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Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer ☆

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ABSTRACT

Metastasis suppressor 1 (MTSS1) may play an important role in cancer metastasis. Firstly, this study assessed MTSS1 expression levels within breast cancer patients to reveal any clinical relevance. Secondly, we aimed to clarify the cellular function of MTSS1 in breast cancer cells. MTSS1 expression levels were assessed in a cohort of breast cancer specimens (normal $n = 33$; cancer $n = 127$), through quantitative PCR analysis and immuno-histochemical techniques. The influence of MTSS1 was further examined via biological overexpression and knockdown within breast cancer cell lines. We report that patients with tumours expressing reduced levels of MTSS1 had a poorer prognosis ($p = 0.042$). High levels of MTSS1 correlated with an increased patient overall survival ($p = 0.0108$) and disease-free survival ($p = 0.012$). Furthermore, overexpression of MTSS1 significantly suppressed ($p < 0.01$) the invasive, migratory, growth and adherence properties of a human breast cancer cell line. In contrast, knockdown of MTSS1 dramatically enhanced these properties. We conclude that MTSS1 is a prognostic indicator of disease-free survival in breast cancer patients and demonstrates the ability to play a role in governing the metastatic nature of breast cancer cells.

1. Introduction

The most deadly aspect of cancer is its ability to metastasise from the primary tumour to secondary sites. The metastatic cascade consists of a series of sequential, interrelated steps that are not as yet completely understood. However, it is known that these metastatic events are modulated by many factors, including metastasis activators and suppressors. Metastasis suppressors, by definition, inhibit metastasis at any step of the metastatic cascade without blocking tumourigenicity.[1]

Metastasis suppressor 1 (MTSS1), also known as MIM or MIM-B (missing-in-metastasis) was originally identified as a potential metastasis suppressor gene that was present in non-metastatic bladder cancer cell lines, but was not expressed in a metastatic bladder cancer cell line.[2] This first study suggested that MIM could be a potential metastasis suppressor. It was also demonstrated that MIM-B induced actin-rich protrusions resembling microspikes and lamellipodia at the plasma membrane and promoted disassembly of actin stress fibres.[3] Actin filament assembly is associated with cytoskeletal structure organisation and many forms of cell motility.[4] This alteration in actin dynamics will have serious consequences on the metastatic ability of cancer cells.[5,6] This may help explain the involvement of MTSS1 in the metastasis of cancer cells. However, further evidence is required to substantiate this preliminary claim and to elucidate any potential role of MTSS1 in the regulation of tumour cell behaviour.

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To date, there are contrasting views to whether or not MTSS1 is in fact a metastasis suppressor. MTSS1 has been reported to play a role as a metastasis suppressor in both prostate cancer[7,8] and bladder cancer[2,8,9]; however, some reports suggest that MTSS1 is unlikely to be metastasis suppressor and instead acts as a scaffold protein that interacts with actin-associated proteins to modulate lamellipodia formation.[10] Recently, evidence suggests that MTSS1 is a regulator of carcinogenesis in hepatocellular carcinoma,[11] and is a member of the sonic hedgehog (SHH) signalling pathway that modulates Gli responses during growth and carcinogenesis.[12,13]

Therefore, a role for MTSS1 in tumourigenesis and metastasis is yet to be established. All these controversial data are due in part to the fact that the study of MTSS1 has been restricted to a limited of cancer types, with little support from the clinical aspect. Studies suggest that further analysis of MTSS1 expression or inactivation in tissue samples and its association with different human malignancies will define a novel candidate to be used as a marker of primary tumours or metastasis.[8]

In an attempt to clarify the situation, we sought to determine the relevance of MTSS1 in breast cancer. Here, we provide new insights into the biological functions of MTSS1 and its role in breast cancer. In the present study, we examined MTSS1 expression a cohort of human breast cancer patient specimens and demonstrated an inverse correlation between MTSS1 and patient prognosis and survival. Importantly, we further analysed MTSS1 through a series of expression and inactivation studies to clarify MTSS1 function in breast cancer cells.

2. Materials and methods

2.1. Cell lines and culture

All cell lines used in this study were obtained from the European Collection for Animal Cell Culture (ECACC, Porton Down, Salisbury, UK), unless otherwise stated. Cells were routinely cultured with Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal calf serum, penicillin and streptomycin (Gibco BRC, Paisley, Scotland).

This study used human breast cancer cells (MDA-MB-157, MDA-MB-231, MDA-MB-435s, MDA-MB-436, MDA-MB-453, MCF-7, BT474, BT549 and ZR-751), human colorectal cancer cells (HRT-18 and HT-115), human pancreatic cancer cells (MIA PACA-2), human bladder cancer cells (EJ-138), human melanoma cells (G-361), human hepatocellular carcinoma cells (PLC-PRF-5) and human fibroblast cells (MRC-5, 1BR.3.G and CPFi). Human white blood cells (WBCs), a human epithelial cell line (ECV-304) and a human endothelial cell line (HECV), from Interlab Cell Line Collection (ICLC, Naples, Italy), were also used.

2.2. Human breast specimens

A total of 160 breast samples were obtained from breast cancer patients (33 were background normal breast tissue and 127 were breast cancer tissue). These tissues were collected immediately after mastectomy, and snap-frozen in liquid nitrogen, with the approval of the Local Ethical Committee.

Table 1 – Breast cancer patient clinical data details.

Clinical data	Grouping	Sample number
Tissue sample	Background	33
	Tumour	127
Nottingham Prognostic Index	1 (<3.4)	72
	2 (3.4–5.4)	39
	3 (>5.4)	16
Tumour grade	1	24
	2	44
	3	59
TNM staging	1	73
	2	42
	3	8
	4	4
Survival status	1	92
	2	13
	3	6
	4	16

Background normal mammary tissues were removed from the same patients. The pathologist verified normal background and cancer specimens, and it was confirmed that the background samples were free from tumour deposits. Tumour samples were macro-dissected by the pathologist. The follow-up for the cohort was 10 years. For patient clinical data see Table 1.

