ORIGINAL ARTICLE

# Multiple SNPs Downregulate Gene Expression of Matrix Metallopeptidase 2 in MCF7 Breast Cancer Cells

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**ABSTRACT**

**Introduction:** On a global scale, breast cancer contributes the highest cancer-related deaths in women due to metastasis which renders the treatments ineffective and non-targeted. The members of Matrix Metallopeptidases, particularly Matrix Metallopeptidase 2 (MMP2), are among the key players in breast cancer metastasis. In most cases, MMP2 was markedly upregulated and linked to poor prognosis. In a previous study, *in silico* analyses revealed that several coding single nucleotide polymorphisms (SNPs) of *MMP2* were shown to reduce gene expression and mRNA stability of *MMP2* in Malaysian breast cancer patients. Therefore, to validate the *in silico* predictions, the objective of this study was to determine the effects of multiple coding SNPs of *MMP2* on the gene expression and mRNA stability of *MMP2* in breast cancer cells. **Methods:** In the current study, breast adenocarcinoma MCF7 cells were transfected with *MMP2* wild type and variant containing the coding SNPs. After confirmation of transfection by DNA sequencing, the gene expression level of *MMP2* was evaluated by quantitative reverse transcription polymerase chain reaction (RT-qPCR) whereas mRNA stability of *MMP2* was determined following treatment with actinomycin D. **Results:** *MMP2* wild type and variant were successfully transfected in MCF7 cells based on sequencing and PCR analysis. It was found that the presence of coding SNPs lowered the gene expression level of MMP2, but not the stability of *MMP2* mRNA. **Conclusion:** This study supports the *in silico* effects of *MMP2* coding SNPs on its gene expression in an *in vitro* model.
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## INTRODUCTION

Breast cancer has the highest number of cancer mortality rate in women globally (1). Likewise, in Malaysia, with an age-standardized rate of 20.7 per 100 000, breast cancer is the number one contributor to cancer-related deaths (2). Although the survival rate of breast cancer patients has considerably improved, cancer treatments become futile and non-targeted when metastasis is present at advanced stages of breast cancer. The occurrence of metastasis in breast cancer is modulated mainly by members of Matrix Metallopeptidases (MMPs), including Matrix Metallopeptidase 2 (MMP2), in altering the chemical and physical properties of the extracellular matrix (ECM) (3-5). MMP2, or gelatinase A, is a zinc-dependent proteolytic enzyme that has a number of ECM molecules as its substrates including collagen, elastin, fibronectin, and laminin. As such, it is not surprising that MMP2 was reported to be significantly upregulated during metastasis in breast cancer in clinical, *in vivo*, and *in vitro* studies (6-9). In the *in vivo* setting, MMP2 expression level and its enzymatic activity were increased in breast cancer brain metastasis. Likewise, breast cancer cells became invasive and metastatic following transfection with *MMP2 in vitro* (7,10). As for the clinical studies, MMP2 expression was strongly correlated with poor prognosis of breast cancer patients, and occurrence of distant metastasis as well as local recurrence (11-13).

The expression of MMPs including MMP2 can be regulated during transcription and translation by single base substitutions known as single nucleotide polymorphisms (SNPs) (3,11,14). Transcriptional and translational regulations of MMP2 by SNPs can modify

mRNA structure and stability, as well as translational efficiency which subsequently affect MMP2 gene and protein expression levels (15-16). These modifications will in turn lead to functional alterations that impact disease phenotype, progression, and susceptibility, as well as response to drug therapies (17). Hence, it is not surprising that many genome-wide association studies discovered correlations between *MMP2* SNPs and susceptibility to breast cancer as well as cancer prognosis.

