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Membrane-Type 1 Matrix Metalloproteinase as predictor of survival and candidate therapeutic target in Ewing Sarcoma

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Abbreviations

CSC: Cancer Stem-like Cell

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EFS: Event Free Survival

EMT: Epithelial-to-Mesenchymal Transition

ES: Ewing sarcoma

ES-CSC: Ewing sarcoma cancer stem-like cell

EWSR1: Ewing Sarcoma Breakpoint Region 1

FBS: Foetal Bovine Serum

FFPE: Formalin-fixed paraffin-embedded

ICC: Immunocytochemistry

IHC: Immunohistochemistry

IQR: Inter quartile range

MFI: Median Fluorescence Intensity

MMP: Matrix Metalloproteinase

MMP-2: Matrix Metalloproteinase 2

MMP-9: Matrix Metalloproteinase 9

MMP-14: Matrix Metalloproteinase 14

MSC: Mesenchymal stem cell

MT1-MMP: Membrane-type 1 Matrix Metalloproteinase

PBS: Phosphate-buffered saline

OS: Overall Survival

Abstract

Background

Ewing sarcoma (ES) is the second most common primary bone malignancy, with an urgent need for new treatments. ES is associated with high rates of progression and relapse, driven by drug-resistant cells capable of migration, self-renewal and single-cell tumorigenesis, termed cancer stem-like cells (CSCs). Membrane-type 1 matrix metalloproteinase (MT1-MMP) is a membrane-bound proteolytic enzyme which, via direct and indirect mechanisms, digests four of the main types of collagen. This can be hijacked in malignancy for invasion and metastasis, with high expression predicting decreased survival in multiple cancers. In this study we have examined the hypothesis that MT1-MMP is expressed by ES cells and explored the relationship between expression and outcomes.

Procedure

MT1-MMP expression in ES established cell lines, primary patient-derived cultures and daughter ES-CSCs was characterised by RNA sequencing, western blotting, immunocytochemistry and flow cytometry. Immunohistochemistry was used to detect MT1-MMP in tumour biopsies and the relationship between expression, event-free and overall survival examined.

Results

MT1-MMP was detected at both RNA and protein levels in 5/6 established cell lines, all primary cultures (n=25) and all daughter ES-CSCs (n=7). Immunohistochemistry of treatment naïve biopsy tissue demonstrated that high MT1-MMP expression

predicted decreased event-free and overall survival (p=0.017 and p=0.036 respectively, n=47); this was not significant in multivariate analysis.

Conclusions

MT1-MMP is expressed by ES cells, including ES-CSCs, making it a candidate therapeutic target. The level of MT1-MMP expression at diagnosis may be considered as a prognostic biomarker if validated by retrospective analysis of a larger cohort of clinical trial samples.

1. Introduction

Ewing sarcoma (ES) is a malignant tumour affecting both bone and soft tissue[1]. It is the second most prevalent primary bone malignancy[2], occurring most frequently in those that are 10-24 years old[3]. Unfortunately, outcome for patients with ES is poor; it is a relatively drug resistant disease with a metastatic propensity (~30% of patients have distant metastases at presentation)[4,5]. Multiple factors are associated with unfavourable prognosis, including tumours that are >8cm in size, spinal or pelvic tumours, incomplete response to chemotherapy, high serum lactate dehydrogenase (2x upper limit of normal) and the presence of metastasis at diagnosis[4-8]. Patients with localised disease usually have better outcomes, although 20-30% still die within 5 years due to relapse or development of metastases[5], emphasising the need for improved prognostic tools to better risk-stratify such patients. For the 25 percent of patients that present with non-pulmonary metastasis, outcome is particularly poor, with a 5 year survival of <30%, compared to ~50% with isolated pulmonary metastases[5].

