed in a mixture of 50% acetone and 50% methanol. The sections were then placed in 'Optimax' wash buffer for 5-10 min to rehydrate. Sections were incubated for 20 min in 10% horse serum of blocking solution and probed with the primary antibody. Following extensive washing, sections were incubated for 30 min in the secondary biotinylated antibody (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc.). Following washings, Avidin-Biotin Complex (Vector Laboratories) was then applied to the sections followed by extensive washing. Diaminobenzidine chromogen (Vector Laboratories) was then added to the sections which were incubated in the dark for 5 min. Sections were then counter stained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip. Cytoplasmic staining of the respective proteins was quantified using Optimas 6.0 software as we previously described (32) and is shown here as relative staining intensity. Statistical analysis was carried out using Mann-Whitney U test and significant difference was set at $p<0.05$.

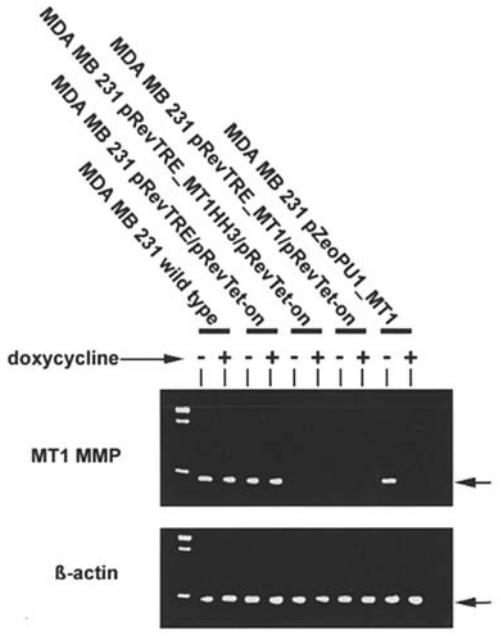

Figure 1. Effects of MT1-MMP knockout on the *in vitro* invasiveness of breast cancer cells. Loss of MT1-MMP from MDA-MB-231 cell line resulted in significant reduction of invasion and loss of their response to HGF.

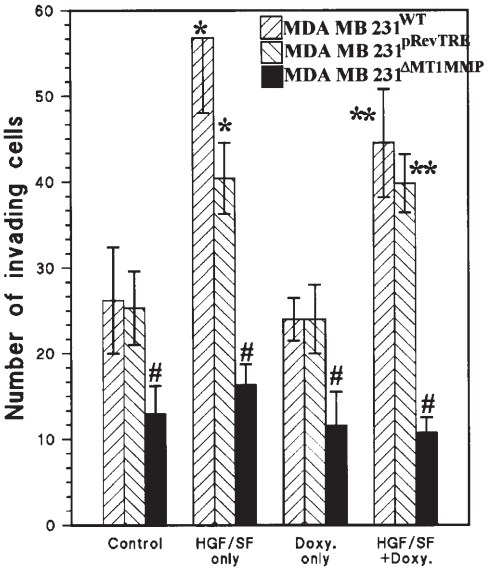

Figure 2. Expression of MT1-MMP in breast cancer cell lines and knockout by ribozyme transgenes. Retroviral transgenes and (pRev-MT1) successfully knocked out the expression of MT1-MMP from MDA-MB-231 breast cancer cells, in the presence or absence of doxycycline. $^{*}p<0.01$ vs respective cell without HGF; $^{**}p<0.01$ vs respective cells without doxycycline; $^{\#}p<0.01$ vs respective wild-type or plasmid control cells.