In fact, *MMP2* c.678G>C synonymous SNP was found to confer a protective effect against breast cancer metastasis in Malaysia breast cancer patients based on statistical analysis, possibly by altering the folding pattern and subsequently the structure of *MMP2* mRNA (18). However, causal SNPs that may have substantial genetic effects on their phenotypes are often difficult to detect as their effects may seem small or negligible when these SNPs are individually genotyped (19). Therefore, further *in silico* analysis using Mfold software was conducted to predict differences in mRNA folding and structure between *MMP2* wild type and its variant alleles. Out of 21 SNPs of *MMP2* that were detected, there were eight SNPs that might cause changes to the secondary structure of *MMP2* mRNA. In particular, c.1499G>A (Exon 10), c.*111G>A (UTR), c.*159T>C (UTR), and c.*400G>A (UTR) SNPs were predicted to cause minor changes to the mRNA structure of *MMP2*, whereas c.678G>C (Exon 5), c.750C>T (Exon 5), c.1806C>T (Exon 12) and c.1842C>G (Exon 12) SNPs were predicted to cause major alterations of *MMP2* mRNA structure. All of the exonic SNPs were synonymous except for c.1499G>A (Arg500His). These structural changes may result in lower mRNA stability. Due to this, the mRNA half-life is shorter leading to faster mRNA degradation and forms the basis of lower *MMP2* gene expression. Hence, the lower *MMP2* gene expression may explain the mechanism behind these synonymous SNPs in conferring protective effect against breast cancer metastasis.

However, this *in silico* prediction needs to be experimentally validated by at least *in vitro* approaches since SNP-induced biological effects cannot be completely predicted based on *in silico* analyses per se (20). Therefore, the aim of the present study was to determine the effects of multiple *MMP2* coding SNPs on its gene expression and mRNA stability in MCF7 breast cancer cell line.

## MATERIALS AND METHODS

### Materials

Two plasmid vector constructs were previously designed and synthesized by Life Technologies Holdings, Singapore, which are MMP2-WT_pcDNA and MMP2-SNP_pcDNA using pcDNA3.1(+)_A009 as the vector backbone (Fig. 1). MMP2-WT gene was synthesized according to the gene reference sequence available on NCBI GenBank (gene accession number: NM_004530.6). The variant MMP2 (MMP2-SNP_pcDNA) contains multiple coding SNPs with their NCBI dbSNP accession numbers as listed in Fig. 1. The cell line used in this study was MCF7 breast adenocarcinoma epithelial cell line which was purchased from American Tissue Culture Collection (ATCC), USA (HTB-22TM) which was maintained with Roswell Park Memorial Institute (RPMI) 1640 media (Nacalai Tesque, Japan) containing 4.5 g/L glucose, L-glutamine, 10% of foetal bovine serum (HyClone, GE Healthcare, USA), and 1% of penicillin/streptomycin (Nacalai Tesque, Japan).

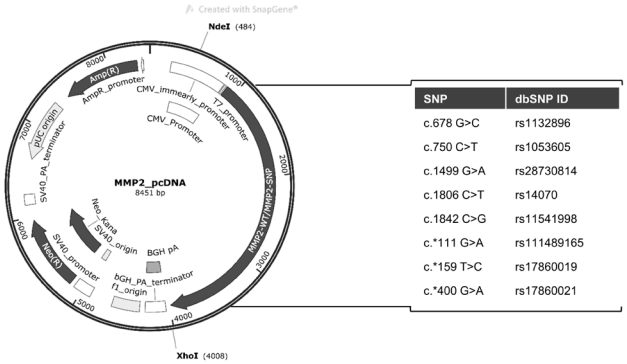


**Figure 1: Plasmid map of MMP2-WT/MMP2-SNP_pcDNA.** The MMP2-SNP plasmid contains the coding SNPs as listed above at their respective nucleotide positions with their accession numbers (NCBI dbSNP). The asterisk symbol represents the nucleotide position at 3′ of the translation termination codon. The plasmid map was created using the SnapGene software.