Drug-refractory and progressive ES may be explained by the presence of a population of cells with stem cell-like properties, so called ES stem-like cells (ES-CSCs), that have the ability to survive chemotherapeutic insult and re-populate the tumour at primary and secondary (metastatic) sites[9]. So called Ewing sarcoma stem-like cells (ES-CSCs) have been identified[10-13] using a number different methods including protein markers CD133[10] and ALDH[11]. As CD133 negative cells have been found to have CSC characteristics, suggesting these methods can miss CSCs[14,15], functional assays have been used more recently[13].

Matrix metalloproteinases (MMPs) are a family of zinc-containing proteases involved in extracellular matrix (ECM) recycling[16]. These include the membrane-bound membrane type-1 matrix metalloproteinase (MT1-MMP, also known as matrix metalloproteinase 14/MMP-14), which is found in 3 forms: as a zymogen (63kDa), the active form (60kDa) and a truncated product following cleavage from the membrane (45kDa)[17]. MT1-MMP digests collagen types I, II and III and other ECM components (proteoglycans and fibronectin) and, through the activation of MMP-2, breaks down collagen type IV, leading to remodelling of the basement membrane[18]. MT1-MMP also promotes cell migration via cleavage of cell surface CD44[19] and the activation of various integrins[20], whilst facilitating monocyte transmigration between tissues and blood vessels through the Intracellular Adhesion Molecule-1 (ICAM-1)-mediated pathway[21]. These properties are consistent with the role of MT1-MMP in invasion and migration[22-27] and development of metastasis *in vivo*[24,28]. The ability of MT1-MMP to regulate epithelial-to-mesenchymal transition (EMT) during development[29] is also hijacked in multiple cancers including synovial sarcoma[30-33], in which adopting an invasive, mesenchymal phenotype aides metastasis[34]. Furthermore, knockdown of caveolin-1, the protein regulating the expression of MT1-MMP at the cell surface, reduces ES metastasis in mouse models[35], whilst the inactivation of ICAM-1 inhibits metastasis and improves outcome in ES[36]. These observations suggest that MT1-MMP may play a role in metastasis in ES which may be effected through ES-CSCs. Consistent with this hypothesis, knockdown of MT1-MMP in tumour-initiating breast cancer cells reduces CSC-like behaviour[37] whilst overexpression of MT1-MMP in squamous carcinoma cells induces a CSC-like phenotype[33].

MT1-MMP is often overexpressed in solid cancers, with sarcomas showing the highest expression of the 31 cancer types included in the TCGA PanCancer atlas[38]. Furthermore, high expression correlates with reduced survival in multiple cancers[39], including the most common bone cancer osteosarcoma, with overall survival (OS) at 5 years of 89% in those with low MT1-MMP expression compared to only 58% in those with high expression (p=0.048)[40]. MT1-MMP expression has been demonstrated by western blotting in a small number of primary and established ES cell lines[41,42], and by immunohistochemistry (IHC) in tissue samples[43]. To date, expression has not been correlated with OS and event free survival (EFS).

In this study we have characterised the expression of MT1-MMP in ES cell lines, primary patient-derived cells and daughter ES-CSCs to assess its potential for future study as a therapeutic or 'theranostic' target. We also aimed to evaluate MT1-MMP expression as a predictor of survival in ES, given the clear need to improve identification of the 20-30% of patients who have a poor outcome despite presenting with localised disease[5].

2. Methods

2.1 Cell Lines and patient-derived cells

All ES cell lines and patient-derived cells[13] studied contain *EWSR1* gene re-arrangements and express CD99 in the cell membrane, characteristic of ES. The ES cell lines A673, SK-N-MC, SKES-1, TC-32, TTC 446 and RD-ES were cultured as previously described[44], as were patient-derived cultures[13]. The dedifferentiated chondrosarcoma cell line (HT1080; MT1-MMP positive control cells, American Type Culture Collection (ATCC), USA) and breast cancer cell line (MCF-7; MT1-MMP negative control cells, ATCC) were cultured in RPMI 1640 media (Sigma-Aldrich,

UK) containing 10% foetal bovine serum (FBS, Sigma-Aldrich), 2mM penicillin-streptomycin (Sigma-Aldrich) and 2mM Glutamine (Sigma-Aldrich). All cells were tested for mycoplasma every four months using the EZ-PCR mycoplasma test kit (Geneflow, UK).