Results

Elimination of MT1-MMP expression in breast cancer cells affects the in vitro invasiveness and migration of breast cancer cells. We constructed three ribozymes using two different vectors, the pU1/ZEO vector and the pRev-Tet/on system. As shown in Fig. 1, both ribozymes successfully eliminated the expression of MT1-MMP mRNA from the breast cancer cells. Although the pRev-MT1-MMP transgene carry a

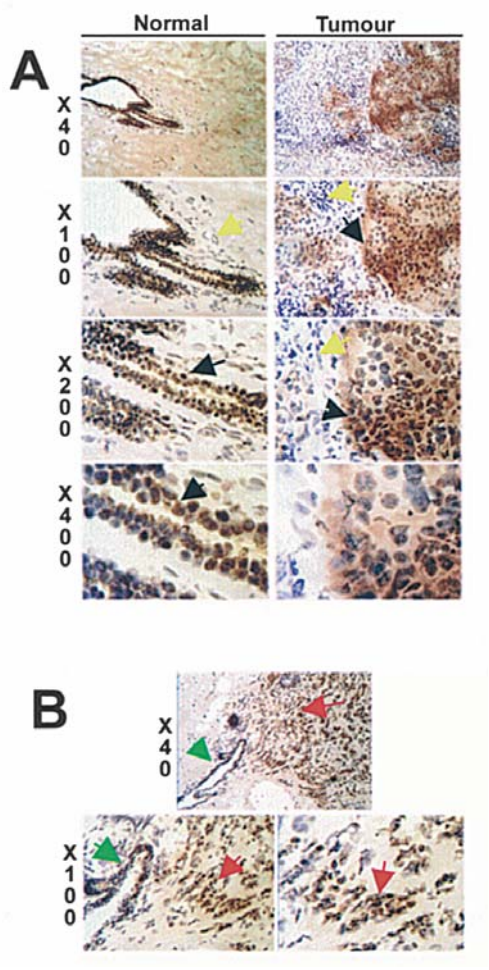

Figure 3. Staining of MT1-MMP in mammary tissues. (A) Left, normal tissues; right, tumour tissue; black arrow, staining in epithelial cells (left panel) or breast cancer cells (right panel); yellow arrows, stromal or infiltrating cells. (B) Tumour tissue with residual normal epithelial cells. Green arrows indicate residual normal epithelial cells with very weak staining of MT1-MMP; red arrows show strongly stained breast cancer cells in the same field.

tetracycline responsive element, the insertion of the U1 promoter in front the ribozyme enables the ribozyme transgene to work without the presence of doxycycline. Thus, both ribozymes were highly effective.

Reduced invasiveness of breast cancer cells after elimination MT1-MMP. The invasiveness of the genetically modified cells was tested, using an *in vitro* invasion model. As shown in Fig. 1, the highly invasive MDA-MB-231 cells responded to HGF with an increased invasiveness. MDA-MB-231^{pRevTRE}, cells transfected with a control vector, responded in a similar fashion to the wild-type (25.3±4.2 without HGF and 40.4±4.1 with HGF, p=0.0049). MDA-MB-231$^{\Delta MT1\text{-}MMP}$, whose expression of MT1-MMP was eliminated, had a significantly reduced spontaneous invasion vs their respective controls, and also lost response to HGF (13±3.1 without HGF and 16.4±2.3 with HGF, p=0.14). Transfection with either control plasmid or the ribozyme transgene did not change the rate of cell growth over a 6-day period.

Staining pattern of MT1-MMP in mammary tissues. Both normal mammary epithelial cells and breast cancer cells stained MT1-MMP (Fig. 3, dark arrows). The staining was seen as both membranous and cytoplasmic in these cells. Neither stromal cells nor infiltrating cells had visible staining (Fig. 3, yellow arrows).

Aberrant expression of MT1-MMP in human breast cancer. Breast tumour tissues had a marginally higher level of MT1-MMP transcript than the normal tissues (Fig. 4A, p=0.15). The same upward trend was retained after the transcript was normalised by CK19 (p=0.23) (Fig. 4A, insert). Although high grade tumours had high levels of the transcript, normalisation with CK19 eliminated the difference (Fig. 4B).

MT1-MMP transcript, nodal status and tumour staging. Although node negative and node positive tumours appear to have similar levels of the MT1-MMP transcript (Fig. 5A), normalised ratio showed a significantly higher levels in node positive tumours (Fig. 5A, insert *p=0.022). There was a steady, but not significant rise of the transcript in late stage tumours (Fig. 5B).

