Sains Malaysiana 53(6)(2024): 1243-1253
http://doi.org/10.17576/jsm-2024-5306-02

Matrix Metallopeptidase 3 Coding SNPs Suppress Cell Invasion in MCF7 Breast Cancer Cells

(Pengekodan Matriks Metalopeptidase 3 SNPs Menekan Pencerobohan Sel dalam Sel Kanser Payudara MCF7)

Shafinah Ahmad Suhaimi[1,4], Soon Choy Chan[2], Pei Pei Chong[3], Norazalina Saad[4,*], De Ming Chau[5] & Rozita Rosli[4]

[1]*Department of Biomedical Sciences, Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam 13200, Kepala Batas, Pulau Pinang, Malaysia*
[2]*School of Liberal Arts, Science and Technology, Perdana University, 50490 Kuala Lumpur, Malaysia*
[3]*School of Biosciences, Faculty of Health and Medical Sciences, Taylor's University, 47500 Subang Jaya, Selangor, Malaysia*
[4]*UPM-MAKNA Cancer Research Laboratory, Institute of Bioscience, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*
[5]*Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

Received: 17 April 2023/Accepted: 1 February 2024

ABSTRACT

Matrix metallopeptidase 3 (MMP3) is among the key players in breast cancer metastasis that contributes to the highest cancer-related deaths in females globally. Previously, *in silico* analyses had shown that several coding single nucleotide polymorphisms (SNPs) of *MMP3* were predicted to alter the secondary structures of *MMP3* and subsequently reduce its mRNA stability. To validate the mentioned hypotheses, this study aimed to determine the effects of six coding SNPs of *MMP3* on its mRNA stability, protein expression level as well as cell invasiveness *in vitro*. In this study, breast adenocarcinoma MCF7 cells were transfected with *MMP3* wild type (MMP3-WT) and a variant containing SNPs (MMP3-Var). Following transfection, protein expression level, mRNA stability and enzyme activity of MMP3-WT and MMP3-Var were evaluated. Finally, the effect of *MMP3* coding SNPs on cell invasiveness in breast cancer was determined. In this study, the mRNA stability, protein expression level and enzymatic activity of MMP3-Var were significantly reduced. Moreover, the presence of *MMP3* coding SNPs led to attenuated invasiveness of transfected MCF7 cells. In conclusion, these findings may contribute to the current understanding of these coding SNPs with metastasis in breast cancer.

Keywords: Carcinoma; *in vitro;* MMP3; mammary; metastasis; stromelysin-1

ABSTRAK

Matriks Metalopeptidase 3 (MMP3) adalah salah satu daripada pemain utama bagi metastasis kanser payudara yang menyumbang kepada kematian berkaitan kanser yang tertinggi dalam kalangan wanita di seluruh dunia. Sebelum ini, analisis *in silico* menunjukkan bahawa beberapa polimorfisme nukleotida tunggal (SNPs) pengekodan *MMP3* diramalkan untuk mengubah struktur sekunder *MMP3* dan seterusnya mengurangkan kestabilan mRNA. Bagi mengesahkan hipotesis tersebut, kajian ini bertujuan untuk mengenal pasti kesan *in vitro* enam SNPs pengekodan *MMP3* ke atas kestabilan mRNA, tahap pengekspresan protein dan kemansangan sel. Dalam kajian ini, sel karsinoma payudara MCF7 telah ditransfeksi dengan *MMP3* jenis liar (MMP3-WT) dan varian mengandungi SNPs (MMP3-Var). Selepas transfeksi, tahap pengekspresan protein, kestabilan mRNA serta aktiviti enzim bagi MMP3-WT dan MMP3-Var telah dinilai. Akhirnya, kesan SNPs pengekodan *MMP3* terhadap kemansangan sel kanser payudara telah ditentukan. Kestabilan mRNA, tahap pengekspresan protein dan aktiviti enzim MMP3-Var menurun dengan signifikan. Tambahan pula, SNPs pengekodan *MMP3* merencat kemansangan sel-sel MCF7 yang telah ditransfeksi. Kesimpulannya, hasil kajian ini boleh menyumbang kepada pemahaman semasa mengenai SNP pengekodan ini dengan metastasis dalam kanser payudara.

Kata kunci: Karsinoma; *in vitro*; mamari; MMP3; metastasis; stromelysin-1

Introduction

In Malaysia, breast cancer has the highest cancer mortality rate, with an age-standardised rate (ASR) of 18.4 per 100,000 (Bray et al. 2018). Despite an increased survival rate of breast cancer patients, metastasis remains as a strong nemesis that causes the treatments to be ineffective and non-targeted. Breast cancer metastasis is often characterized by increased cell invasion and migration due to modification of the extracellular matrix (ECM) which is mainly regulated by the members of matrix metallopeptidases (MMPs) (Li, Pritchard & Yu 2022; Raeeszadeh-Sarmazdeh, Do & Hritz 2020). MMP3 is a zinc-dependent proteolytic enzyme that digests various ECM molecules including collagen, elastin, fibronectin, and laminin. *In vitro*, *in vivo* and clinical studies have shown that MMP3 was significantly overexpressed in breast cancer and was linked to poor prognosis as well as metastasis of breast cancer (Maiti et al. 2021; Nagase, Visse & Murphy 2006). Not only that, upregulated MMP3 was associated with loss of E-cadherin and increased cell migration and invasion in signet-ring cell carcinoma (SRCC) clinical samples (Yamaguchi et al. 2022). The reciprocal interaction between MMP3 and E-cadherin regulates the hallmarks of cancer metastasis such as angiogenesis, migration, invasion as well as epithelial-mesenchymal transition. Due to its key role in cancer progression, recent studies have focused on developing chemical compounds for MMP-3 inhibition and multiple therapy involving oncolytic virus-mediated knock down of MMP3 (Almutairi et al. 2023; Chin et al. 2021; Liang et al. 2021). However, these had only been tested in *in vitro* and *in vivo* cancer models.

MMP3 expression can be regulated transcriptionally with the presence of SNPs that can regulate gene and protein expressions by altering mRNA stability, mRNA structure and translational efficiency (Gebert et al. 2020; Li & Chen 2023; Sampieri, León-Córdoba & Remes-Troche 2013). These alterations can lead to structural and functional changes that influence susceptibility and progression to disease, as well as the phenotypic expression of the disease (Kotnis, Sarin & Mulherkar 2005). Therefore, the relationship between *MMP3* SNPs, particularly in the promoter regions, and susceptibility to breast cancer has been investigated. For example, the 5A/6A SNP (rs3025058) at *MMP3* promoter has been associated with risk of developing breast cancer as well as metastasis and patient survival in various populations (Suhaimi, Chan & Rosli 2020). In particular, the 6A allele reduced the promoter activity and subsequently the gene expression of *MMP3*. In addition, *in silico* analyses showed that *MMP3-707 A>G* (rs522616) and *MMP3-375 C>G* (rs617819) SNPs at the promoter regions enhanced the promoter activity which led to an increased expression of *MMP3* (Banik et al. 2022).