2.3. RNA preparation and reverse transcription-polymerase chain reaction

Total cellular RNA was isolated from the homogenised breast samples and human cell lines using the ABgene Total RNA Isolation Reagent (Advanced Biotechnologies Ltd., Epsom, Surrey, UK). RNA concentration and quality were determined through spectrophotometric measurement (WPA UV 1101, Biotech Photometer, Cambridge, UK).

cDNA was generated from 0.25 μg of each RNA sample and a reverse transcribed using a transcription kit (Sigma, Poole, Dorset, UK). The quality of DNA was verified using β-actin primers (sense: ATGATATCGCCGCGCTCGTC; antisense: CGCTCGGTGAGGATCTTCA). MTSS1 mRNA levels were assessed using MTSS1 primers (sense: TCAAGAACAGATGGAAGAATGG; Antisense: TGCGGTAGCGGTAATG–TG). PCR was performed in a GeneAmp PCR system 2400 thermocycler (Perkin–Elmer, Norwalk CT, USA). Conditions for PCR were 40 s at 95 °C for denaturation, 40 s at 61 °C for annealing and 40 s at 72 °C for elongation (35 cycles). PCR products were then loaded onto a 0.8% agarose gel and electrophoretically separated. The gel was then visualised under ultraviolet light following ethidium bromide staining.

2.4. Real-time quantitative polymerase chain reaction (QPCR)

The iCycler IQ system (BioRad, Camberley, UK) was employed,[14,15] to quantify the level of MTSS1 transcripts in the breast specimens (shown as copies/μl from internal standard). Breast cDNA samples were then examined for MTSS1 transcript expression, along with a set of standards and

negative controls (MTSS1 QPCR primers – sense: ATATCCCAGGATGCCTTC; antisense: ACTGAACCTGACCGTA–CACGGTTCTCGCTTCTCTTT). The QPCR technique utilised the Amplifluor system™ (Intergen Inc., England) and QPCR master mix (ABgene, Surrey, England), in conjunction with a universal probe (UniPrimer™). Real-time QPCR conditions were 95 °C for 15 min, followed by 60 cycles at 95 °C for 20 s, 55°C for 30 s and 72 °C for 20 s. The results of the test molecules were normalised against levels of β-actin, using a β-actin quantitation kit from Perkin–Elmers (Perkin–Elmer, Surrey, England, UK). The epithelial content within the tumours was taken into account by normalising MTSS1 levels against cytokeratin19 (CK19 primer details – sense: CAGGTCCGAGG–TTACTGAC; antisense: ACTGAACCTGACCGTACACACTTTCT-GCCAGTGTGTCTTC).

2.5. Immuno-histochemical staining of breast specimens

Frozen sections of breast tumour ($n = 32$) and background tissue ($n = 32$) were cut at a thickness of 6 μm using a cryostat. 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 a horse serum blocking solution and probed with the MTSS1 antibody (1:80) (Abnova, Caltag-Medsystems Ltd., Buckingham, UK). This study employed controls that omitted the primary and secondary antibodies. Furthermore, control tests were also conducted using purified IgG from non-immunised sheep (Sigma, Poole, Dorset, UK). Following extensive washings, sections were incubated for 30 min in the secondary biotinylated antibody (1:100) (Multilink Swine anti-goat/mouse/rabbit immunoglobulin, Dako Inc.). Following washings, the Avidin Biotin Complex (Vector Laboratories, Peterborough, UK) was then applied to the sections, followed by extensive washing steps. Diamino benzidine chromogen (Vector Labs, Peterborough) was then added to the sections, and incubated in the dark for 5 min. Sections were then counterstained in Gill's haematoxylin and dehydrated in ascending grades of methanol before clearing in xylene and mounting under a cover slip. Staining was independently assessed by the authors.

2.6. Generation of a MTSS1-overexpressing cell line

The full sequence of MTSS1 was amplified from PLC/PRF-5 cDNA, using the standard PCR procedure and a master mix with proof reading enzyme (sense primers: ATGGAGGCTGTG-ATTGAG; antisense: CTAAGAAAAGCGAGGGG). This MTSS1 sequence was then T–A cloned into pEF6/V5-His-TOPO vector (Invitrogen, Paisley, UK), and then electroporated into the MDA-MB-435s breast cancer cell line with the aim of inducing the expression of MTSS1 into a cell line that does not normally express it. Multiple clones were used, assessed and sequenced, all details of these procedures have been adapted from the reports described previously.[14,16] Multiple clones were employed in all the functional studies and results represent the summarised data of multiple clones. The MDA-MB-435s cells thus prepared and expressing MTSS1 were termed in the study as MDA-MTSS1-Exp. The control group of cells contained the same plasmid vector (minus the MTSS1 sequence) and was termed MDA-pEF-control.

2.7. Knockout of MTSS1 expression using ribozyme transgenes

Anti-MTSS1 ribozyme transgenes were employed to knockdown the expression of MTSS1 in the MCF-7 breast cancer cell line, and were generated using the methods previously described.[16,17] Briefly, the secondary structure of human MTSS1 was generated using Zucker's RNA mFold software. The ribozymes that specifically target MTSS1 were generated using touchdown PCR with the appropriate primers (sense: CTGCAG AGGCTTTTTAGATCTTCCGACTGATGAGTCCGTGAGGA; antisense: ACTAGTTAACCCACCTTCAGACCATTTCGTCCTCACGGA CT). The resulting correct inserts were purified and cloned into the pEF6/V5-His-TOPO vector, and then electroporated into the MCF-7 breast cancer cell line, which highly expressed MTSS1. MCF-7 cells with MTSS1 eliminated were thus termed MCF-MTSS1-KO. Following selection and knockout confirmatory processes described previously;[16] the effect of MTSS1 loss from these breast cancer cells was examined through a series of *in vitro* studies described in this methods section.

2.8. Western blot confirmation of MTSS1 overexpression and knockdown

MTSS1 protein expression was assessed in the human breast cancer cell line lysates through standard SDS–PAGE and Western Blot analysis. Currently, there is only one commercially available against MTSS1 (Abnova, Caltag-Medsystems Ltd., Buckingham, UK). GAPDH expression was used as an internal control (Santa-Cruz Biotechnologies, California, USA). Protein expression was assessed and quantified using Uvitech analysis software (Uvitech, Cambridge, UK).