2.2 Total RNA sequencing

Total RNA libraries were prepared from 1µg of RNA (RNA integrity number >9) extracted from ES primary patient-derived cultures. RNA was sequenced using the Illumina® HiSeq3000, FASTQ files were downloaded and reads pre-processed using cutadapt[45], and mean normalised read counts generated as previously described[13,46].

2.3 Western Blot

MT1-MMP protein expression was detected by Western blot as described previously[47]. Briefly, membranes were probed for 16 hours at 4°C with antibodies raised to the catalytic domain of MT1-MMP/MMP-14 (1µg/ml, MAB3328, clone LEM-2/15.8, Merck-Millipore, USA) and the loading control anti-β-actin (10ng/ml, ab8226, Abcam, UK). Secondary antibody Goat Anti-Mouse IgG (H+L) – HRP conjugate (0.16µg/ml, Bio-Rad, USA) was incubated at room temperature for 60 mins. Membranes were imaged with the Chemidoc System (Bio-Rad) using Immobilon Forte Western HRP Substrate (Millipore, USA).

2.4 Cell surface expression of MT1-MMP

2.4.1 Flow Cytometry

Cells pellets containing $1x10^5$ cells were suspended in 100µl of flow cytometry buffer (0.5% bovine serum albumin, 2mM EDTA (Sigma-Aldrich) in PBS) containing 5µl of

Human Seroblock (Bio-Rad) for 10 min at room temperature. Cells were incubated in triplicate with MT1-MMP primary antibody (0.1µl/µl Human MMP-14/MT1-MMP PE-conjugated Antibody – FAB9181P, clone 128527, R&D systems) or corresponding isotype control antibody (0.1µl/µl, Mouse IgG2B Control PE conjugated – IC0041P, R&D systems) for 30 minutes in the dark, at room temperature. Cells were fixed in 1% paraformaldehyde (in PBS Sigma-Aldrich) for 20 min at room temperature and then analysed using the Attune acoustic focusing cytometer (Applied Biosystems) recording 10,000 events. The percentage of MT1-MMP positive cells and the median fluorescence intensity (MFI) was determined using FlowJo v10 (BD Biosciences) and data expressed relative to the isotype control. For each sample the inter-quartile range (IQR) of fluorescence intensity was determined to evaluate the heterogeneity of MT1-MMP expression.

2.4.2 Immunocytochemistry

Cells were centrifuged onto slides (1000g for 3 min, followed by 3000g for 1 min, Rotix 32A Hettich zentrifugen, Hettich Lab, Germany), fixed in 4% paraformaldehyde (w/v in PBS; Sigma-Aldrich) for 10 min at room temperature and incubated with peroxidase-blocking solution (Dako, Agilent Technologies, USA) for 5 min at room temperature. Cells were incubated with MT1-MMP (Anti-MMP-14 catalytic domain, 125ng/ml clone LEM-2/15.8, MAB3328, Merck-Millipore) or isotype control antibodies (Mouse $IgG_1\kappa$ antibody, 125ng/ml, MG1-45, ab18447, Abcam) for 60 min at room temperature, followed by 30 min incubation at room temperature with the secondary antibody (Dako EnVision+ System- HRP Labelled Polymer Anti-mouse, Agilent Technologies). Dako DAB+ substrate chromogen system (5 min at room temperature, Agilent Technologies) was used to visualise antibody binding. Slides were counterstained with Haematoxylin and imaged using a Zeiss Axioplan

191 microscope and Zeiss AxioCam (Zeiss, Germany). Staining was quantified using H-
192 scores[48]; counting the number of positive cells from 100 cells in three-independent
193 fields of view, and recording the intensity of staining of positive cells with a score of
194 +1, +2 or +3.