Likewise, even though they are less commonly studied, the SNPs in the coding regions may also contribute to the regulation of MMP3 expression. In fact, Chan (2013) discovered that out of 15 identified SNPs of *MMP3* in Malaysian breast cancer patients, further *in silico* analysis discovered that 6 coding SNPs may cause structural and functional effects to MMP3. The reported SNPs which are c.1164C>T, c.1200A>G, c.133A>G, c.288T>C, c.306C>G and c.*129T>C, were predicted to change the secondary structure of *MMP3*. The alteration is expected to reduce mRNA stability of *MMP3*, which leads to lowered protein expression level of MMP3 that will in turn functionally attenuate the invasiveness of breast cancer. However, the findings of *in silico* analyses need to be validated via actual experiments such as the *in vitro* approach. Hence, the objective of this study was to determine the functional effects of multiple coding SNPs of *MMP3* on mRNA stability and protein expression level of MMP3 as well as on cell invasiveness in MCF7 breast cancer cell line.

Materials and Methods

Materials

Two plasmid vector constructs (MMP3-WT_pcDNA and MMP3-Var_pcDNA) were previously designed and synthesized by Life Technologies (Singapore) according to the gene bank sequence of *MMP3* mRNA (NM_002422.5) (Figure 1). The sequence variation between MMP3-WT_pcDNA and MMP3-Var_pcDNA at each SNP position is included in Table 1. The SNPs that are present in MMP3-Var_pcDNA were chosen based on the *in silico* analysis conducted by Chan (2013) to predict SNPs that might alter the mRNA folding and structure of *MMP3* using the Mfold software. The cell line used in this study was MCF7 breast adenocarcinoma cell line.

Amplification and Extraction of Plasmid Constructs

Glycerol stocks of competent *Escherichia coli* JM109 cells transformed with the plasmid constructs were grown on Luria-Bertani (LB) agar (Sigma-Aldrich, Germany) containing 50 µg/mL ampicillin (Nacalai Tesque, Japan) overnight at 37 °C. Next, single colonies from the agar were incubated overnight in LB (Miller) broth (Sigma-Aldrich, Germany) containing 50 µg/mL ampicillin. The

E. coli broth cultures were then subjected to plasmid DNA extraction using Presto Endotoxin Free Mini Plasmid Kit as described in the manufacturer's protocol (Geneaid Biotech, Taiwan). The extracted plasmid DNA constructs were qualitatively validated by restriction enzyme digest, based on the size of the plasmid DNA. Plasmid DNA containing MMP3-WT and MMP3-Var were digested with *Not*I and *Sac*I restriction enzymes (Thermo Fisher Scientific, USA) according to the manufacturer's protocol with incubation of the reactions at 37 °C for 30 min. The digested samples were run on 0.7% agarose gels (Vivantis, USA) for 45 min at 75 V voltage and viewed under the ultraviolet light (Gel Doc XR+, Bio-rad, USA).

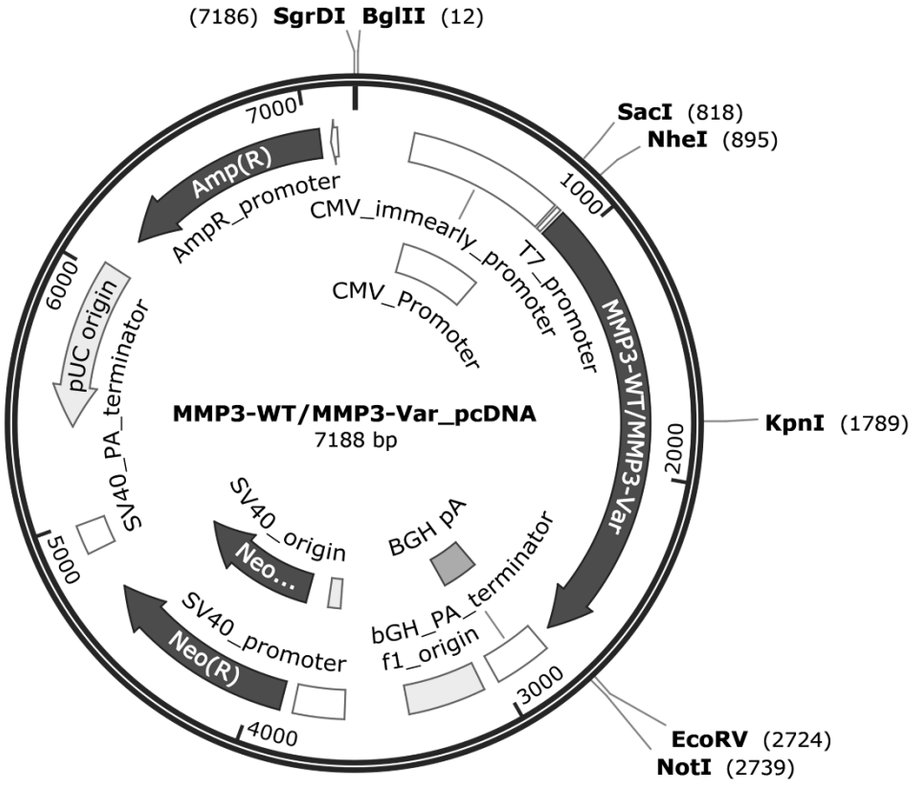

FIGURE 1. Plasmid Map of MMP3-WT/MMP3-Var_pcDNA (7188 bp). The synthetic gene of *MMP3* (1833 bp) was cloned into pcDNA 3.1(+) A009 using *Nhe*I and *Eco*RV cloning sites

TABLE 1. Sequence variation of *MMP3* at its respective SNP position. The c. abbreviation refers to the coding regions of *MMP3* whereas the asterisk in c.*129 T>C polymorphism represents the position of nucleotide 3' of the 3' untranslated region (UTR)