2.9. Tumour cell growth assay

The effects of MTSS1 modification on breast cancer cell growth rates were assessed using an *in vitro* growth assay. Cells were seeded in triplicate into 24-well plates at a density of 30,000 cells per well. Plates were then incubated for 0, 24, 48, 72 and 96 h before being fixed in 4% formaldehyde (v/v) and stained with 0.5% (w/v) crystal violet. The crystal violet stain was then extracted using 10% acetic acid (v/v) and cell density was determined by measuring the absorbance of this solution using a Bio-Tek ELx800 multi-plate reader (Bio-Tek Instruments Inc., Winooski, VT).

2.10. Cell adhesion assay

The adhesive properties of the MTSS1-modified cells to an artificial basement membrane were quantified using the *in vitro* Matrigel adhesion assay adapted from a previously described method.[18] Briefly, MCF-7 wild-type, pEF control and MTSS1-KO breast cancer cells were seeded at a density of 50,000/well (in triplicate) into a 96-well plate that had been previously coated with 5 μg of Matrigel artificial basement membrane. MDA-MB-435s wild-type, MDA-pEF control and MDA-MTSS1-Exp cells were seeded at 40,000/well. These cells

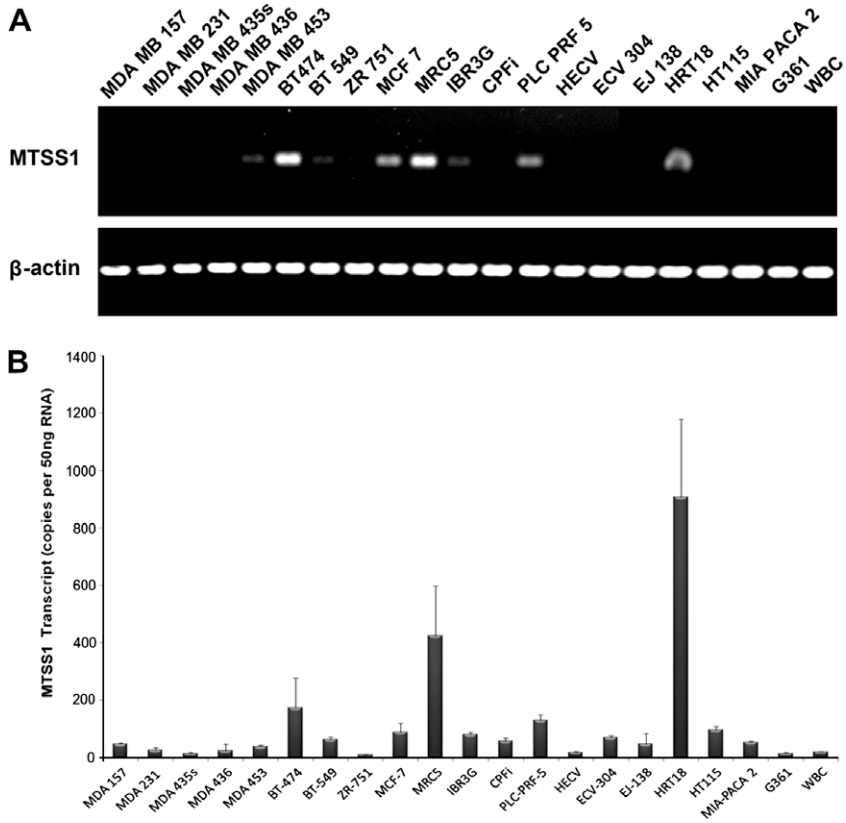

Fig. 1 – (A) RT-PCR analysis of MTSS1 mRNA expression within a panel of human cell lines. MTSS1 was found to be expressed at moderate levels within the MCF-7 and BT474 breast cancer cells; HRT-18 colorectal cancer cells; PLC-PRF-5 liver cancer cell line and the MRC5 stromal fibroblast cell line. (B) Quantitative-PCR assessment of MTSS1 expression. Quantification of MTSS1 transcripts in cell lines revealed that high MTSS1 levels corresponded with those identified through PCR ($n = 3$). QPCR data confirmed observations from the initial PCR data.

were then incubated for 45 min to allow cells to adhere, before being subjected to vigorous washing (×4) in BSS, to remove non-adherent cells. Adherent cells were then fixed in 4% formaldehyde (v/v) and stained with 0.5% (w/v) crystal violet. The number of stained, adherent cells was counted in several random fields under 40× objective magnification.

2.11. *Wounding/motility assay*

The migratory properties of these cells were assessed to determine the impact of the forced expression or knockout of MTSS1 on the invasive nature of these breast cancer cells. This technique to measure cell motility has been described in a previous study.[19] The cells were seeded at a density of 50,000/well into a 24-well plate and allowed to reach confluence. The layer of cells was then scraped with a fine gauge needle to create a wound of approximately 200 μm. The movement of cells to close the wound was recorded and analysed as described previously using a time-lapsed video system.[19] After the addition of a treatment, the cells motile qualities were monitored and recorded on a video for 120 min. Wound closure/cell migration was evaluated with motion analysis and line morphometry software (Optimus 6). Results were exported to a spreadsheet (Excel) for further evaluation and interpretation.

2.12. *Tumour cell invasion assay*

We quantified the invasive nature of the MTSS1-modified cancer cells using the standard invasion assay procedure as described previously.[20] Transwell chambers, equipped with a 6.5 mm diameter polycarbonate filter insert (pore size 8 μm) (Becton Dickinson, Labware, Oxford, UK), were pre-coated with 50 μg/insert of solubilised tissue basement membrane, Matrigel (Collaborative Research Products, Bedford, Massachusetts, USA). MCF-7 wild-type, MCF-pEF control and MCF-MTSS1-KO breast cancer cells were seeded at a density of 60,000/insert and allowed to invade for 4 d. MDA-MB-435s wild-type, MDA-pEF control and MTSS1-Exp breast cancer cells were seeded at a density of 15,000/insert and allowed to invade for 3 d. Following incubation, cells that had invaded through the basement membrane were fixed (4% formaldehyde), and then stained with crystal violet. For analysis, the cells were counted in 10 fields/insert (×40 magnification); to determine the mean number of invaded cancer cells.