### Plasmid Constructs Amplification and Extraction

In this study, the glycerol stocks of competent *E. coli* JM109 cells transformed with the plasmid constructs were streaked onto 40 g/L Luria-Bertani (LB) agar (Sigma-Aldrich, Germany) containing 50 µg/mL ampicillin (Nacalai Tesque, Japan). After an incubation overnight at 37 °C, single colonies from LB agar were cultured in 25 g/L LB (Miller) broth (Sigma-Aldrich, Germany) with 50 µg/mL ampicillin. Following an overnight incubation, the plasmid constructs were extracted using Presto Endotoxin Free Mini Plasmid Kit as instructed in the manufacturer's protocol (Geneaid Biotech, Taiwan). The concentration of extracted plasmid DNA was measured at absorbance of 260 nm whereas the purity of the extracted plasmid DNA was determined based on absorbance ratio of 260/280 nm and 260/230 nm (Eppendorf BioSpectrometer® Kinetic, Germany).

### Restriction Enzyme Digest

Following plasmid DNA extraction, the size of the plasmid DNA was qualitatively confirmed by restriction enzyme digest. In this experiment, MMP2-WT_pcDNA and MMP2-SNP_pcDNA were digested with NdeI and XhoI restriction enzymes (Thermo Fisher Scientific, USA) for 30 minutes at 37 °C. The undigested and digested samples were electrophoresed on 0.7% (w/v) agarose gel in 1x Tris-Acetate-EDTA (TAE) buffer at 75 V for 45 minutes and viewed under the ultraviolet light (Gel Doc

XR+, Bio-Rad, USA). Florosafe DNA Stain (1st BASE, Singapore) was added to agarose before gel solidification and 100 bp DNA ladder (SMOBIO Technology, Taiwan) was loaded in the well to view DNA and measure the size of DNA, respectively.

**Stable Transfection**

MCF7 breast cancer cells were seeded in a 24-well plate (5x $10^4$ cells/well) and incubated at 37°C in a 5% $CO_2$ and humidified condition. After an overnight incubation, the cells were transfected with MMP2-WT_pcDNA or MMP2-SNP_pcDNA using jetPRIME® *in vitro* DNA transfection reagent (Polyplus-transfection®, France) according to the provided instructions. The transfected cells were positively selected using the neomycin resistance gene (Neo(R)) in the plasmid as the selection marker against G418 antibiotic. To confirm that all un-transfected cells died after exposure to selection antibiotic (G418), cells that were mock transfected with only the transfection reagent acted as the negative control. At 24 hours post-transfection, the media was replaced with fresh complete RPMI 1640. At 48 hours post-transfection, the media was substituted with fresh complete RPMI 1640 containing 0.8 mg/mL G418 (Nacalai Tesque, Japan). At 2 to 3 days interval, the media was changed with fresh media containing G418 until all mock transfected cells died, and the selected cells were then expanded and cryopreserved. MCF7 cells that were not transfected are referred to as parental MCF7 cells.

**Total RNA Extraction and cDNA Synthesis**

Using FavorPrep™ Tissue Total RNA Mini Kit (Favorgen, Taiwan), the transfected and parental MCF7 cells were subjected to total RNA extraction as instructed in the provided protocol. The absorbance ratio at 260/230 nm and 260/280 nm were used as an indicator of total RNA purity whereas the absorbance value at 260 nm was used to measure the concentration of extracted total RNA (Eppendorf BioSpectrometer® Kinetic, Germany). Following total RNA extraction, complementary DNA (cDNA) was synthesized using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Japan) according to the manufacturer's instructions. During cDNA synthesis, no reverse transcriptase (NRT) control which omitted the addition of reverse transcription was included to detect the presence of genomic DNA contamination. The synthesized cDNA was then utilized in DNA sequencing and real time quantitative polymerase chain reaction (RT-qPCR).