SNP	NCBI SNP ID	Position	Wild Type (MMP3-WT)	Variant (MMP3-Var)
c.133 A>G	rs679620	Exon 2	A	G
c.288 T>C	rs602128	Exon 2	T	C
c.306 C>G	rs41380244	Exon 2	C	G
c.1164 C>T	ss244234690	Exon 8	C	T
c.1200 A>G	ss244234692	Exon 8	A	G
c.*129 T>C	ss244234694	3' UTR	T	C

STABLE TRANSFECTION

MCF7 cells were plated in a 24-well plate with cell density of 5×10^4 cells per well and incubated overnight at 37 °C in a 5% CO_2 and humidified incubator. The cells were then transfected with the extracted plasmid DNA constructs using jetPRIME® *in vitro* DNA transfection reagent (Polyplus-transfection®, France) and in accordance with the manufacturer's protocol. MCF7 cells that were mock transfected with the transfection reagent serve as the negative control. After replacing the media with fresh media containing 0.8 mg/mL G418 antibiotic (Nacalai Tesque, Japan) for every 2-3 days, the transfected cells were selected and expanded after all the mock transfected cells were dead. Cell transfection of MMP3-Var in the MCF7 cells was confirmed by DNA sequencing.

DNA SEQUENCING

Prior to DNA sequencing, total RNA extraction of transfected and parental MCF7 cells was performed using FavorPrep™ Tissue Total RNA Mini Kit (Favorgen, Taiwan) as described in the manufacturer's protocol. The extracted RNA was then converted to complementary DNA (cDNA) with ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) according to the manufacturer's instructions. The synthesized cDNA was then subjected to polymerase chain reaction (PCR) and subsequently DNA sequencing. The primer sequences for Amplicon 1 (forward 5'- GGCAAGACAGCAAGGCATAG-3'and reverse 5'-AGGTCCATAG AGGGACTGAATG-3') and Amplicon 2 (forward 5'-ATGATGATGAACAATGGACAAAGG-3' and reverse 5'- TCACTTGTCTGTTGCACACG-3') of *MMP3* were designed and synthesized by 1st BASE (Singapore). PCR amplification was performed with exTEN 2x PCR Master Mix (1st BASE, Singapore) according to the following PCR cycling conditions: (1) 3 min at 95 °C, (2) 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 60-90 s, and (3) 72 °C for 10 min (Eppendorf Mastercycler® Gradient, Germany). The PCR products were sent to 1st BASE Laboratories (Malaysia) for DNA sequencing using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA). The obtained nucleotide sequences were aligned to MMP3-Var sequence for similarities using the BLASTN program (National Institute of Health, USA). The presence of MMP3-WT in MCF7 cells was confirmed qualitatively by the presence of amplicons during electrophoresis.

mRNA STABILITY OF MMP3

This assay was conducted in accordance with Ayupe and Reis (2017) and Li et al. (2019) with modifications. MCF7 transfected cells were plated in a 6-well plate with cell density of 3×10^5 per well and grown overnight at 37 °C in a 5% CO_2 and humidified incubator. Next, the cells were treated with 5 µg/mL actinomycin D (MP Biomedicals, USA) and total RNA was extracted at timepoints of 0, 3, 6, 9, and 12 h and converted to cDNA as described earlier. The synthesized cDNA was subjected to RT-qPCR on CFX96™ Real Time PCR Detection System (Bio-Rad, USA) as per manufacturer's protocol of Quantinova™ SYBR® Green PCR Kit (Qiagen, Germany). The SYBR Green primers for *MMP3* (forward 5'-CAGGCTTTCCCAAGCAAATAG-3' and reverse 5'-CCAACTGTGAAGATCCAGTAAAG-3') were designed and synthesized by 1st BASE, Singapore. The experiments were performed in triplicates. At each time point, the Cq values of *MMP3* were normalized to the Cq values of *MMP3* at t=0 h (ΔCq). The relative expression of *MMP3* was calculated as $2^{-\Delta Cq}$ in which the expression level at 0 h was set at 1. Based on the plotted graphs of normalized mRNA expression of *MMP3* against duration of actinomycin D treatments (hour), the half-lives of *MMP3* mRNA were determined based on a one-phase exponential decay model using GraphPad Prism version 7 (GraphPad Software, USA).

PROTEIN EXPRESSION LEVEL OF MMP3

Total protein concentrations of the transfected cell lysates were determined with the bicinchoninic acid (BCA) assay kit (Nacalai Tesque, Japan) as per manufacturer's protocol. Next, 20 µg of denatured protein samples were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% Mini-PROTEAN TGX precast protein gels (Bio-Rad, USA) for 95 min at 100 V. The protein samples on the gel were transferred to polyvinylidene difluoride (PVDF) membranes using a semi dry method (Trans-Blot® Turbo, Bio-Rad, USA) based on the manufacturer's instructions and blocked for an hour at room temperature. Next, the membranes were incubated with primary antibodies of β-actin (rabbit monoclonal, 1:1000 dilution, Cell Signaling Technology, USA) or MMP3 (rabbit polyclonal, 1:2000 dilution, Cusabio Technology, USA) overnight at 4°C with gentle agitation. Afterwards, the membranes were incubated with horse radish peroxidase (HRP)-linked anti-rabbit IgG antibody (1:1000 dilution, Cell Signaling Technology, USA) for 2 h

at room temperature with gentle agitation. After washing, for chemiluminescence detection, the membranes were incubated with WesternBright ECL chemiluminescence reagent (Advansta, USA) for 2 min and visualized using a FluorChem E System (ProteinSimple, USA). The densitometry analysis of MMP3 and β-actin bands was performed using ImageJ software (Schneider, Rasband & Eliceiri 2012). The normalized MMP3 protein expression levels were reported as fold change relative to MMP3-WT. The experiments were performed in triplicates.

ENZYMATIC ACTIVITY OF MMP3

This assay was conducted using MMP3 Activity Fluorometric Assay Kit (Sigma-Aldrich, USA). The experiments were performed in triplicates. MCF7 parental and transfected cells were seeded in 6-well plates at a cell density of 3×10^5 cells per well and grown for 24 h at 37 °C in a 5% CO_2 and humidified incubator. Subsequently the cells were serum-starved overnight, collected, centrifuged for 5 min at 1000 × g and resuspended in ice-cold MMP3 Assay Buffer. The cell lysates were then proceeded to the assay as per manufacturer's instructions. The ΔRFU values (RFU(T_1) – RFU (T_0)) for different concentrations of the MCA standard were plotted, and the enzymatic activity (mU/mL) of MMP3 in MCF7 cells was calculated.