2.13. *Statistical analysis*

The results were assessed using non-paired (two-sided) Student's *t*-test, one-way ANOVA test and also with a Kaplan–Meier survival curve (*p*-values by Cox Proportion Hazardous

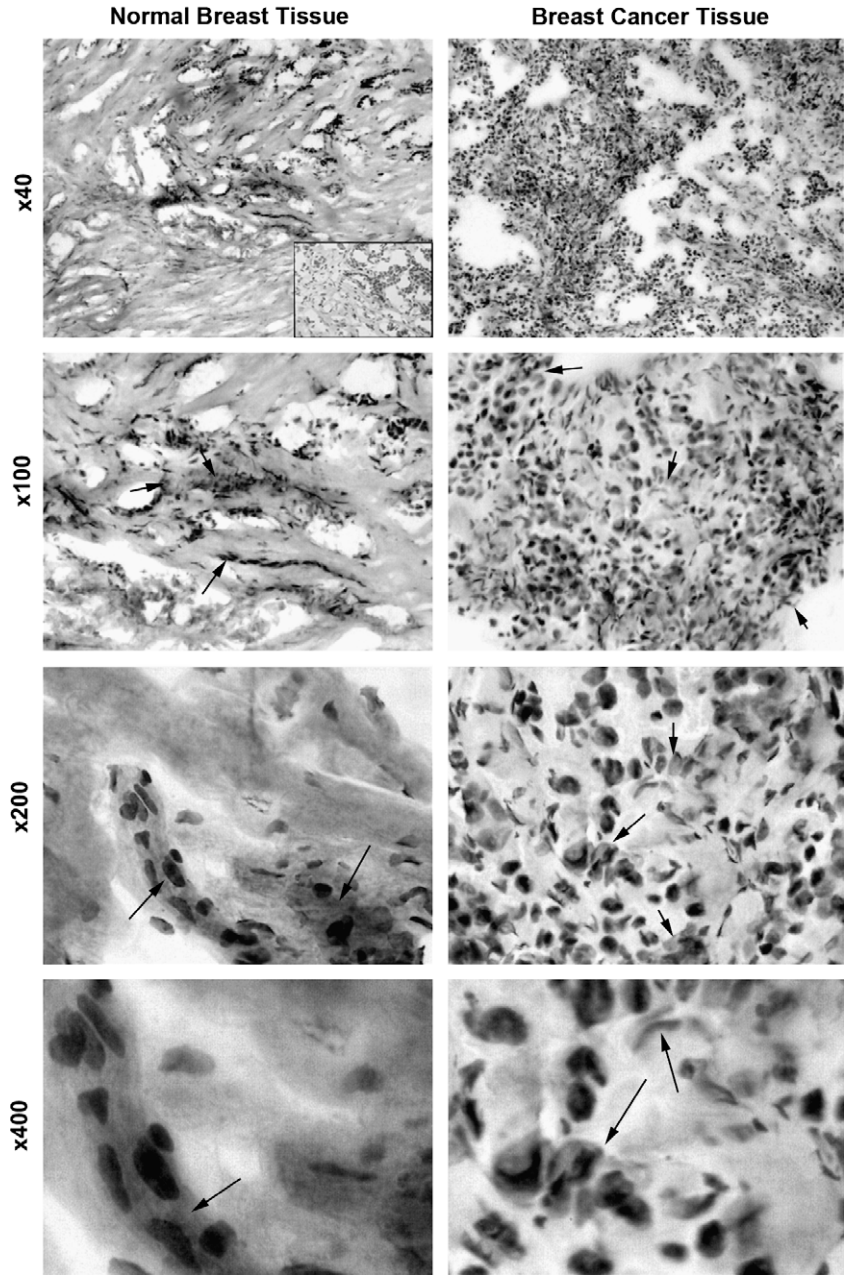

Fig. 2 – Immuno-histochemical Staining of Human Breast Specimens. (Left panel) Normal background breast tissue. The MTSS1 protein was found to be well stained in the normal breast epithelial cells (indicated by black arrows). Normal mammary tissue was also stained in the absence of a MTSS1 primary antibody to act as a negative control (top left insert). (Right panel) Breast cancer tissue. Staining of breast cancer cells for MTSS1 was seen to be negative/weakly positive in the breast tumour specimens (black arrows in right panel), when compared against the intense epithelial staining of the normal breast tissues.

Analysis). MTSS1 mRNA values obtained in the QPCR study are given as mean transcript copy number per 50 ng of RNA ± SD. A p-value <0.05 was defined as statistically significant.

3. Results

3.1. *MTSS1 mRNA expression in human cell lines*

A variety of 21 human normal and cancer cell lines were examined for the presence of MTSS1 through RT-PCR (Fig. 1A) and Quantitative-PCR (Fig. 1B). Of the nine human breast cancer cells examined, MTSS1 was most strongly expressed in the BT474 and MCF-7 cell lines, which are known for having low-invasive potential.[20] The MDA-MB-157, MDA-MB-231, MDA-MB-435s, MDA-MB-436 and ZR-751 breast cancer cell lines were negative for MTSS1 transcript. The HRT-18 colon cancer cell line and the PLC/PRF/5 liver cancer cell line also strongly expressed MTSS1. Interestingly, MTSS1 was found to be expressed at high levels in the MRC5 human stromal fibroblasts, and also in the IBR.3.G fibroblasts.