**DNA Sequencing**

The synthesized cDNA was subjected to polymerase chain reaction (PCR) using exTEN 2x PCR Master Mix (1st BASE, Singapore) based on the manufacturer's protocol. The components of PCR reaction were 1x PCR Master Mix, 0.2 µM forward and reverse primers, 100 ng of cDNA and nuclease-free water. The primers were designed by 1st BASE Laboratories (Malaysia) to obtain three amplicons of MMP2 gene based on MMP2 gene with accession number of NM_004530.6. The first set of primers (forward 5'-CTGCATCCAGACTTCCTCAG and reverse 5'-GGTGCAGCTCTCATATTTGTTG) amplified Amplicon 1 with length of 1373 bp, the second set of primers (forward 5'-GCAAGTTTCCATTCCGCTTC and reverse 5'- TAGGCACCCTTGAAGAAGTAG) amplified Amplicon 2 with length of 1036 bp, whereas the third set of primers (forward 5'- GATGTCCAGCGAGTGGATG and reverse 5'- CCACTCAGTAGGTGTCTTTATTC) produced Amplicon 3 with length of 1090 bp.

The reactions were then used in the following PCR cycling conditions: (1) initial denaturation for 3 minutes at 95°C, (2) 35 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 60 to 90 seconds, and (3) final extension at 72°C for 10 minutes (Eppendorf Mastercycler® Gradient, Germany). For qualitative confirmation of transfection and verification of specific amplification, the PCR products were electrophoresed on 2.0% agarose gel at 60 V for 70 minutes and imaged under the Gel Doc XR+ (Bio-Rad, USA) with 1 kb DNA ladder (SMOBIO Technology, Taiwan) to estimate the size of MMP2 amplicons. PCR products of MMP2-SNP with a single band indicate that PCR amplification was specific and were used for DNA sequencing by 1st BASE Laboratories (Malaysia). DNA sequencing was performed using ABI PRISM 3730xl Genetic Analyzer (Applied Biosystems, USA). Via the BLASTN program (National Institute of Health, USA), the similarities between MMP2-SNP and obtained nucleotide sequences based on sequence alignments were determined.

**Real Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

For this experiment, synthesized cDNA of transfected and parental MCF7 cells which were prepared as described earlier was subjected to RT-qPCR using Luna® Universal qPCR Master Mix (New England Biolabs, USA). Based on the manufacturer's protocol, each reaction consisted of 1x Master Mix, 0.2 µM forward and reverse SYBR Green primers, 100 ng of cDNA and nuclease-free water. The primers for MMP2 (forward 5'-CCGTGGTGAGATCTTCTTCTTC and reverse 5'-GCCTCGTATACCGCATCAATC) were designed and synthesized by 1st BASE, Singapore, whereas the primers for the reference gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were commercially obtained (Genecopoiea, USA). The reactions were run in triplicates on CFX96™ Real Time PCR Detection System (Bio-Rad, USA) with the following PCR cycling conditions: 60 seconds at 95°C for initial denaturation, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The relative expression of normalized MMP2 gene was calculated using the ΔΔCq formula as described by Livak and Schmittgen (21). The MMP2 primers were also used in the mRNA stability assay.

**mRNA Stability Assay**

This assay was performed according to the methods described by Li et al., Wada and Becskei, as well as Ayupe and Reis with modifications (22-24). For this assay, MCF7 transfected cells were seeded in a 6-well plate (3 x $10^5$/well) and grown at 37°C in a 5% CO2 and humidified setting. After an overnight incubation, the cells were treated with 5 µg/mL actinomycin D (MP Biomedicals, USA) and total RNA extraction was performed at 0, 3, 6, 9, and 12 hours post-treatment. The purity and concentration of extracted RNA were determined, and the extracted RNA was reverse transcribed to cDNA as described earlier. As per manufacturer's instructions, the synthesized cDNA was used for RT-qPCR using Quantinova™ SYBR® Green PCR Kit (Qiagen, Germany). The reactions contained 1x Master Mix, 0.7 µM forward and reverse primers of MMP2, 100 ng of cDNA and nuclease-free water. Using CFX96™ Real Time PCR Detection System (Bio-Rad, USA), the reactions were run in triplicates, and the following PCR cycling setting was employed: 2 minutes at 95°C for initial heat activation, followed by 40 cycles of 5 seconds at 95°C and 10 seconds at 60°C. In this assay the relative expression of MMP2 was quantitated as $2^{-\Delta Cq}$ using the ΔCq formula. MMP2 expression was quantitated relatively to the expression level at 0 h which was set at 1, and the graph of relative MMP2 mRNA expression against duration of actinomycin D treatment (hour) was plotted. Using a one-phase exponential decay model, the mRNA half-lives of MMP2 were computed via GraphPad Prism version 7.