CELL INVASION OF MCF7 PARENTAL AND TRANSFECTED CELLS

This assay was conducted as described by Pijuan et al. (2019) with several modifications. Prior to cells plating, MCF7 parental and transfected cells were serum starved overnight. Next, in 24-well plates, pre-chilled polycarbonate Transwell inserts with 8 µm pore size and 6.5 mm membrane diameter (Corning, USA) were coated with 100 µL of 3 mg/mL Matrigel Basement Membrane Matrix (BD Biosciences, USA) per well. The plates were then incubated in 5% CO_2 incubator at 37 °C for 4 h. After 4 h, 100 µL of cell suspension containing 7×10^4 of cells were seeded on the Matrigel-coated inserts. RPMI 1640 media containing 10% FBS, which act as chemoattractant, were subsequently added to the well plate and the cells were incubated overnight at 37 °C in a 5% CO_2 and humidified incubator. For the migration assay, cells were seeded onto inserts without the Matrigel coating, whereas as negative control, chemoattractant was not added to the well plate. After incubation, the cells were fixed with freezer-cold 100% methanol for 15 min at room temperature and stained with 0.5% crystal violet (Sigma-Aldrich, Germany) for 10 min at room temperature. The images of cells were then captured using an inverted microscope and the inserts were submerged in 100% methanol for 20 min at room temperature for elution of crystal violet. The absorbance values of the samples were measured at 570 nm and normalized to the absorbance values of the negative control (without chemoattractant) (Feoktistova, Geserick & Leverkus 2016). The experiments were performed in triplicates.

STATISTICAL ANALYSES

The statistical analyses were performed using SPSS Statistics 22 (IBM, USA). Relative protein expression levels as well as mRNA half-lives of MMP3 were subjected to independent samples *t* test. Meanwhile, enzymatic activity of MMP3 and cell invasion of MCF7 cells were analyzed using one-way analysis of variance (ANOVA) and Tukey's post hoc tests. These tests were conducted with the assumptions that there was homogeneity of variance and normal distribution of data. The difference between the samples were considered as statistically significant when p-value < 0.05.

RESULTS AND DISCUSSION

CONFIRMATION OF MMP3 TRANSFECTION INTO MCF7 CELL LINE

In the Malaysian breast cancer patients, several coding SNPs of *MMP3* were found to alter its mRNA structure and reduce its mRNA stability based on *in silico* analysis. Hence, this study aimed to determine the functional effects of these *MMP3* coding SNPs on invasiveness of breast cancer *in vitro*. Single digest of *MMP3*-containing plasmid constructs with *Not*I resulted in the presence of a single band with the length of approximately 7200 bp (Figure 2(A)). Restriction enzyme double digests of MMP3-WT and MMP3-Var plasmid constructs produced two DNA fragments with the length of 5200 bp and 1900 bp (Figure 2(A)). In this study, breast adenocarcinoma MCF7 cell line was selected as the endogenous expression of *MMP3* was reported to be absent (Figueira et al. 2009; Kousidou et al. 2004), which was also observed in this study (Figure 2(B)). After transfection, following agarose gel electrophoresis of *MMP3* amplicons, MMP3-WT and MMP3-Var were present in MCF7 cell line with predicted amplicon sizes of 847 bp (Amplicon 1, A1) and 1111 bp (Amplicon 2, A2) (Figure 2(B)). After

confirming the presence of MMP3-WT and MMP3-Var in the transfected cells via gel electrophoresis, the amplicons or PCR products of MMP3-Var were subjected to DNA sequencing. All coding SNPs of *MMP3*, which are c.133 A>G, c.288 T>C, c.306 C>G, c.1164 C>T, c.1200 A>G and c.*129 C>T, were detected in the transfected MCF7 cells (Figure 2(C)). The first three mentioned SNPs (c.133 A>G, c.288 T>C, c.306 C>G) are present in Amplicon 1 (A1) of *MMP3* whereas the latter three SNPs (, c.1164 C>T, c.1200 A>G, c.*129 C>T) are present in Amplicon 2 (A2) of *MMP3*.

mRNA STABILITY OF MMP3

For the mRNA stability of *MMP3*, the mRNA half-lives of MMP3-WT and MMP3-Var were 6.45 ± 1.10 h and 3.07 ± 0.15 h, respectively (Figure 3(A) and 3(B)). The mRNA half-life of MMP3-Var was significantly lower than MMP3-WT ($p < 0.05$). The reduced mRNA stability was predicted to reduce protein expression in MMP3-Var transfected MCF7 cells, which was determined in this study.