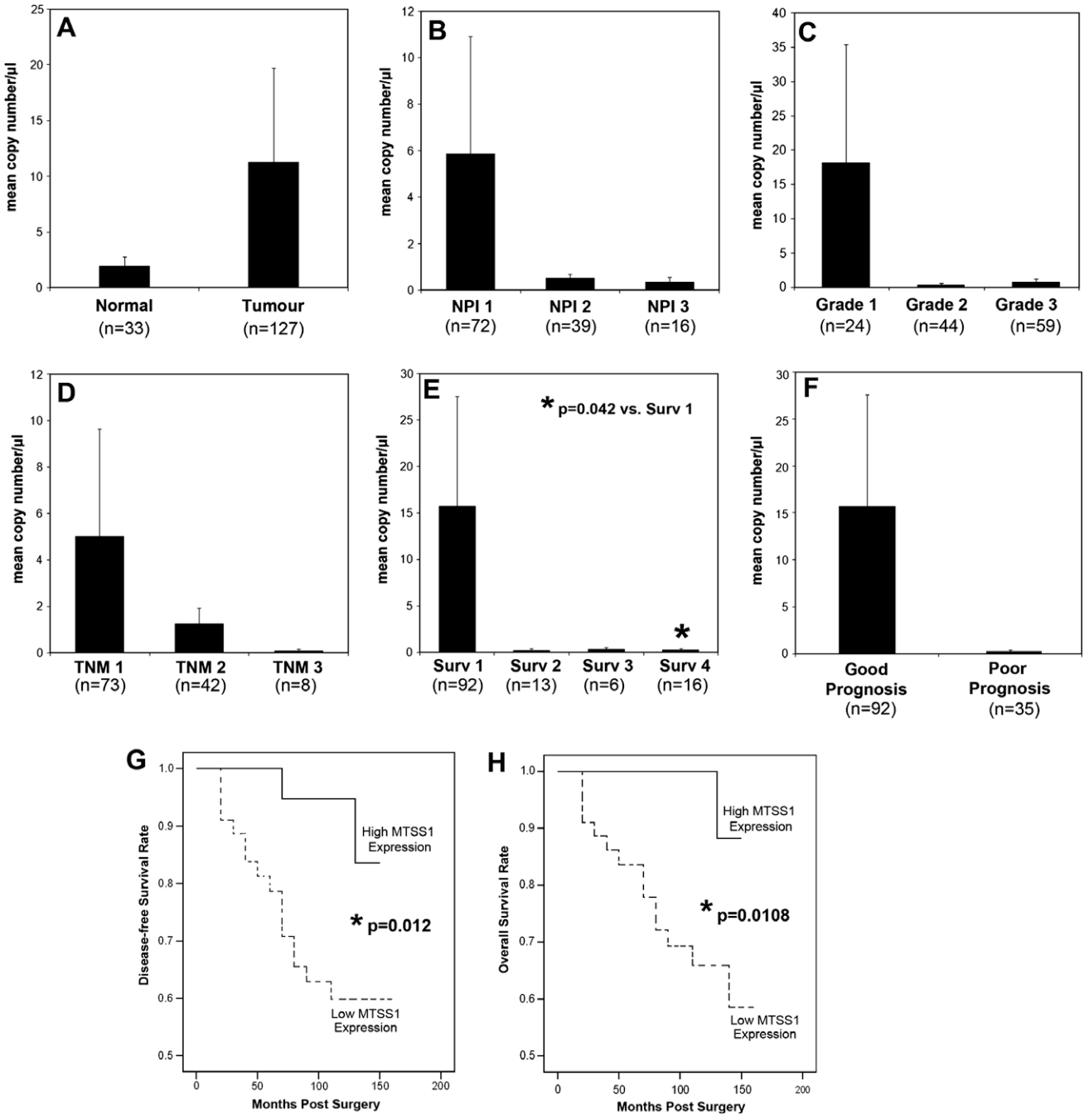

Fig. 3 – Quantitative-PCR analysis of MTSS1 expression levels in breast cancer patient specimens. (A) Tumour versus background tissue from breast cancer patients; (B) Nottingham Prognostic Index of patient prognosis; (C) tumour grade; (D) tumour-node-metastasis classification of patients; (E) patient survival status; (F) patient prognosis; (G) Kaplan–Meier disease-free survival analysis and (H) Kaplan–Meier overall survival analysis.

3.2. *Immuno-histochemical staining of human breast specimens*

MTSS1 immunostaining was observed in the human breast tissue sections ($n = 32$ pairs). MTSS1 was strongly expressed in the normal mammary tissue (Fig. 2 – left panels), and its distribution was mainly confined to the ductal epithelium and myoepithelial cells. Interestingly, it was noted that stromal fibroblasts also displayed MTSS1 immunoreactivity. Upon the analysis of breast tumour tissues we reveal that MTSS1 protein levels were dramatically reduced/absent in the breast cancer cells from the tumour tissue specimens, particularly in those patients who had recurrence, metastasis to another site or had died as a result of breast cancer (Fig. 2– right panels).

3.3. Quantitative-PCR analysis of MTSS1 transcripts in mammary tissues and their clinical/pathological relevance

No significant difference between the normal background tissues (1.96 ± 0.8) and the tumour tissues (11.26 ± 8.4) was observed (Fig. 3A).

3.3.1. MTSS1 expression in relation to patient prognosis indices

The levels of MTSS1 transcripts were analysed against the Nottingham Prognostic Index (NPI) (Fig. 3B), which divided the patients into three groups: the NPI-1 group (NPI score <3.4) represented patients with a good prognosis; the NPI-2 group (NPI score 3.4–5.4) represented patients with a moderate prognosis; whereas the NPI-3 patients (NPI score >5.4) had a poor prognosis. There is a trend that suggests patients with a NPI-2 moderate and a NPI-3 poor prognosis (0.516 ± 0.19 and 0.35 ± 0.21, respectively), had significantly reduced levels of MTSS1, when compared to the MTSS1 levels in the patients of the NPI-1 good prognosis group (5.88 ± 5.1), this observation was not statistically significant.

3.3.2. MTSS1 and tumour grade

The grade 2 (0.38 ± 0.14) and grade 3 (0.75 ± 0.45) tumours had reduced levels of MTSS1 compared to well differentiated grade 1 tumours (18.2 ± 17.2), although these values did not reach statistical significance (Fig. 3C).

3.3.3. MTSS1 and tumour staging

We also analysed MTSS1 expression in relation to tumour staging, TNM classified after surgery (Fig. 3D). TNM group 4 was excluded from the analysis due to low sample number in the group. Patient TNM grouping revealed that TNM-2 (1.25 ± 0.7) and TNM-3 (0.08 ± 0.08) patients had lower levels of MTSS1, compared to the TNM-1 group (5.01 ± 4.65). Again, we showed the trend that MTSS1 levels are reduced in patients with a poorer outcome; however, results did not reach statistical significance.