**Statistical Analysis**

The relative gene expression and mRNA half-lives of MMP2 were statistically analysed in an independent samples t test using SPSS Statistics 22 (IBM, USA). The t tests were conducted following the assumptions that the data was normally distributed and there was homogeneity of variance. The difference between the MMP2-WT and MMP2-SNP were considered as statistically significant when p-value < 0.05.

**RESULTS**

**MMP2-WT and MMP2-SNP constructs were successfully transfected in MCF7 cells**

In the current study, following extraction of plasmid DNA, the estimated size of the plasmid DNA was determined by restriction enzyme digest. This parameter is important in ensuring the successful transfection of the plasmid DNA constructs. Based on Fig. 2, the estimated sizes of digested DNA also matched the sizes of plasmid DNA. Single digest of plasmid DNA with *Xho*I resulted in the presence of a linearized plasmid that matched the size of the plasmid DNA of approximately 8.5 kb. Furthermore, double digest of plasmid DNA with NdeI and XhoI also led to the presence of two DNA fragments which corresponded to the predicted sizes of 5.0 kb and 3.5 kb based on the position of *Xho*I and *Nde*I sites in the plasmid DNA.

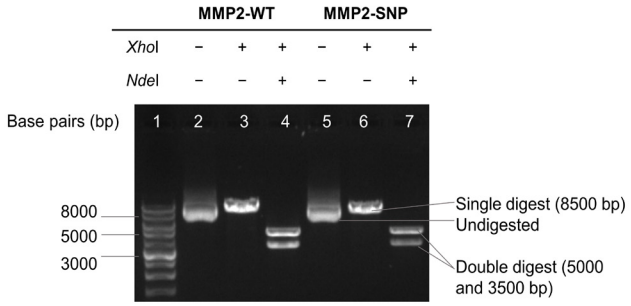


**Figure 2: Restriction digest analysis of MMP2-WT and MMP2-SNP plasmid constructs by agarose gel electrophoresis.** Lane 1: 1 kb DNA marker; Lanes 2-4: MMP2-WT plasmid in its circular form (undigested), linear form following digest with only *Xho*I (8500 bp) and double fragments following double digest with *Xho*I and *Nde*I, respectively (5000 and 3500 bp); Lanes 5-7: MMP2-SNP plasmid in its circular form, linear form following digest with only XhoI (8500 bp) and double fragments following double digest with *Xho*I and double digested with *Xho*I and *Nde*I, respectively (5000 and 3500 bp). The undigested plasmid serves as the negative control, which migrated faster than the linearized plasmids (single digest) due to its supercoiled and circular forms.

The successful transfection of both plasmid DNA constructs was confirmed based on gel electrophoresis and DNA sequencing of MMP2 amplicons (Fig. 3). Sanger sequencing is a conventional method to determine the DNA sequence in a cost-effective and straightforward approach. Moreover, with modern system of capillary-based Sanger sequencing, data analysis and validation have been greatly improved. Despite that, one of the limitations of Sanger sequencing is that it can only accurately provide a sequence read that ranges between 700 to 1000 base pairs. For that reason, gene amplification of *MMP2* was performed to produce 3 amplicons in our study, since the length of *MMP2* gene sequence is 3096 base pairs. Based on Fig. 3, MMP2-WT and MMP2-SNP were successfully transfected into MCF7 cells. The presence of all amplicons with their predicted sizes of 1373, 1036 and 1090 bp, respectively in Fig. 3a is the initial confirmation of successful transfection. The presence of a single band for each amplicon verified the specificity of the PCR primers whereas the NRT control ruled out the presence of genomic DNA contamination. Moreover, *MMP2* amplicons were not detected in the MCF7 parental cells, corroborating the absence of endogenous *MMP2* expression with previous studies.