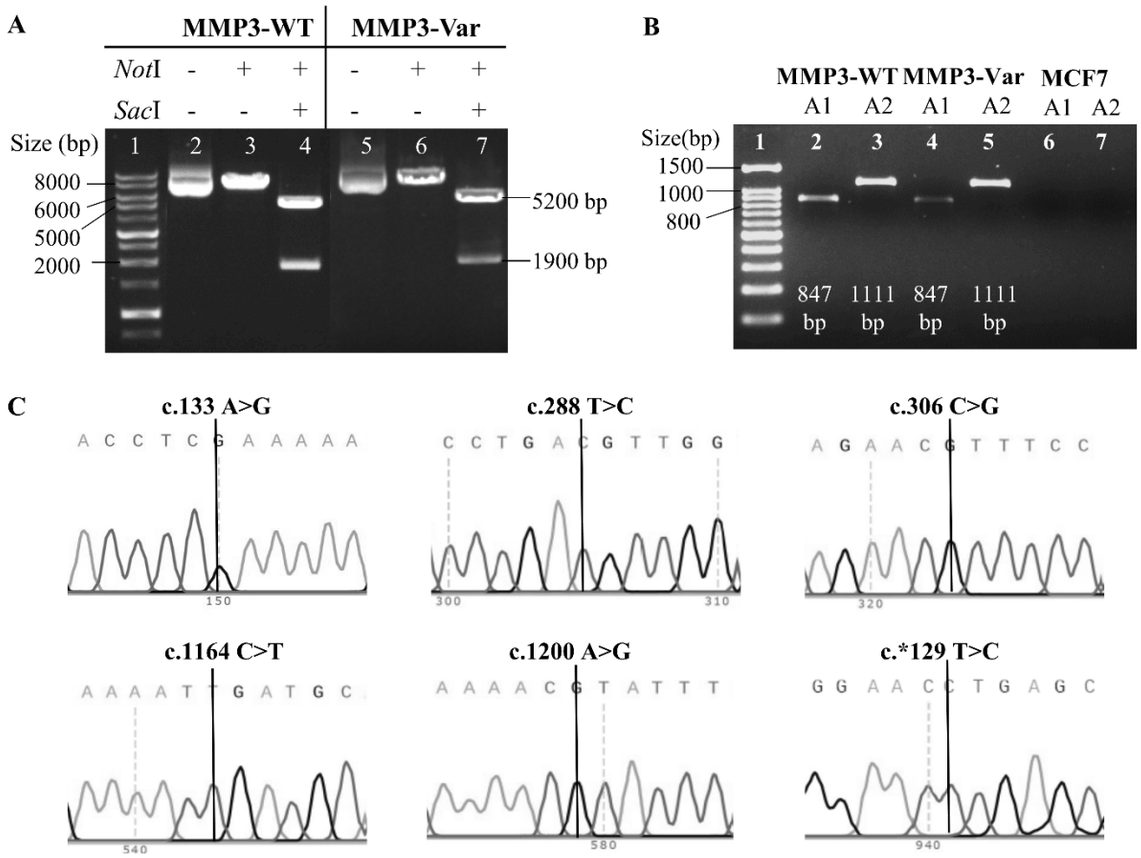

FIGURE 2. Transfection of *MMP3* plasmid constructs in MCF7 breast cancer cells. A: The restriction digest analysis of the plasmids by agarose gel electrophoresis. Lane 1: 1 kb DNA marker; lanes 2-4: undigested, single digest and double digest forms of MMP3-WT_pcDNA; lanes 5-7: undigested, single digest and double digest of MMP3-Var_pcDNA. The single digest was performed using *Not*I whereas the double digest used *Not*I and *Sac*I restriction enzymes. B: Confirmation of transfection of MMP3-WT and MMP3-Var in MCF7 cells based on *MMP3* amplification. Lane 1: 100 bp DNA marker; lanes 2-3: MMP3-WT amplicons; lanes 4-5: MMP3-Var amplicons; lanes 6-7: *MMP3* amplicons in MCF7 parental cells. A1 and A2 refer to *MMP3* amplicons with lengths of 847 bp and 1111 bp, respectively. C: Confirmation of MMP3-Var transfection based on DNA sequencing chromatogram. A total of 6 SNPs were detected in which c.133 A>G, c.288 T>C and c.306 C>G were present in amplicon 1 of *MMP3* (A1) whereas c.1164 C>T, c.1200 A>G and c.*129 C>T were detected in amplicon 2 of *MMP3* (A2). The asterisk in c.*129 C>T denotes the position of nucleotide 3' of the 3' untranslated region (UTR)

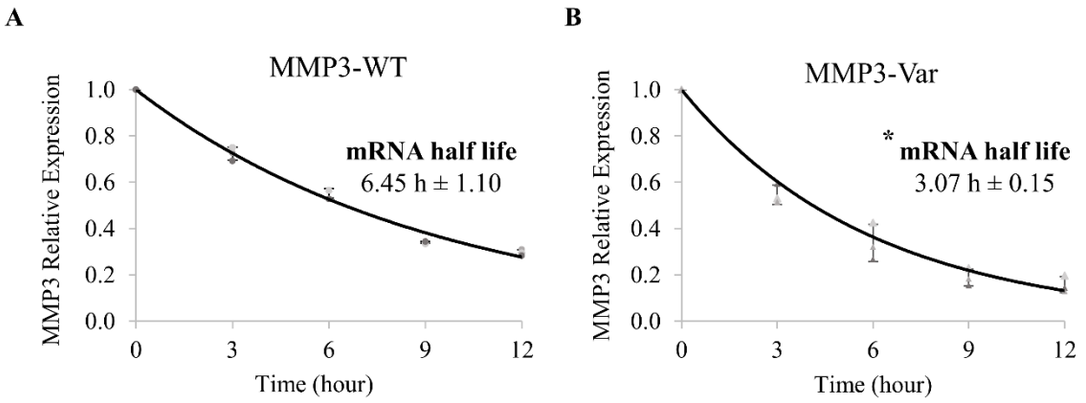

FIGURE 3. mRNA stability of (A) MMP3-WT and (B) MMP3-Var in MCF7 transfected cells. *MMP3* expression at 0 h of actinomycin D treatment was set at 1. The mRNA half-lives of *MMP3*, which were calculated from the decay curves, were displayed as mean ± standard deviation (SD). The mRNA half-life of MMP3-Var was significantly lower than the mRNA half-life of MMP3-WT, as represented by the asterisk (*) ($p < 0.05$)

PROTEIN EXPRESSION AND ENZYMATIC ACTIVITY OF MMP3

In this study, endogenous protein expression of MMP3 was absent in the MCF7 parental cell line, whereas MMP3 was upregulated in both transfected cells (Figure 4(A)). In particular, the protein expression level of MMP3 in cells transfected with MMP3-Var was significantly lower than the wild type, with 0.859 ± 0.046-fold change (Figure 4(B)). In this study, only 35-kDa active MMP3 was present in MCF7 transfected cells. MMP3 is produced and secreted as a 54-kDa proenzyme. It is activated extracellularly when the signal and propeptide domains are removed, resulting in a 45-kDA active MMP3 (Figure 4(C)). In this study, there was no presence of the 56-kDa proenzyme form, suggesting that all or most MMP3 were in active form (Figure 4(C)). Surprisingly, the 45-kDA active MMP3 was also not detected. Instead, there were immunoreactive bands that corresponded to a 35-kDa protein. Even though the 35-kDa form of MMP3 was not commonly reported, it was detected in HepG2 human hepatic carcinoma cells and human liver tissues (Si-Tayeb et al. 2006). Interestingly, it has only a part of the hemopexin domain (Figure 4(C)) and was only detected in the nuclei.

The smaller form of active MMP3 may be produced as a result of alternative splicing of pro-MMP3 transcript, alternative promoter usage or intracellular cleavage of pro-MMP3 (Si-Tayeb et al. 2006). Nuclear localization of MMP3 was reported to enhance genetic and epigenetic defects that may promote tumorigenesis by inducing apoptosis. Thus, the downregulated protein expression of the nuclear MMP3 by the coding SNPs might have led to inhibition of apoptosis that subsequently prevents tumorigenesis.