3.4. MTSS1 expression, clinical outcome and long-term survival

Patients were divided into four subgroups with a 10-year median follow-up period. Survival group 1 contains those patients who are alive, well and free of recurrence; survival group 2 patients had local recurrence; survival group 3 patients had recurrence and metastases at other sites and survival group 4 patients had died as a result of breast cancer. ANOVA-based analysis of the quantity of MTSS1 transcript revealed a highly significant correlation between the levels of MTSS1 and survival status ($p = 0.042$) (Fig. 3E). We report that survival group 4 breast cancer patients who had died of breast cancer (0.242 ± 0.22) had significantly lower levels of MTSS1 ($p = 0.042$), compared to survival group 1 (15.7 ± 11.9). Survival group 2 and 3 patients (0.23 ± 0.22 and 0.35 ± 0.23, respectively) also had reduced MTSS1 levels compared to group 1 patients, although these decreases did not reach statistical significance. Patients who had recurrence, metastasis to another site or had died as a result of breast cancer were collectively compared with the good prognosis group (Fig. 3F), with the trend remaining (0.252 ± 0.15 versus 15.7 ± 11.9) ($p = 0.088$).

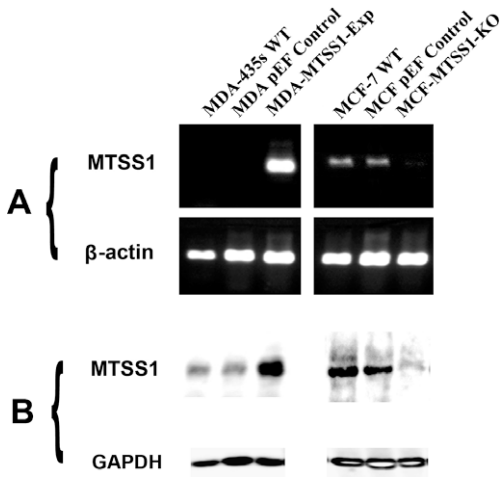

Fig. 4 – (A) RT-PCR assessment of MTSS1 'forced' expression or knockdown within the breast cancer cell lines. MTSS1 was overexpressed in the MDA-MTSS1-Exp cell line; whereas MTSS1 expression levels were reduced in the MCF-MTSS1-KO cells. (B) Western blotting confirmation of MTSS1 protein levels. Results obtained above at the mRNA level were confirmed at the protein level.

Using the Kaplan–Meier Survival Curve method and Cox Proportion Hazardous Analysis, it was revealed that the patients with high levels of MTSS1 expression in their tumours showed a better prognosis, in terms of disease-free survival (Fig. 3G), than those with lower expression levels ($p = 0.012$). Furthermore, comparison of MTSS1 levels and overall patient survival (Fig. 3H) also revealed that patients with high MTSS1 levels possessed a better chance of overall survival compared to those whose tumours expressed low levels of MTSS1 ($p = 0.0108$). Finally, multivariate analysis using NPI, grade, TNM, nodal status, ER status and MTSS1 as variants has shown that nodal status ($p = 0.033$), ER status ($p = 0.010$) and MTSS1 ($p = 0.036$) are independent factors for the overall survival.

3.5. Creation of breast cancer cell sub-lines with differential pattern of MTSS1 expression

The MDA-MB-435s invasive breast cancer cell line was a good candidate for MTSS1 expression as we demonstrate that the wild-type MDA-MB-435s cell line did not express the MTSS1 mRNA. Whereas the MCF-7 breast cancer cell line was also a good candidate for knockdown as the low-invasive cell line expressed moderate levels of MTSS1. MTSS1 was absent in the MDA-MB-435s wild-type (MDA-wild-type) and empty vector control (MDA-pEF-control) cells are now clearly detected within the cells forced to express it (MDA-MTSS1-Exp) (Fig. 4A). Likewise, MTSS1 which was strongly present within the wild-type and control MCF-7 cells was reduced in the MCF-MTSS1-KO cells. These experiments were replicated at the protein level through Western blotting (Fig. 4B). Thus,