195 **2.4.3 Immunohistochemistry**

196 ES tissues collected between 22/02/2001 and 19/05/2017 from patients with a
197 median age of 18.5 years (range 5-64 years) were obtained from the Newcastle
198 Biobank (IRAS 233551, REC 17/NE/0361). Tumours were positive for CD99, with
199 *EWSR1* gene rearrangements, and confirmed as ES by specialist sarcoma
200 pathologists. The median follow up and time to a first event was 1900 and 623 days
201 respectively. Treatment-naïve biopsy tissue taken at diagnosis with matched clinical
202 information was available for 47 patients. Paired diagnosis and metastasis biopsy
203 tissue was collected from 7 patients, whilst metastatic tissue only was available from
204 2 patients. Antigen retrieval of formalin-fixed paraffin-embedded (FFPE) tumour
205 sections (4µm) and on-slide control paraffin-embedded cell line sections (HistoCyte
206 laboratories, UK) was performed using Ventana cell conditioning 1 reagent (Roche)
207 (100°C for 32 min).

208 Tissues were incubated with MT1-MMP catalytic domain antibody (2µg/ml,
209 MAB3328, clone LEM-2/15.8 – Merck Millipore, in Discovery antibody diluent
210 (Roche)) for 60 min at room temperature, prior to incubation for 30 min at room
211 temperature with UltraView Universal HRP Multimer (Ventana, USA).

212 Slides were scanned using the Aperio CS2 and analysed using Aperio ImageScope
213 x64 (both Leica Biosystems). The Aperio Membrane v9 algorithm (Leica Biosystems)

was used to detect membranous staining over the whole sample which was quantified using the H score[48] (see above).

2.5 Statistical analysis

Significance in difference of MT1-MMP expression between primary patient-derived cells was calculated using the one-way ANOVA test with Tukey's post-hoc analysis (Graphpad, Prism software), whilst differences in intensity of expression and the number of positive cells between populations within primaries were analysed using paired t-tests (SPSS, IBM).

Results were linked to clinical outcome data in R (R version 3.4.0). The prognostic value of MT1-MMP was evaluated using a univariate and multivariate Cox model, the optimal cut-point in the data being determined using the Harrell's C index[13,49]. The Cox model was then performed using the defined cut-point and Kaplan Meier (KM) plots generated using the Survminer package and ggplot.

3. Results

3.1 MT1-MMP is expressed in established ES cell lines

MT1-MMP expression at the RNA level was detected in 6/6 cell lines (Figure 1A), whilst expression of the 60kDa activated form of MT1-MMP protein was only observed in 5/6 ES cell lines (Figure 1B), with TTC-466 negative for MT1-MMP on western blotting and ICC (Figure 1C). Cell surface expression of MT1-MMP, detected by ICC, was decreased compared to total cellular MT1MMP expression (Figure 1B); all cell lines had an intensity score of 0 or 1+ and only TC-32 expressed MT1-MMP in more than 50% of cells. This suggests MT1-MMP appears to be predominantly expressed intracellularly in ES cell lines.

3.2 MT1-MMP RNA and protein expression in patient-derived ES cell cultures

MT1-MMP RNA (Figure 2A) and total cellular protein, using western blotting (Figure 2B), were detected in 15/15 and 23/23 patient-derived ES cell cultures respectively. Furthermore, cell surface MT1-MMP was detected using ICC in 20/21 cultures (Figure 2A, 2C), with a mean H-score of 158±16 (range=0-281) and mean of 87±6% (range=0-100) cells positive for MT1-MMP; the intensity of cell-surface expression was significantly higher than in established cell lines ($p=0.02$). Variation in the level of heterogeneity was displayed within individual primary cell cultures; in some cell cultures expression was homogenous (Figure 2D) whereas in other cultures, expression was heterogenous (Figure 2E) with different intensities of expression present within the same culture. There was no correlation between H-scores and western blotting ($R=-0.10$, $p=0.972$).