In addition, it has also decreased enzymatic activity of MMP3, as shown in Figure 4(D). For the enzymatic activity of MMP3 in MCF7 cells, MCF7 parental cells had the lowest MMP3 activity of 0.216 ± 0.032 mU/mL (Figure 4(D)). MMP3 wild type had the highest enzymatic activity of 0.855 ± 0.046 mU/mL. Meanwhile, the enzymatic activity of MMP3-Var was 0.466 ± 0.050 mU/mL. It is worth noting that enzymatic activity of MMP3-Var was significantly lower than MMP3-WT. Both MMP3-WT and MMP3-Var had significantly higher activity of MMP3 than MCF7 parental cells. These also confirm that even though only a part of the hemopexin domain is intact in the 35-kDa active MMP3, it is still able to exhibit its proteolytic activity as the catalytic domain still remains. This finding was also discovered by Si-Tayeb et al. (2006) in which the 35-kDa active MMP3 had its proteolytic activity during casein zymography.

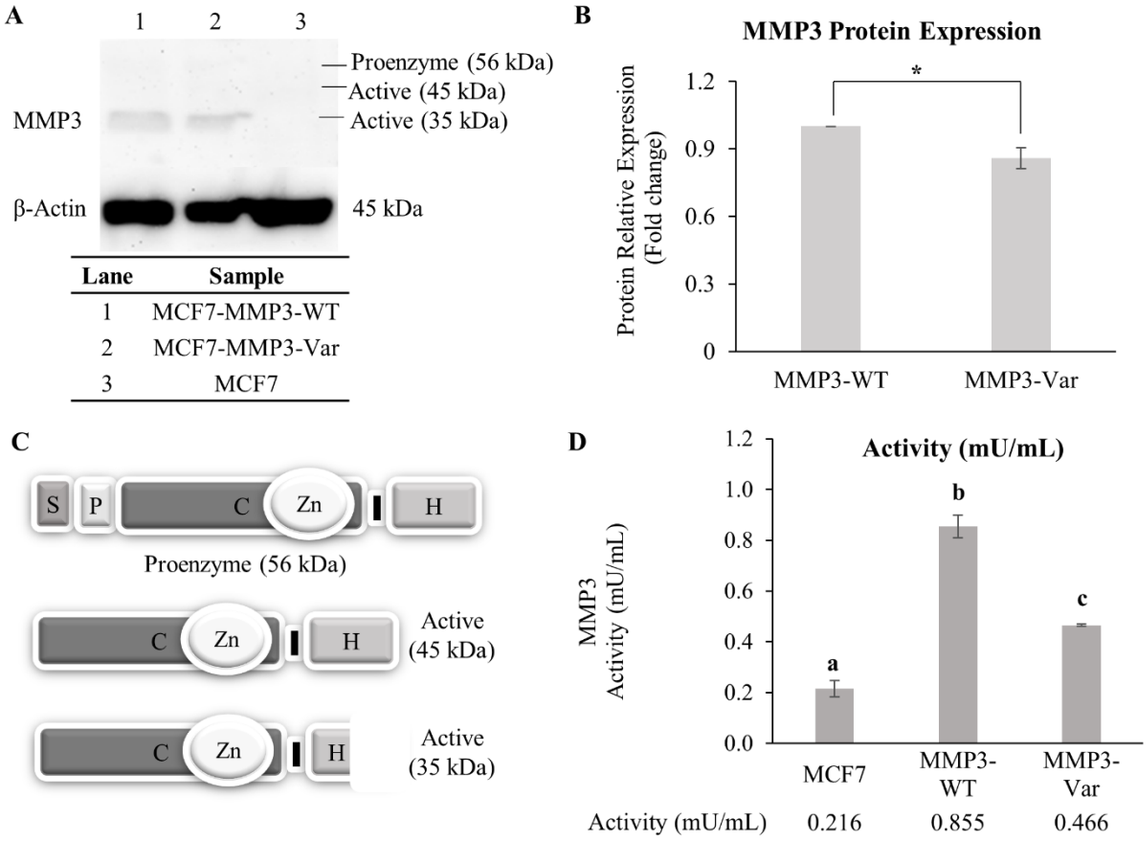

FIGURE 4. The protein expression and enzymatic activity of MMP3. A: Protein expression of MMP3 in MCF7 cells. Lanes 1-2: MCF7 cells transfected with MMP3-WT and MMP3-Var, respectively; lane 3: MCF7 parental cells. β-actin (45 kDa) serves as a loading control. B: Relative protein expression level of MMP3 by fold change. MMP3 band was normalized to β-actin and was relatively quantitated to MMP3-WT. Protein expression of MMP3-Var was significantly lower than MMP3-WT, as denoted by the asterisk (*) ($p < 0.05$). C: Enzymatic structures of pro-MMP3 (56 kDa) and active MMP3 (45 kDa and 35 kDa). S=Signal domain, P=Propeptide domain, C=Catalytic domain, H=Hemopexin domain. D: Enzymatic activity of MMP3 (mU/mL) in MCF7 parental and transfected cells. The MMP3 activity was calculated based on the changes of RFU values after 1 h of incubation with the substrate. Statistical differences between parental cells (MCF7), MMP3-WT and MMP3-Var transfected cells, which are represented by different letters (a-c), were significant when $p < 0.05$, n = 3

THE EFFECT OF MMP3 CODING SNPs ON CELL INVASIVENESS IN MCF7 BREAST CANCER CELLS

In a previous study involving Malaysian breast cancer patients, it was predicted that the presence of these coding SNPs will reduce the MMP3 expression level since several coding SNPs were found to confer a protective effect against breast cancer metastasis (Chan 2013). The ability of cancer cells to cross the basement membrane and invade secondary organs is one of the hallmarks of metastasis (Welch & Hurst 2019). Therefore, in the final part of this study, the effects of these coding SNPs on the invasiveness of breast cancer were investigated. In the Transwell invasion assay, the term 'invasion' is often used to describe migration of cells across basement membrane or ECM coatings whereas the term 'migration' describes the migration of cells across the Transwell insert without the presence of any ECM molecule. The MCF7 parental cells had the highest migration rate, with absorbance value of 0.4049 ± 0.010 (Figure 5(A)). Meanwhile, the effects of MMP3-WT and

MMP3-Var on the migration rate of MCF7 cells were quite similar with normalized absorbance values of 0.318 ± 0.009 and 0.282 ± 0.037, respectively. There was no statistically significant difference in migration rates of both transfected cells. In contrast, MCF7 parental cells had the lowest invasion rate with normalized absorbance value of 0.049 ± 0.003 (Figure 5(B)). Both MCF7 cells transfected with MMP3-WT and MMP3-Var had higher invasion rate than the parental cells, which were 0.140 ± 0.008 and 0.075 ± 0.007, respectively (Figure 5(B) and 5(C)). Moreover, compared to the wild type, MMP3-Var significantly reduced invasion rate of MCF7 cells.