Further analysis of *MMP2* amplicons by Sanger sequencing confirmed the successful transfection of MCF7 cells with MMP2-SNP_pcDNA, as indicated by the presence of all coding SNPs (Fig. 3b). In particular, the coding SNPs of c.678G>C and c.750C>T were detected in Amplicon 1, c.1499G>A SNP was found in Amplicon 2, whereas the rest of studied coding SNPs (c.1806C>T, c.1842C>G, c.*111G>A, c.*159T>C, and c.*400G>A) were discovered in Amplicon 3 of MMP2 gene, as predicted. After confirmation of successful

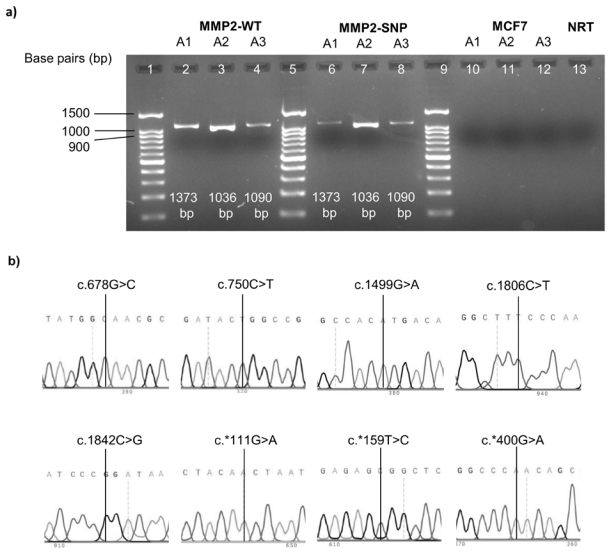


**Figure 3: Successful transfection of MMP2-WT and MMP2-SNP in MCF7 cells. a)** The amplification of *MMP2* in MCF7 cells. Lanes 1,5,9: 100 bp DNA ladder; Lanes 2-4: MMP2-WT amplicons; lanes 6-8: MMP2-SNP amplicons; lanes 10-12: MCF7 parental cells; lane 13: No-reverse transcriptase control (NRT). A1 refers to Amplicon 1 (1373 bp), A2 refers to Amplicon 2 (1036 bp), and A3 refers to Amplicon 3 (1090 bp). The samples were run in a 2% agarose gel for 70 minutes at 60 V**. b)** DNA sequence chromatograms of *MMP2* coding SNPs at their respective positions. The c.678G>C and c.750C>T SNPs were detected in Amplicon 1, c.1499G>A SNP was detected in Amplicon 2, whereas the remaining SNPs (c.1806C>T, c.1842C>G, c.*111G>A, c.*159T>C, and c.*400G>A) were detected in Amplicon 3 of *MMP2*.

transfection based on PCR and Sanger sequencing data, the gene expression and mRNA stability of *MMP2* were determined.

### *MMP2* coding SNPs reduced its gene expression but not the mRNA stability

The gene expression levels of *MMP2* in transfected and parental MCF7 cells were relatively quantitated via RT-qPCR in which MCF7 parental cells serves as the control for the relative measurement. Based on both qualitative and quantitative analyses, endogenous expression of *MMP2* was found to be absent as Cq value of *MMP2* in the parental cells was 33.89 ± 0.25, with GAPDH serves as the reference gene (Fig. 4a, 4b and 4c). Following transfection, MMP2-WT was overexpressed by approximately 110-fold ± 17.6 compared to the parental cells (Fig. 4d). On the other hand, the presence of coding SNPs lowered the expression level, which was 1.6-fold ± 0.2 relatively to the parental cells. Moreover, the mRNA expression level of *MMP2* containing SNPs was significantly lower than *MMP2* wild type ($p < 0.05$).