Expression of MT1-MMP is linked with clinical outcome. Using the Nottingham prognostic index as a predictive factor for prognosis, no significant difference was seen between predicted groups (Fig. 6A). However, significantly high ratio

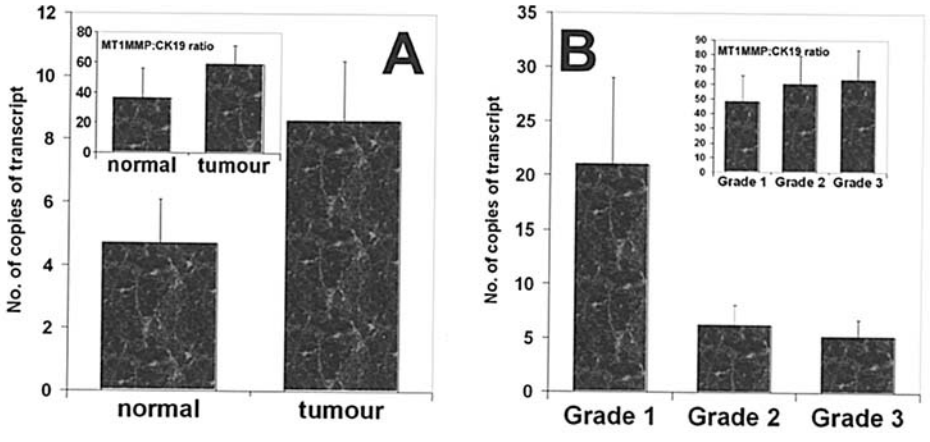

Figure 4. Quantitative analysis of MT1-MMP in breast cancer tissues (A) and in tissues with different tumour grade (B). Inserts, MT1-MMP:CK19 ratio.

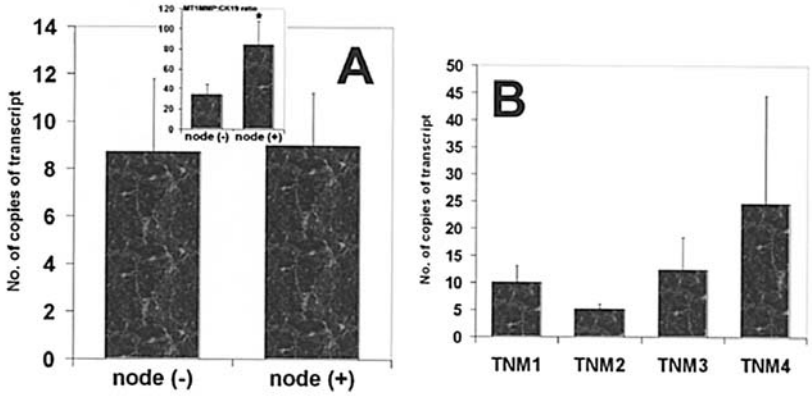

Figure 5. MT1-MMP transcript in node negative and positive tumours (A) and in relation with tumour staging (B). Inserts, MT1-MMP:CK19 ratio.

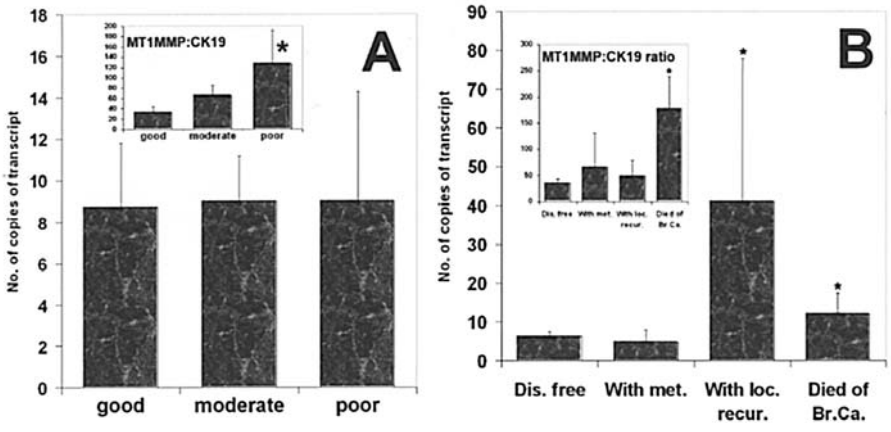

Figure 6. MT1-MMP transcript and its relationship with predicted clinical outcome (based on the Nottingham prognostic index) (A) and with clinical outcome (B). Inserts, MT1-MMP:CK19 ratio.