In this study, cell invasion only occurred in MCF7 cells expressing MMP3 (Figure 5(C)). Moreover, the transfected cells had higher invasion but lower migration rates in comparison to the parental cells (Figure 5(A) and 5(B)). This suggests the role of tumor micro-environment in influencing the mode of migrations used by cancer cells between amoeboid-like migration which does not involve proteolysis of ECM proteins and proteolytic-dependent migration (Wu et al. 2021). In this study, the transfected cells were able to transition smoothly to proteolytic-dependent migration during cell invasion perhaps due to overexpression of MMP3. Moreover, MMP3-Var transfected MCF7 cells have significantly reduced invasive potential compared to MMP3-WT transfected MCF7 cells. It is possible that the presence of c.288 T>C and c.133 A>G SNPs may be involved in reducing cancer cell invasion as it was previously reported that distant and lymph node metastases were less likely to be developed in breast cancer patients with these SNPs (Chan 2013). In order for distant and lymph node metastases to take place, cancer cells need to migrate to lymph node and distant sites by modifying and degrading the basement membrane. This was observed in the invasion of MMP3-WT transfected cells and to a much lesser extent, MMP3-Var transfected cells.

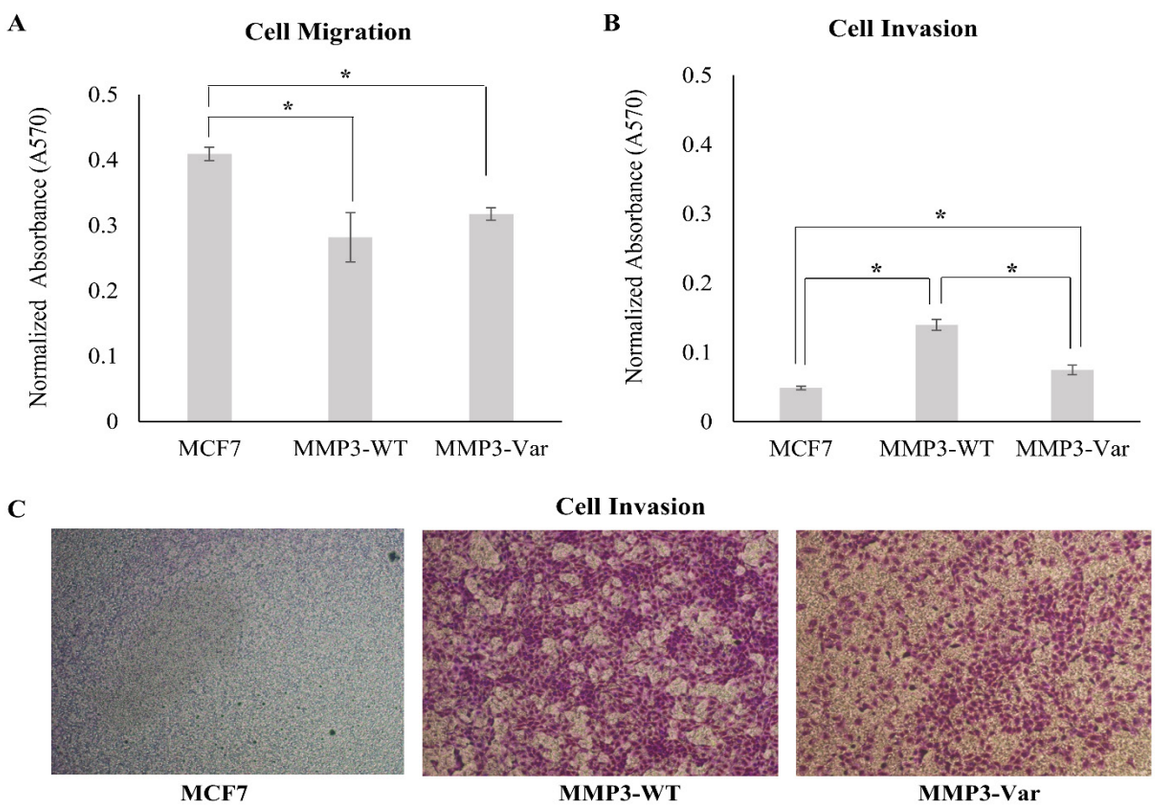

FIGURE 5. The effect of *MMP3* SNPs on cell invasiveness in MCF7 breast cancer cells. A-B: (A) Cell migration and (B) cell invasion of MCF7 parental and transfected cells. Cell migration and invasion assays were conducted similarly with the presence of chemoattractant (10% FBS) except that Matrigel (3 mg/mL) coating was present on the Transwell inserts in (B). The absorbance values at 570 nm were normalized with the absorbance values of samples without the presence of chemoattractant. The asterisks denote statistically significant differences between the samples ($p < 0.05$). C: Microscopic images of invasive MCF7 cells with the presence of chemoattractant (10% FBS) at 40x magnification. The violet stained cells indicate invasive cells

However, in this study, as the effects of MMP3 SNPs were only investigated on mRNA stability, protein expression and enzymatic activity of MMP3 as well as cell invasion, their effects against breast cancer metastasis were not extensively studied. Therefore, to fully validate the predicted effects of MMP3 SNPs on breast cancer metastasis, an extensive analysis involving components of tumor micro-environment including cadherins and integrins as well as other hallmarks of metastasis such as epithelial-mesenchymal transition (EMT) and angiogenesis should be performed.

Conclusions

In this study, *in vitro* effects of *MMP3* coding SNPs on cell invasiveness in MCF7 breast cancer cells were determined. The coding SNPs exerted its effects that resulted in reduced mRNA stability, protein expression level and enzymatic activity of MMP3. This led to suppression of cell invasion as observed in MMP3-Var transfected MCF7 cells, as opposed to the enhanced cell invasion by MMP3 wild type. In conclusion, this *in vitro* study successfully confirmed the hypothesized effects of *MMP3* coding SNPs in breast cancer, which may provide a better understanding of their association with breast cancer metastasis.

Acknowledgements

The authors would like to thank Universiti Putra Malaysia (UPM) for funding this study under the Putra Grant (Grant number: GP-IPS/2008/9622300). The authors would also like to acknowledge the Ministry of Education, Malaysia and Universiti Sains Malaysia (USM) for providing a scholarship to Shafinah Ahmad Suhaimi during this study.

References

Almutairi, S., Kalloush, H.M.d., Manoon, N.A. & Bardaweel, S.K. 2023. Matrix metalloproteinases inhibitors in cancer treatment: An updated review. *Molecules* 28: 5567.