Over 12 hours of treatment with actinomycin D, the mRNA stability of *MMP2* variant containing SNPs was marginally lower than *MMP2* wild type, with mRNA half-lives of 11.15 ± 0.42 and 11.38 ± 1.96 hours respectively (Fig. 4e and 4f). Furthermore, the comparison was not statistically significant ($p > 0.05$).

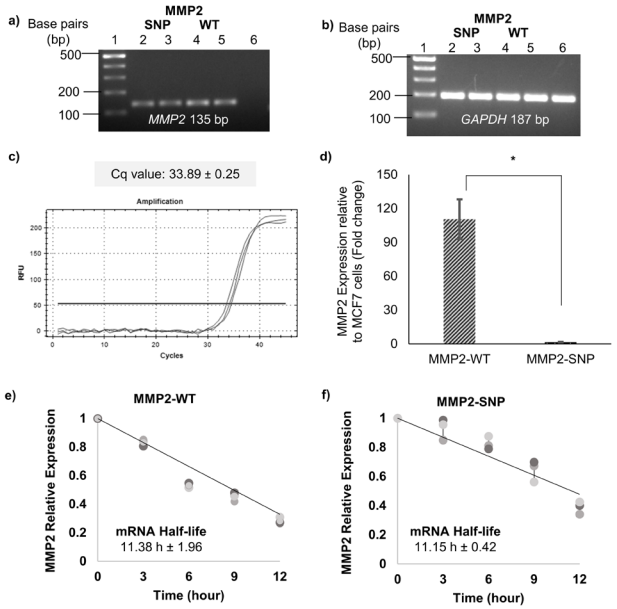


**Figure 4: The effects of *MMP2* coding SNPs on mRNA expression and stability of *MMP2* in MCF7 cells.** a) mRNA expression of *MMP2* (135 bp) in MCF7 parental and transfected cells (MMP2-WT and MMP2-SNP) based on agarose gel electrophoresis. Lane 1: 100 bp DNA ladder; Lanes 2-3: MCF7 cells transfected with MMP2-SNP; Lanes 4-5: MCF7 cells transfected with MMP2-WT; Lane 6: MCF7 parental cells. b) mRNA expression of *GAPDH* (187 bp) which serves as the reference gene in MCF7 parental and transfected cells (MMP2-WT and MMP2-SNP) based on agarose gel electrophoresis. Lane 1: 100 bp DNA ladder; Lanes 2-3: MCF7 cells transfected with MMP2-SNP; Lanes 4-5: MCF7 cells transfected with MMP2-WT; Lane 6: MCF7 parental cells. In a) and b), the samples were run in 2% agarose gels for 70 minutes at 60 V. For MCF7 transfected cells, two replicates were run for each group (MMP2-SNP and MMP2-WT). (c) Amplification graphs of *MMP2* in MCF7 parental cells based on RT-qPCR analysis. (d) Relative mRNA expression of MMP2-WT and *MMP2* variant containing SNPs (MMP2-SNP). The Cq values were normalized to *GAPDH* gene then quantitated relatively to MCF7 parental cells. The difference between MMP2-WT and MMP2-SNP, as represented by the asterisk, is considered statistically significant when $p < 0.05$ (n=3). e) The mRNA half-lives of MMP2-WT and f) MMP2-SNP. MCF7 cells were transfected with MMP2 and treated with actinomycin D in a time-dependent manner. The expression level of *MMP2* at 0 h was set at 1, and the *MMP2* expression was quantitated relatively to the expression level at 0 h. The mRNA half-lives of *MMP2* were calculated by GraphPad software and presented as mean ± standard deviation (SD).