Five cultures derived from patients with a range of clinical characteristics were then examined by flow cytometry to further quantify surface expression and identify sub-populations with high expression. By flow cytometry, 87% (range 74-93%) of cells within the 5 primary cultures were positive for MT1-MMP (Figure 2A).

Heterogeneity of expression was assessed by comparing the level of expression detected by flow cytometry; expression of MT1-MMP was significantly more heterogeneous in CCRG1-L-017 and CCRG1-L-072 compared to CCRG1-L-003, and CCRG1-L-023 ($p<0.005$, Figure 2F). ICC and flow cytometry both demonstrate that MT1-MMP is expressed at the surface of many ES primary patient-derived cells (Figure 2A). Variation between RNA, total cell protein and surface expression implies differences between cultures in the processing and trafficking of MT1-MMP.

3.3 MT1-MMP is expressed in ES CSCs

After identifying that the majority of primary patient-derived cells express MT1-MMP at the cell surface, we sought to determine the level and pattern of expression within the driver ES-CSCs, to further determine MT1-MMPs suitability as a candidate therapeutic target. It had been possible to isolate and culture sufficient daughter ES-CSC from 2 parental patient-derived cell cultures. RNA sequencing of ES-CSCs revealed high expression of MT1-MMP in 7/7 cultures studied (Figure 3A).

MT1-MMP cell surface protein expression in the patient-derived ES-CSCs was confirmed by ICC. As seen with RNA sequencing, MT1-MMP was detected by ICC in 100% (7/7) of examined ES-CSC cultures, with 87% (range 14-100 ±12%) of ES-CSCs positive for MT1-MMP and a mean H-score of 219 (range 14-300±39.8) (Figure 3A). ES-CSCs derived from the CCRG1-L-017 parent culture demonstrated increased intensity of MT1-MMP surface expression ($p=0.001$) (Figure 3B). In contrast, there was a trend towards lower expression between CCRG1-L-023 and it's 3 daughter CSC cell populations ($p=0.19$) (Figure 3C), suggesting heterogeneity in MT1-MMP expression in ES-CSC cultures from different parental cell populations. Cell surface expression was further examined via flow cytometry of two ES-CSCs derived from the CCRG1-L-023 culture (CCRG1-L-023-01 and CCRG1-L-023-02). As suggested by ICC, flow cytometry confirmed lower cell surface expression of MT1-MMP in these ES-CSCs than the parent primary (CCRG1-L-023-01 vs CCRG1-L-023, $p=0.014$; CCRG1-L-023-02 vs CCRG1-L-023, $p=0.005$), although a higher percentage of cells expressed MT1-MMP (CCRG1-L-023-01, 94.7% vs parent, 89.2%, $p=0.003$; CCRG1-L-023-02, 94.5% vs parent, 89.2%, $p=0.007$) (Figure 3C). The IQRs of fluorescence intensity of ES-CSCs were significantly lower (CCRG1-L-023-01=3083, CCRG1-L-023-02=2491) than the parent (CCRG1-L-023=3331) (CCRG1-L-023 01 vs parent, $p=0.011$; -02 vs parent, $p<0.001$) suggesting increased

homogeneity in the intensity of expression by ES-CSCs when compared to the parent culture (Figure 3D).

Although intensity of expression does not appear to be consistently increased in ES-CSCs compared to their parent culture, MT1-MMP is still expressed at the surface of high numbers of ES-CSCs (87% by ICC, 95% using flow cytometry), suggesting MT1-MMP may be a viable therapeutic target given it is expressed on all cells, including the driver ES-CSCs.

3.4 MT1-MMP is expressed in ES tissues and predicts clinical outcome

To evaluate the prognostic potential of MT1-MMP, protein expression was examined in a cohort of treatment naïve ES taken at diagnosis (Supplementary Table S1) from a representative patient population (Table 1)[4,50]; metastasis at diagnosis was predictive of decreased EFS and OS (p=0.001, p=<0.001 respectively).