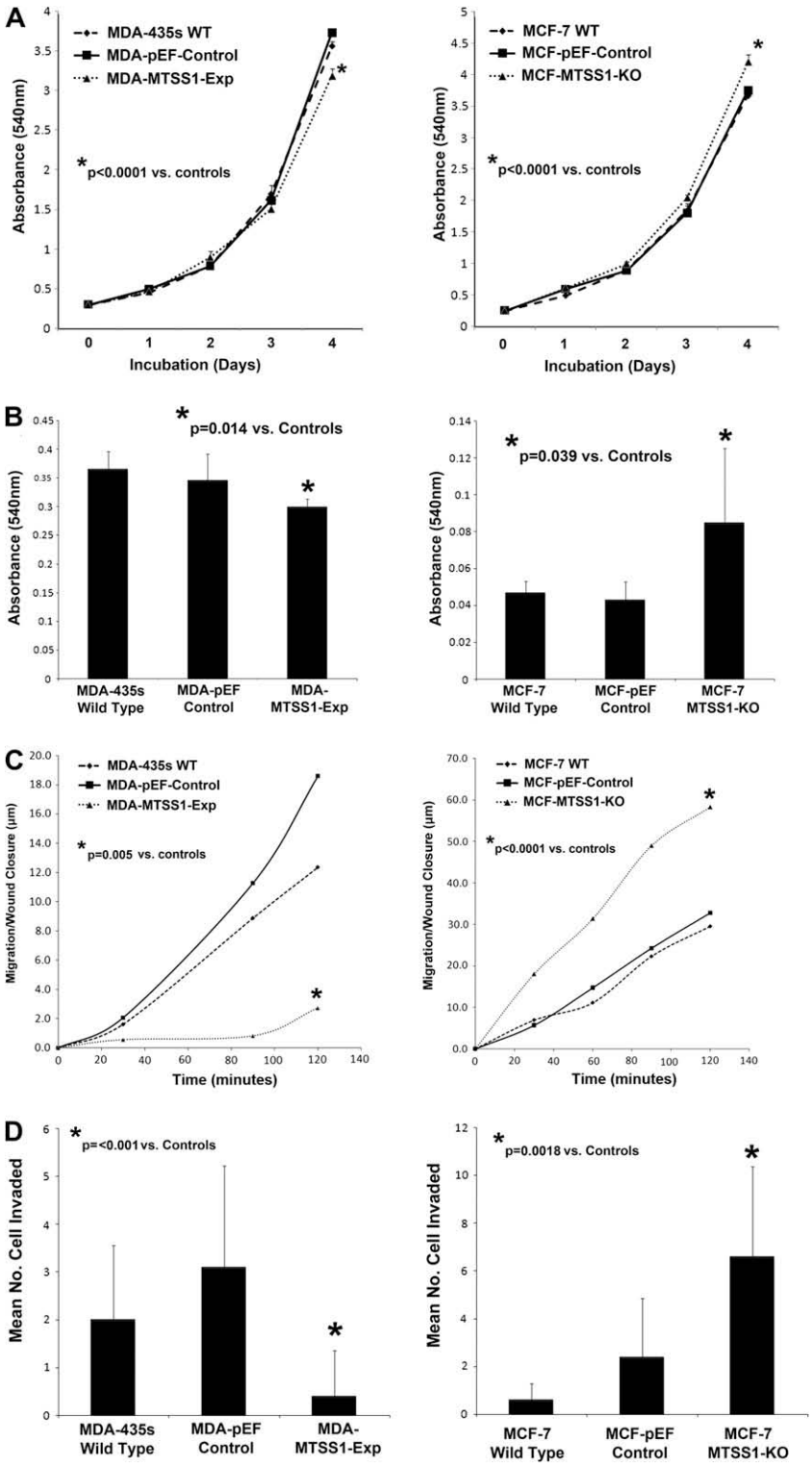

Fig. 5 – (A) Tumour cell growth assay. (Left panel) Overexpression of MTSS1 significantly decreased cell growth rate. (Right panel) Conversely, knockdown of MTSS1 expression resulted in a significant increase in growth rate. (B) Tumour cell adhesion assay. (Left panel) A reduction in the adhesive nature of the MDA-MB-435s cells was observed through the forced expression of MTSS1. (Right panel) Suppression of MTSS1 expression levels enhanced the adhesive properties of the MCF-7 breast cancer cell line. (C) Tumour motility assay. (Left panel) Overexpression of MTSS1 dramatically suppressed the motile properties of these cells. (Right panel) An increase in migration rate was observed through the knockdown of MTSS1 expression. (D) Tumour cell invasion assay. (Left panel) Tumour cell invasion was significantly suppressed through overexpression of MTSS1 within the invasive MDA-MB-435s cells. (Right panel) In contrast, knockdown of MTSS1 dramatically enhanced the invasive nature of the MCF-7 breast cancer cells. *Note:* All experiments were performed independently a minimum of three times.

we have successfully created cell lines that demonstrate MTSS1 over- or under-expression, as appropriate. The properties of these new MTSS1-modified cell lines were ready for the analysis through a series of *in vitro* studies.

3.6. *Regulation of MTSS1 expression had an impact on breast cancer cell aggressiveness*

3.6.1. *Enhanced MTSS1 expression suppresses breast cancer cell growth, adhesion, motility and invasion*

We reveal that overexpression of MTSS1 protein has an effect on the growth rate of cells. MDA-MTSS1-Exp breast cancer cells had a minor yet significantly reduced rate of growth ($p < 0.0001$) compared to the control group (Fig. 5A). The adhesive nature of these breast cancer cells to bind and attach to a replica basement membrane was also examined (Fig. 5B). Breast cancer cells overexpressing MTSS1 showed a significant reduction in adhesive ability ($p = 0.014$).

The presence of MTSS1 also significantly reduced the motile nature of breast cancer cells (Fig. 5C). The MDA-MB-435s breast cancer cells demonstrated their motile properties in the wound closure motility assay; however, the presence of MTSS1 within the cells significantly suppressed cell migration to close the wound compared to the controls ($p = 0.005$).

The MDA-MB-435s cell line is an invasive cell line that does not normally express MTSS1 (Fig. 1). The growth assay mentioned above demonstrates that following 4 d incubation, there was a significant difference that could add experimental error into experiments using long incubation times. However, at 3 d incubation (invasion assay allows 72 h incubation), there was no significant difference in growth rate between MTSS1-modified cells and controls. Thus, invasion data obtained are valid. We report that upon forced expression of MTSS1 there was a change in the aggressive nature of these breast cancer cells (Fig. 5D). The forced expression of MTSS1 in this cell line resulted in a dramatic reduction in the degree of invasion ($p < 0.001$ versus controls). Importantly, our data suggest that the presence of MTSS1 may suppress or limit the invasive nature of cancer cells.

3.6.2. *Knockdown of MTSS1 expression increased the growth, adhesive, motile and invasive properties of breast cancer cells*

Here, using the MCF-7 cell model in which MTSS1 expression has been reduced by way of ribozyme transgenes, we observed that the MCF-7 cell models displayed an opposite trend of functional changes compared with the MDA-MB-435s model: i.e. reduced MTSS1 expression (MCF-MTSS1-KO) cells displayed an accelerated growth rate ($p < 0.0001$, versus control, Fig. 5A), increased adhesive properties ($p = 0.039$, Fig. 5B) and enhanced the motility ($p < 0.0001$, Fig. 5C). Upon suppression of MTSS1 expression within the MCF-7 cells there was also a dramatic change in the invasiveness of these breast cancer cells (Fig. 5D). MCF-MTSS1-KO breast cancer cells were significantly more invasive than the control breast cancer cells with MTSS1 present ($p = 0.0018$). These data demonstrate that the presence of MTSS1 may limit breast cancer cell invasion. However, it is important to note that MCF-7 cells were allowed to invade for 4 d, and as the growth assay demonstrated above, 4 d incubation was long enough to observe differences in growth rate and cell number. This factor may therefore influence the statistical data obtained from the MCF-7 invasion assay.

4. Discussion

Although metastasis suppressor 1 (MTSS1) is suggested to be an exciting new metastasis suppressor; there is little information available on this factor. The knowledge on MTSS1 is particularly weak from a clinical aspect. Supported by a series of cellular function tests, the present study indicates for the first time that MTSS1 acts as a powerful inhibitor to the aggressiveness of breast cancer cells and has strong prognostic and survival relevance in patients with breast cancer.