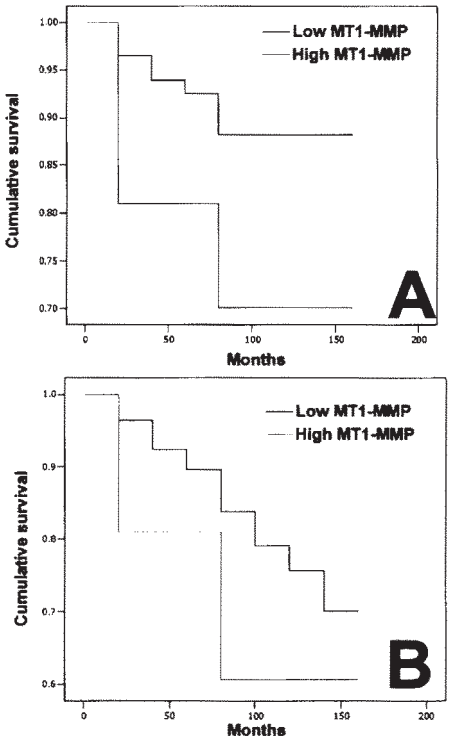

Figure 7. MT1-MMP and long-term survival (A, overall; B, disease-free survival).

was seen with the poor prognostic group (Fig. 6A, insert p=0.019). Over a 10-year follow-up period, patients who died of breast cancer had a significantly higher levels of MT1-MMP transcript (p=0.009) and MT1-MMP:CK19 ratio (p=0.0041, compared with patients who remained disease-free).

Patients with high levels of MT1-MMP had a shorter overall survival [117.6 (80.5-154.6, 95% CI) months], compared with those with low levels [128.8 (120.4-137.2, 95% CI) months, p=0.082] (Fig. 7A). Similarly, patients with high levels of MT1-MMP had a shorter disease-free survival [124.1 (114.9-133.2, 95% CI) months], compared with those with low levels [109.2 (72.1-146.3, 95% CI) months, p=0.15] (Fig. 7B, p=0.12).

Discussion

The current study showed that expression of MT1-MMP in human breast cancer is aberrant. Aggressive tumours, for example node positive tumours, have high level of MT1-MMP expression. High levels of expression of MT1-MMP are also associated with a shorter survival, although the difference is yet to reach statistical significance. These observations have pointed MT1-MMP as a potential prognostic indicator in clinical breast cancer.

MT1-MMP has been known to activate other MMPs including MMP2 and MMP13 which may contribute to the

degradation of extracellular matrix. Furthermore, MT1-MMP is also acting as bridging molecule between matrix proteins such as collagen and integrins. Collectively, MT1-MMP facilitates the binding between tumour cells and matrix and degradation of matrix by cancer cells. These cellular events would therefore contribute to the migration and invasion of cancer cells which eventually leads to increased aggressiveness of breast tumours.

The current study has provided some clear evidence that high levels of MT1-MMP in breast tumours are correlated with the outcome of patients with breast cancer. This is reflected by the following observations: high levels of MT1-MMP in the predicted poor prognostic group, in node positive tumours and in patients who developed recurrence and who died of breast cancer related causes. In addition, patients with high MT1-MMP tumours tend to have shorter overall and disease-free survival, although the difference is marginal in statistical terms. It has been shown in a smaller study in gastric cancer (n=25) that MT1-MMP is linked to a poor prognosis (33). In colon cancer (n=90), high levels of MT1-MMP was shown to be a prognostic factor (34). Although few studies have reported the aberrant staining pattern of MT1-MMP in breast cancer, the current study is the first to demonstrate the quantitative changes of MT1-MMP mRNA in breast cancer. The present study is also the first to show a correlation between MT1-MMP and the long-term survival of the patients with breast cancer. It has been recently reported by Pantel's group that the inducer of MT1-MMP, EMMPRIN (extracellular matrix metalloprotease inducer) is highly raised in breast cancer in a large tissue array based analysis (6). The study has shown a relationship between high expression of EMMPRIN and decreased tumour-free survival. The above evidence, together with literature reports using *in vitro* and *in vivo* models, strongly indicate that MT1-MMP is a prognostic factor in human breast cancer and a potential therapeutic target. To further verify this point, we constructed a hammerhead ribozyme transgene target at human MT1-MMP.