ICC of primary ES cultures demonstrated heterogeneous MT1-MMP expression between cells, IHC confirmed this heterogeneity (Figure 4A) and provided insight into the distribution of these cells within tissues. The level of MT1-MMP expression varied between samples, but high expressing cells were frequently grouped in pockets, with Figure 4A showing examples of such pockets in samples with predominantly low and medium levels of expression, whilst Figure 4B demonstrates more homogenous expression patterns.

Analysis of diagnosis tissues (n=47) revealed high MT1-MMP expression, defined as a H score >105, was associated with significantly decreased EFS (KM *p*=0.017, HR=2.63, HR*p*=0.0224) (Figure 5A) and OS (KM P=0.036, HR=2.37, HR*p*=0.0418) (Figure 5B). The independent prognostic value of MT1-MMP expression was then evaluated in a multivariate analysis including the following risk factors: pelvic/spinal

primary location, age, and the presence of metastases at diagnosis. High MT1-MMP expression was associated with reduced EFS and OS (HR=2.32, p=0.054 and HR=2.10, p=0.086 respectively), although this did not reach signficance (Figure 5C).

In keeping with the role of MT1-MMP as a driver of intravasation at the primary tumour leading to dissemination and metastasis, increased MT1-MMP expression was frequently noted in the perivascular region in diagnosis tissue and paired metastases, with the same pattern present in both tumours (Figure 4C). Other diagnosis tissue and paired metastases displayed a slightly different pattern, in which the primary showed perivascular invasion, whilst the metastases displayed pockets of high expression dispersed throughout the tissue, not perivascular in location (Figure 4D).

Of the 9 samples of metastatic tissue available, all expressed MT1-MMP (mean H-score=91.0, ±15.3 range 29-163), further emphasising MT1-MMPs potential as a therapeutic target. There was no difference in MT1-MMP expression between treatment-naïve diagnosis tissue and their paired metastasis, which had been exposed to chemotherapy (n=7, p=0.819).

4. Discussion

In the largest study to date, not only have we shown that MT1-MMP is expressed at the cell surface of ES cells consistent with previous studies[41-43], but we have demonstrated that high MT1-MMP expression is predictive of both reduced EFS ($p=0.001$) and OS ($p<0.001$). We have confirmed the expression of MT1-MMP in ES, using multiple methods to examine RNA and protein expression, in 6 established cell lines, 25 primary patient-derived cultures and 56 ES, as well as in 7 ES-CSC cultures. ES is thought to arise in mesenchymal stem cells (MSCs)[51-53], in which the controlled increases in expression of MT1-MMP during development[54] and it's crucial role in differentiation and migration[55-57] are well documented; as such it could be that ES inherently expresses high levels of MT1-MMP and is pre-programmed to be a stem-like cancer.

The most important finding is the identification of MT1-MMP as a candidate therapeutic target in ES. The high percentage of ES-CSCs expressing MT1-MMP, combined with the low levels of expression in normal tissues[58,59], make it an interesting therapeutic target that might be combined with contemporary chemotherapy to overcome the drug resistant metastatic disease. This might be achieved by using BT1718, a mertansine bi-cyclic peptide conjugate currently in phase IIa clinical trials with specific affinity to MT1-MMP[60-63], overcoming problems arising from cross-reactivity with other MMPs in previous targeted therapeutics[64,65]. MT1-MMP cleaves the peptide, releasing the drug; as most normal tissues do not express MT1-MMP, systemic exposure to the cytotoxic is limited, expanding the therapeutic window. High levels of the drug accumulate within the tumour, meaning the few cells not expressing MT1-MMP are also exposed. There is a clear need to eradicate the driver CSCs in ES[13]; it may be that the higher concentrations of

cytotoxic achieved within the tumour with such drugs may overcome some of the CSC's mechanisms of drug resistance, such as increased ATP-binding cassette transporters[66], killing ES-CSCs more effectively. Furthermore, we have shown that MT1-MMP is also expressed in metastatic ES; this is important given it is patients with distant failure who are offered early phase trials of novel therapeutics. Currently, patients need a H score of ≥150 for entry to the trial[67]; 19.1% of patients studied met this criteria, rising to 29.1% when excluding patients whose disease did not progress and hence would not have been eligible for the trial. MT1-MMP may also represent a viable option for targeted imaging for the same reasons, making it a viable target for 'theranostic' agents.