Our initial studies examined MTSS1 expression within a variety of human normal and cancer cell lines; and breast tissue from breast cancer patients. We report that two of the nine breast cancer cells, MCF-7 and BT474, expressed moderate levels of MTTS1. Both these cell lines are considered to be of a low/non-invasive nature. A few other aggressive cells lines, namely MDA-MB-231 and MDA-MB-435s, are negative in MTSS1 expression. This is an interesting pattern and may partly indicate that the levels of MTSS1 are inversely correlated with aggressiveness. This is reflected in our subsequent expression modification studies in which we created two cell models with differential pattern of MTSS1: the MCF-7 model and MDA-MB-435s model. The invasive MDA-MB-435s, MTSS1-negative, cell line was 'forced' to overexpress MTSS1. While the non-invasive, and MTSS1-positive, MCF-7 cell line had its MTSS1 expression levels 'knocked-down'. We employed a range of biological function assays *in vitro* to assess the effect of MTSS1-modification on the metastatic nature of these breast cancer cell lines. Our approaches have proved that the forced expression of MTSS1 within the breast cancer cells greatly reduced the aggressive nature of these cells by reducing the invasive, adhesive, growth and motile properties of these MDA-MB-435s tumour cells. In contrast, the elimination of MTSS1 expression within the MCF-7 cells had the reverse effect, and this low-invasive cell line displayed significant increases in tumour cell migration, invasion, growth and adhesion. Therefore, our findings suggest that MTSS1 may play a role in deciding the metastatic nature and development of breast cancer cells.

This important link is further supported by the significant correlation between MTSS1 and the clinical outcome and long-term survival of the patients with breast cancer. It appears that MTSS1 may represent an indicator of survival in breast cancer, as patients with a good prognosis expressed elevated levels of MTSS1. MTSS1 mRNA levels were dramatically reduced in patients who had died of breast cancer compared to the high levels observed in patients who were alive and well with no recurrence. Our survival analysis also reported that, over a mean 10 year follow-up, patients whose tumours expressed high levels of MTSS1 were statistically more likely to survive, cancer-free, for longer than those patients with a low MTTS1 level. These results demonstrate that the degree of MTSS1 expression may be relevant in terms of breast cancer patient survival. In view of our data, we suggest that MTSS1 acts as a survival indicator for breast cancer patients, as we demonstrate that patients expressing

high/elevated levels of MTSS1 have a favourable prognosis, in contrast to those patients with low/reduced levels of MTSS1 and a poor prognosis.

Our study reveals that MTTS1 expression levels are down-regulated in the breast cancer tissues of patients with a poor prognosis. The mechanism for the down-regulation of MTSS1 may involve DNA methylation.[8] Although the relevance of such down-regulation to tumour progression, in particular, metastasis, has not yet been confirmed, several lines of evidence have indicated a role of MTSS1 in cell morphogenesis. Overexpression of MTSS1 triggers distinct cell shape changes such as increase in the formation of membrane ruffles, lamellipodia and filopodia-like structures.[3,7,10,13,21]

However, one would have to exert a degree of caution when making such a link. In the present study, a non-invasive cell line (ZR-751) is also negative for MTSS1. We could probably argue that the MTSS1-aggressiveness link can have exceptions and may also depend upon other factors, such as other actin-regulating proteins. This is also reflected in the previous studies in that MTSS1 has been shown as a metastatic suppressor in both bladder[2,9] and prostate cancers.[7,8] In contrast, MTSS1 expression was maintained in metastatic cell lines,[10] and basal cell carcinomas.[12] This regulation suggests that MTSS1 levels are likely to be controlled during developmental programming, and that MTSS1 expression may be altered in cancer cells, leading to changes in the signalling and architecture of the cytoskeleton. This evidence suggests that MTSS1 may be pivotal in the carcinogenesis of many cancer types. These previous studies thus indicate that the role of MTSS1 may be more diverse than initially anticipated.

One study assessed immunoreactivity within bladder cancer patient samples and reports that down-regulation of MTSS1 expression may correlate with the transition of tumour cells from distinct epithelium-like morphology to less differentiated carcinomas.[22] Importantly, our immuno-histochemical studies of normal mammary tissue demonstrate that MTSS1 was strongly expressed by the normal breast epithelia from breast cancer patients. However, immuno-histochemical staining of the patient tissues strongly suggests that MTSS1 levels were reduced within the tumour tissue of patients. Another report reveals that an incremental increase in MTSS1 expression was detected in normal liver specimens compared against matched hepatocellular carcinoma tumour tissue specimens.[11] Elevated MTTS1 expression was observed at the early stage disease, suggesting that MTTS1 may play an important role in promoting the early development of hepatocellular carcinoma; and may therefore serve as a biomarker for the prediction of early tumour development of hepatocellular carcinoma. Thus, it is likely that 'to be' or 'not to be' a metastasis suppressor for MTSS1 is highly dependent upon tumour type.

It is crucial to identify the mechanisms and functional consequences of MTSS1 action before we can fully appreciate the direct implications of MTSS1 expression in cancers including breast cancer. Studies report that the mechanism of metastasis suppression by MTSS1 may be due to actin binding, re-organisation and/or changes in cell adhesion or tyrosine phosphorylation upon loss of MTSS1.[3,22–24] The importance of actin activity on tumourigenesis and enhanced metastatic potential of several types of malignancies is well established.[25] Therefore, MTTS1 involvement in breast cancer invasion and metastasis is most likely through actin dynamics and a membrane-deformation activity.[26]

Furthermore, we have recently obtained evidence, which suggests that MTSS1 interacts with plakoglobin (γ-catenin) to exert its biological effect. Plakoglobin exists within cells in association with desmoplakin in the desmosomes, which are in turn coupled to the cytoskeleton linking to the plasma membrane. A recent study demonstrated that plakoglobin overexpression caused (i) a reduction in E-cadherin, which led to increased cell mobility and migration and (ii) an increase in c-Myc levels which leads to uncontrolled proliferation.[27] Therefore, MTSS1 may play a role in controlling plakoglobin levels, and a loss of MTSS1 may result in enhanced plakoglobin expression. This lead is currently under intensive investigation.

In conclusion, our study indicates that MTSS1 demonstrates the ability to modulate metastatic ability in breast cancer cells. We also report that MTSS1 expression levels may act as an indicator of patient survival. Therefore, metastasis suppressor 1 displays prognostic value for breast cancer patients.

Conflict of interest statement

None declared.

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