The ribozyme transgene constructed in the current study is highly active. It has successfully eliminated MT1-MMP mRNA from breast cancer cells. Although we attempted to construct a regulated transgene by using a Tet-regulated vector, we have failed to show doxycycline regulated activation of the ribozyme but succeeded in having a highly active, non-regulated activation of the ribozyme. This is the result of insertion of the U1 promoter upstream of the ribozyme, which enabled transcription independent of the tetracycline response elements. Using cells thus generated, we have provided evidence that elimination of MT1-MMP rendered the cancer cells to be less invasive *in vitro*. This work has added further to the value of targeting MT1-MMP in breast cancer. Other methods to inhibit or deactivate MT1-MMP have been reported. For example, Ro-28-2653, an inhibitor with high selectivity for MMP2, MMP9, and MT1-MMP has been shown to inhibit the *in vitro* invasion and reduce the growth of breast tumours of breast cancer cells (19). Targeting matrix related proteins has been challenging. Despite early surge of interest in MMP inhibitors, including TIMPs, antibodies, matrix related proteins/peptides, a successful clinical development has not been achieved yet. This may relate to matters regarding MMP. For example, MMPs are often acting in coordinate fashion, MT1-MMP and MMP2 in this case. Targeting a single one may not be sufficient. We have recently shown that targeting another MMP, MMP7 (matrilysin) yielded a similar reduction of *in vitro* invasiveness in the same cell line (25). It is indicated therefore that an approach targeting multiple MMPs may be necessary to have an impact when considering cancer therapies.

In the present study, we used cytokeratin-19 as a means to normalise the epithelial cellularity of mammary tissues. This relatively stable cytoskeletal associated protein has been widely used as a marker of cancer cells in tissues other than mammary gland, such as lymph nodes and bone marrow and has been valuable in identifying the metastatic cells in these tissues (35,36). The marker is not only useful in identifying cancer cells, it has been recognised as an epithelial marker. Presently, there is no universely accepted marker in normalising epithelial/cancer cell cellularity in clinical tumours, and CK19 is by no means a satisfactory one. However, we present these data in order to raise an interest and debate. Using CK19 to normalise the cellularity is an improvement as observed in the current study.

The source of MT1-MMP in mammary tissues and, indeed, in tissues of other origins has been a point of dispute. For example, some investigators have reported that MT1-MMP is exclusively expressed in breast cancer cells (8,9,18) while others show it is highly expressed in stromal cells or in both cell types (14,15). We have clearly identified staining of MT1-MMP in both stromal and breast cancer cells. This further indicates that activation of MMP2 by MT1-MMP may operate in two ways, activation of stromal MMP2 by stromal made MT1-MMP and by MT1-MMP expressed by cancer cells, both contribute to the degradation of matrix surrounding tumour cells. A number of substrates have been reported that can be degraded by MT1-MMP, including extracellular matrix proteins, CD44, and tissue transglutaminase. In the same cohort, we did not observe a correlation between MT1-MMP and CD44 and tissue transglutamases (data not shown), both were aberrantly expressed in the same cohort (37,38).

In conclusion, we report that MT1-MMP expression is raised in human breast cancer. A high level of MT1-MMP is associated with nodal involvement, with poor prognosis and high incidence of breast cancer related mortality. Furthermore, a ribozyme transgene to human MT1-MMP successfully knocked out the expression of MT1-MMP mRNA and resulted in reduction of invasion of breast cancer cells. The study has indicated the potential prognostic and therapeutic value of MT1-MMP in human breast cancer.

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