This study suggests that MT1-MMP may be predictive of both reduced OS and EFS. This is unsurprising given that high expression is associated with decreased survival in a large number of other cancers[39], including osteosarcoma[40]. It is reassuring that the other known risk factors reached significance, further suggesting that the dataset is representative. Going forward, it will be important to prospectively validate the prognostic power of MT1-MMP in a second cohort and interesting to examine whether expression of MT1-MMP can identify patients with localised disease who do badly. In the interim, it would also be beneficial to study MT1-MMP as a prognostic marker retrospectively in tissue obtained from clinical trial patients given they will have been homogenously treated and have clearly documented risk factors.

In this study, ES expression of MT1-MMP was heterogeneous as previously described[43]. By IHC, many tumours appeared to show increased MT1-MMP expression in the perivascular region in keeping with published preclinical models of invasive cancer cell mechanisms[68]. This phenomenon could represent several scenarios. As it is already established that MT1-MMP may play an important role in

dissemination of ES[35], this could represent part of the metastatic process. It is plausible that cells upregulate MT1-MMP to invade through blood vessel walls; the importance of invadopodia for transmigration has been demonstrated[69], as has importance of MT1-MMP for perivascular invasion in a glioblastoma mouse model[68]. It could also represent cells undergoing EMT prior to moving into the blood vessels as reported in other sarcomas[70], with MT1-MMP already being identified as a driver of this process in both embryogenesis[29] and cancer[30-33]. Although the high-expressing, perivascular cells appear Ewing-like in their morphology, without co-localisation studies and staining for CD99, it is impossible to confirm this, although the fact they were stained for CD99 during the diagnostic process is reassuring. Additionally, staining for vascular markers, such as CD31, is required to confirm that the vascular-like structures are actually blood vessels. It may be that, despite appearing ES-like, these high-expressing cells are actually endothelial cells, which are known to express MT1-MMP during angiogenesis[71,72]. This would still be a positive finding, increasing the understanding of the establishment of vasculature during tumorigenesis. Further study is clearly required to elucidate the role of MT1-MMP in ES migration and metastasis, as well as the interaction of tumour cells within the tumour microenvironment.

In this study, there were inconsistencies in the level of MT1-MMP expression detected across the different techniques used. This is likely secondary to intracellular regulation pathways which are not yet fully understood and require further study. This may in part be related to the causative fusion protein; caveolin-1 positively regulates MT1-MMP expression and is a direct transcriptional target of EWS-FLI1[35]. It could be theorised that ES tumours caused by alternative fusion proteins may therefore have lower MT1-MMP expression; this was not the case in our series

however, with the 3 lowest expressing ES tumours all caused by EWS-FLI1. Other possible explanations include post-transcriptional and epigenetic regulation, such as microRNA regulation as previously described[73]. As MT1-MMP is secreted as a pro-peptide[17], there could also be variation in the level of activation, reducing surface expression as detected via ICC and flow cytometry.

In conclusion, we propose that MT1-MMP is of significant potential as both a therapeutic target and predictor of outcome in ES. Patients with relapsed ES should be considered for clinical trials of MT1-MMP targeted therapeutics. A second, independent series should be conducted to validate MT1-MMP expression as a predictor of survival in ES.

5. Conflict of Interest Statement

There are no conflicts of interest to declare.