Ayupe, A.C. & Reis, E.M. 2017. Evaluating the stability of mRNAs and noncoding RNAs. In *Enhancer RNAs. Methods in Molecular Biology*, edited by Ørom, U. New York: Humana Press. pp. 139-153.

Banik, P., Majumder, R., Mandal, A., Dey, S. & Mandal, M. 2022. A computational study to assess the polymorphic landscape of matrix metalloproteinase 3 promoter and its effects on transcriptional activity. *Computers in Biology and Medicine* 145: 105404.

Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A. & Jemal, A. 2018. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians* 68: 394-424.

Chan, S.C. 2013. Identification and analysis of single nucleotide polymorphisms in matrix metallopeptidase 2 and 3 genes in Malaysian breast cancer patients. PhD Thesis, Universiti Putra Malaysia (Unpublished).

Chin, L.T., Liu, K.W., Chen, Y.H., Hsu, S.C. & Huang, L. 2021. Cell-based assays and molecular simulation reveal that the anti-cancer harmine is a specific matrix metalloproteinase-3 (MMP-3) inhibitor. *Computational Biology & Chemistry* 94: 107556.

Feoktistova, M., Geserick, P. & Leverkus, M. 2016. Crystal violet assay for determining viability of cultured cells. *Cold Spring Harbor Protocols* 2016:pdb.prot087379.

Figueira, R.C., Gomes, L.R., Neto, J.S., Silva, F.C., Silva, I.D.C.G. & Sogayar, M.C. 2009. Correlation between MMPs and their inhibitors in breast cancer tumor tissue specimens and in cell lines with different metastatic potential. *BMC Cancer* 9: 20.

Gebert, M., Jaskiewicz, M., Moszynska, A., Collawn, J.F. & Bartoszewski, R. 2020. The effects of single nucleotide polymorphisms in cancer RNAi therapies. *Cancers* 12: 3119.

Kotnis, A., Sarin, R. & Mulherkar, R. 2005. Genotype, phenotype and cancer: Role of low penetrance genes and environment in tumour susceptibility. *Journal of Biosciences* 30: 93-102.

Kousidou, O.C., Roussidis, A.E., Theocharis, A.D. & Karamanos, N.K. 2004. Expression of MMPs and TIMPs genes in human breast cancer epithelial cells depends on cell culture conditions and is associated with their invasive potential. *Anticancer Research* 24: 4025-4030.

Li, X., Xue, Y., Liu, X., Zheng, J., Shen, S., Yang, C., Chen, J., Li, Z., Liu, L., Ma, J., Ma, T. & Liu Y. 2019. ZRANB2/SNHG20/FOXK1 axis regulates vasculogenic mimicry formation in glioma. *Journal of Experimental & Clinical Cancer Research* 38: 68.

Li, S., Pritchard, D.M. & Yu, L.G. 2022. Regulation and function of matrix metalloproteinase-13 in cancer progression and metastasis. *Cancers* 14: 3263.

Li, Z. & Chen, L. 2023. Predicting functional consequences of SNPs on mRNA translation via machine learning. *Nucleic Acids Research* 51: 7868-7881.

Liang, M., Wang, J., Wu, C., Wu, M., Hu, J., Dai, J., Ruan, H., Xiong, S. & Dong, C. 2021. Targeting matrix metalloproteinase MMP3 greatly enhances oncolytic virus mediated tumor therapy. *Translational Oncology* 14: 101221.

Maiti, A., Okano, I., Oshi, M., Okano, M., Tian, W., Kawaguchi, T., Katsuta, E., Takabe, K., Yan, L., Patnaik, S. & Hait, N.C. 2021. Altered expression of secreted mediator genes that mediate aggressive breast cancer metastasis to distant organs. *Cancers (Basel)* 13: 2641.

Nagase, H., Visse, R. & Murphy, G. 2006. Structure and function of matrix metalloproteinases and TIMPs. *Cardiovascular Research* 69: 562-573.

Pijuan, J., Barceló, C., Moreno, D.F., Maiques, O., Sisó, P., Marti, R.M., Macia, A. & Panosa, A. 2019. *In vitro* cell migration, invasion, and adhesion assays: From cell imaging to data analysis. *Frontiers in Cell and Developmental Biology* 7: 107.

Raeeszadeh-Sarmazdeh, M., Do, L.D. & Hritz, B.G. 2020. Metalloproteinases and their inhibitors: Potential for the development of new therapeutics. *Cells* 9: 1313.

Sampieri, C.L., León-Córdoba, K. & Remes-Troche, J.M. 2013. Matrix metalloproteinases and their tissue inhibitors in gastric cancer as molecular markers. *Journal of Cancer Research and Therapeutics* 9: 356-363.

Schneider, C.A., Rasband, W.S. & Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9: 671-675.

Si-Tayeb, K., Monvoisin, A., Mazzocco, C., Lepreux, S., Decossas, M., Cubel, G., Taras, D., Blanc, J.F., Robinson, D.R. & Rosenbaum, J. 2006. Matrix metalloproteinase 3 is present in the cell nucleus and is involved in apoptosis. *American Journal of Pathology* 169: 1390-1401.

Suhaimi, S.A., Chan, S.C. & Rosli, R. 2020. Matrix metallopeptidase 3 polymorphisms: Emerging genetic markers in human breast cancer metastasis. *Journal of Breast Cancer* 23: 1-9.

Welch, D.R. & Hurst, D.R. 2019. Defining the hallmarks of metastasis. *Cancer Research* 79: 3011-3027.

Wu, J.S., Jiang, J., Chen, B.J., Wang, K., Tang, Y.L. & Liang, X.H. 2021. Plasticity of cancer cell invasion: Patterns and mechanisms. *Translational Oncology* 14: 100899.

Yamaguchi, K., Yoshihiro, T., Ariyama, H., Ito, M., Nakano, M., Semba, Y., Nogami, J., Tsuchihashi, K., Yamauchi, T., Ueno, S., Isobe, T., Shindo, K., Moriyama, T., Ohuchida, K., Nakamura, M., Nagao, Y., Ikeda, T., Hashizume, M., Konomi, H., Torisu, T., Kitazono, T., Kanayama, T., Tomita, H., Oda, Y., Kusaba, H., Maeda, T., Akashi, K. & Baba, E. 2022. Potential therapeutic targets discovery by transcriptome analysis of an *in vitro* human gastric signet ring carcinoma model. *Gastric Cancer* 25: 862-878.

*Corresponding author; email: norazalina@upm.edu.my