## DISCUSSION

In the current study, the predicted effects of *MMP2* coding SNPs on mRNA expression and stability of MMP2 were determined *in vitro*. After the plasmid DNA constructs were verified, MMP2-WT_pcDNA and MMP2-SNP pcDNA were transfected into MCF7 breast cancer cell line. In this study, MCF7 breast cancer cells are aptly suitable for transfection as previous studies had reported the absence of endogenous *MMP2* expression (25-27). Indeed, MMP2 was not endogenously expressed in this study. Based on the relative expression of MMP2-WT and MMP2-SNP, the presence of coding SNPs in MMP2 variant influenced the gene expression of *MMP2*, confirming the biological and functional effects

of these coding SNPs. However, these effects could be regulated by several ways involving mRNA splicing, stability, and structure as well as protein conformation and translational efficiency (15-16). As for our study, we hypothesized that the functional changes were due to the altered structure of *MMP2* mRNA, causing the structure to be more unstable and consequently degrade faster than *MMP2* wild type. Therefore, the mRNA stability of *MMP2* was studied following treatment of the transfected cells with actinomycin D in a time-dependent manner.

Actinomycin D is commonly employed as an inhibitor of eukaryotic RNA polymerases via DNA intercalation at GC-rich regions, leading to inhibition of gene transcription (24). However, as there was no significant difference between the mRNA half-lives of MMP2-WT and MMP2-SNP in the assay, the downregulation of *MMP2* expression by its coding SNPs might be regulated by factors other than its mRNA stability. Nonetheless, the insignificant comparison between MMP2-WT and MMP2-SNP might also be due to the shortcomings of utilizing actinomycin D. For example, chemical inhibitor-based mRNA stability assay mainly focuses on the effect of mRNA degradation on its stability and expression, even though mRNA expression can be influenced by mRNA synthesis as well (28). It is possible that the reduced *MMP2* mRNA expression level is due to a decrease of mRNA synthesis that is largely regulated by transcription factors, instead of increased mRNA decay. Furthermore, mRNA stability assays using chemical inhibitors are conducted with the assumption that mRNA decay follows a one phase exponential model since it is spontaneous, though this mostly applies to short-lived mRNAs (23,28). Hence, mRNAs with long half-lives might be difficult to study using this approach since actinomycin D is toxic, making it is less possible to observe over a long period of treatment (28).

Indeed, in our study, cell viability and concentration of extracted RNA following 12 hours of treatment with actinomycin D was substantially low, which made it challenging to determine the mRNA half-lives of *MMP2* over an extended period. Moreover, as analysed via the GraphPad software, even though mRNA stability of MMP2-WT fit the exponential model, the mRNA stability of MMP2-SNP fit the linear regression model (data not shown). Although it is not certain why such a difference was observed between MMP2-WT and MMP2-SNP, nevertheless direct comparison between *MMP2* wild type and variant may not be feasible. Based on current findings, it was not possible to confirm whether *MMP2* mRNA is short- or long-lived. Similarly, previous literature reported inconsistent results regarding the mRNA half-life of *MMP2* which varied between 6 to 49 hours (29-31). Therefore, for an accurate measurement of degradation rate, different approaches to study mRNA stability should be conducted such as the pulse labelling of RNA.

## CONCLUSION

Nevertheless, *MMP2* was significantly downregulated due to the presence of its multiple coding SNPs. Although the role of these SNPs in lowering its mRNA stability is still ambiguous, the current study successfully validated the predicted effects of *MMP2* coding SNPs on its gene expression in an *in vitro* setting. This is a small but significant effort to better comprehend the association between *MMP2* and its coding SNPs with breast cancer metastasis. To further validate the predicted 'protective' functional effect of these SNPs against breast cancer metastasis, a more extensive study needs to be performed. These include the use of a 3-dimensional model of cell culture using ECM proteins as scaffolds to mimic the *in vivo* microenvironment of breast cancer, and the investigation of MMP2 interactions with cell adhesion molecules such as cadherins and integrins. Moreover, it is also imperative to study the impact of these SNPs at the MMP2 protein expression level, its regulation by TIMP-2 and MT1-MMP, as well as its function as an enzyme that contributes to the hallmarks of breast cancer metastasis.

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