6. Acknowledgements

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7. Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Legends

Figure 1 MT1 MMP expression in established ES cell lines. *A) Summary of quantitative data; mean RNA reads detected by total RNA sequencing demonstrated MT1-MMP RNA expression in all cell lines, whilst ICC showed low expression of MT1-MMP in 3/3 cell lines examined B) Western blots demonstrate MT1-MMP expression in 6/6 ES established cell lines C) Representative ICC pictures demonstrating variable MT1-MMP expression in comparison to the controls used throughout. Black arrows show indicate positive cells.*

Figure 2 MT1-MMP expression in primary cell cultures *A) Summary of quantitative MT1-MMP expression data in primary patient-derived cell cultures – ICC detected expression of MT1-MMP in 20/21 primary cultures tested, whilst flow cytometry demonstrated expression in 5/5 primary cultures examined. All 15 primaries that underwent total RNA sequencing demonstrated high expression of MT1-MMP RNA. B) Western blots demonstrate MT1-MMP expression in 23/23 primary ES cultures. C) Representative ICC pictures demonstrating variable MT1-MMP expression levels, with representative images of each intensity. Arrows indicate representative cells. D) ICC pictures demonstrating both homogenous intensity of MT1-MMP expression, with arrows indicating cells of interest. E) ICC pictures demonstrating both heterogenous intensity of MT1-MMP expression, with arrows indicating cells of interest. F) Boxplot of the interquartile ranges of MT1-MMP expression as detected by flow cytometry; CCRG1-L-017 and CCRG1-L-072 were significantly more heterogenous than CCRG1-L-003 and CCRG1-L-023. CCRG1-L-020 was not performed in triplicate so statistics not performed. *=p<0.05, **=p<0.005, ***=p<0.001, ****=p<0.0001*

Figure 3 MT1-MMP expression in ES-CSCs. *A) Summary of quantitative MT1-MMP expression data in ES-CSCs and their parent primaries. High levels of MT1-MMP RNA expression was detected in all cultures examined with total RNA sequencing. B) Representative ICC pictures demonstrating MT1-MMP expression. D) Left graph shows the median fluorescence intensity of ES-CSCs compared to their parent cells– both s3 and s5 clones demonstrate significantly reduced expression compare to parent culture (p=0.014 and p=0.005 respectively); Middle graph shows the percentage of positive cells in ES-CSCs compared to their parent clone – both s3 and s5 clones contain significantly more positive cells (p=0.003 and p=0.007 respectively); Right graph is a boxplot of the interquartile ranges of MT1-MMP expression - 5366-02 s3 and s5 showed significantly less variation than the 5366-02 parent culture (p=0.011 and p<0.001 respectively). *=p<0.05, **=p<0.005, ***=p<0.001, ****=p<0.0001*

Figure 4 MT1-MMP expression in treatment naïve ES biopsy tissue. *A) Photomicrographs show examples of biopsies with low and high overall MT1-MMP expression, both demonstrating heterogenous expression. B) Photomicrographs of the tumours with both highest and lowest overall MT1-MMP expression, along with clinical outcome. C) Photomicrographs demonstrate a paired primary and metastasis from the same patient, with both appearing to show increased expression in the peri-vascular region. D) Photomicrographs show a paired primary and metastasis from the same patient, with the primary appearing to have increased peri-vascular expression whilst the metastasis has pockets of very high expression which do not appear to be peri-vascular in location.*

Figure 5 Survival analysis. *A) Kaplan Meier (KM) plot demonstrating high MT1-MMP expression is associated with decreased EFS (p=0.017). B) KM plot demonstrating high MT1-MMP expression is associated with decreased OS (p=0.036).The tables below the graphs demonstrate the number of patients present in each risk group at each time point. C) Table shows hazard*

ratios HR and p values of risk factors at both the univariate and multivariate levels for both EFS and OS, as calculated using Cox regression.

Table 1 Summary of patient clinical details *Table 1 displays the demographic information of the patient samples available for analysis as a percentage, with percentages quoted in the literature in the second column [4,50].*

Supplementary Table S1 Patient clinical details with the percentage of cells with an intensity score of 0, +1, +2 or +3.

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