DIFFERENTIAL EXPRESSION OF MMP-13 IN BREAST CANCER CELL LINES WITH DIFFERENT TUMOROGENICITY AND PHENOTYPES

Leleesha Samaraweera
Department of Biology, Fordham University, Bronx, NY.

ABSTRACT

The Matrix Metalloprotease-13 (MMP-13) is an enzyme involved in degrading the extra cellular matrix, which is observed to be elevated in the cancers during metastasis. This study involved, investigation of expression of MMP-13 in a normal human mammary cell line (MCF 10A), a non invasive breast cancer cell line (MCF 7) and two highly invasive breast cancer cell lines (MDA-MB 231 and HTB 122). The RT-PCR analysis of transcript levels showed high levels of expression in MCF-7 and MDA-MB 231 compared to MCF 10A, whereas HTB-122 did not show expression under identical incubation conditions. Amplification of MMP-13 was however detected in HTB-122 when amplification was performed for 50 cycles. Western blot analysis of the proteolyzed MMP-13 in cell extracts of the MCF 10A, MCF 7 and MDA-MB 231 cell lines showed higher expression levels than the levels detected in HTB 122. The observed differences in the transcripts and the MMP-13 enzyme levels could be due to different post transcriptional regulation mechanisms in the different cell lines.

INTRODUCTION

Malignant breast carcinomas have the ability to invade normal tissues and spread to other sites giving rise to metastasis. Cancer invasion and metastasis requires the degradation of the basement membrane and the Extra Cellular Matrix (ECM), which enable tumor cells to migrate. The majority of the destruction of the matrix components during metastasis is carried out by stimulated release of Matrix Metalloproteinases (1). In a normal mammary gland, constitutive expression of MMPs is low, except during times of development and pregnancy (1). Matrix metalloproteinases (MMPs) are a family of Zinc dependant endopeptidases classified into four subfamilies, based on their substrate specificity and structural similarity. In humans a total of 26 MMPs have been identified

(2). MMP-13 also known as Collagenase-3 is a member of collagenase subfamily initially identified in breast cancer. It was later found to be over expressed in other malignant tumors, suggesting that MMP-13 is associated with aggressive tumors (3). MMP-13 is capable of preferentially degrading collagen type II. It is also capable of degrading other types of ECM proteins including collagen type IV, X and XIV and fibrillin (4). The findings that, MMP-13 is over expressed in several malignant tumors and with its capability to degrade wide range of ECM proteins indicate it plays a major role in tissue remodeling events accompanying tumor progression. Therefore, expression of MMP-13 can be used as a marker for tumor progression. Furthermore, understanding the regulation of MMP-13 expression can lead to the identification of potential targets to develop therapeutic agents for treatment of tumor invasion and metastasis.

Regulation of the MMP-13 is exerted at many levels, and involves both transcriptional and post trancriptional regulation. It has been reported that MMP-13 is synthesized as a proenzyme which become activated at the cell surface by a proteotytic cascade involving MMP-14 and MMP-2 (5), and Plasmin (6). The expression of MMP-13 is induced by Interleukin-α, Interleukin-β (7) and TGF-β. Its expression is inhibited by variety of compounds including thyroid hormones, Glucocorticoids, Progesterone and Androgens (8). Expression of MMP-13 has been studied in both breast cancer cell lines and breast tumors. In breast carcinomas, MMP-13 is produced by fibroblast-like cells located in the stromal compartment of the breast cancer tissue (9), whereas other studies have indicated that it is also synthesized by epithelial tumor cells (10). Analysis of MMP-13 expression in breast cancer cell lines by RT-PCR showed that MMP-13 was expressed in highly invasive MDA-MB 231 cell line, but not expressed in slightly invasive MCF-7,

T-47D and BT-20 cell lines (7). The previous studies on both, carcinomas and the breast cancer cell lines shows that expression of MMP-13 is dependant on the tumorogenicity of the breast cancer cell lines. The goal of this project is to investigate the dependence of the expression of MMP-13 in both mRNA and protein level in breast cancer cell lines with different tumorogenicity and phenotypes. Table 1 shows the different cell lines used in this study.

Cell line	ATCC #	Invasiveness	Origin
MCF 10A	**CRL-10317**	**Normal**	**Normal human breast cells**
MCF 7	**HTB-22**	**Non invasive**	**Human breast adenocarcinoma (with estrogen receptor)**
MDA-MB 231	**HTB 26**	**Highly invasive**	**Human breast adenocarcinoma (with no estrogen receptor)**
BT 549	**HTB 122**	**Highly invasive**	**Breast ductal carcinoma (with no estrogen receptor)**

Table 1. Different breast cancer cell lines and their properties used in this study.

MATERIALS AND METHODS

Cell cultures

The MCF-10A, MCF-7, MDA-MB-231 and HTB-122 cell lines were generously provided by Dr. R. Kandpal. MCF-10A cells were grown in 1:1 DMEM:F12 media (Giboco) with 5% Horse Serum (Gibco), 20mM Hepes, 10 ng/ml EGF (Epidermal Growth Factor) (Invitrogen), 50 U/ml peniclillin, 50 μg/ml streptomycin (antibiotic), 146 μg/ml L-glutamine (Gibco), 10 μg/ml insulin (Invitrogen), 0.1 μg/ml Cholera toxin (Sigma) and 500 ng/ml Hydrocortisome (Sigma). MCF-7, MDA-MB-231 and HTB-122 cells were grown in DMEM (Gibco) supplemented with 2mM L-glutamine (Gibco), 1mM sodium pyruate (Gibco), 25 U/ml penicillin and 25 μg/ml streptomycin (Gibco) and 10% fetal bovine serum (Hyclone). All cells were cultured at 37˚C / 7% CO_2.

Antibodies

The monoclonal MMP-13 antibodies were purchased from Sigma. This antibody was generated in rabbit against the hinge region of MMP-13. The mouse anti-rabbit antibody conjugated to Alkaline Phosphatase (AP) was purchased form Promega. The monoclonal anti-GAPDH antibody, generated in mouse was purchased from Ambion. The monoclonal Anti-mouse antibody conjugated to AP was purchased from Promega.

RNA Extraction

The RNA from all the cell cultures were purified using the Tri Reagent (Molecular Research Center Inc.) according to the manufacturer's protocol.

Primers and RT-PCR

The primers were designed using *Homo sapiens* MMP-13 mRNA (NCBI accession number NM_002427) to amplify a segment of MMP-13 spanning from exon-7 to exon- 9, which gives a 324 bp product from cDNA and a 3441 bp product from genomic DNA. The forward and the reverse primers used for MMP-13 amplification were, 5′ CTGCATCCTCAGCAGGTTG 3′ (nucleotides, 959-977 of NM_002427) and 5′ GTCTCGGATAGTCTTTATCC 3′ (nucleotides,1264-1283 of NM_002427) respectively. Actin primers, forward 5′CTGACTGACTACCTCATGAAG 3′ (nucleotides, 637-657 of NCBI accession number AK 223055) and reverse 5′ GAGGAGCAATGATCTTGATCT 3′ (nucleotides, 1061-1081 of NCBI accession number AK 223055), were used to amplify a 444 bp PCR product from β-Actin transctripts as an external control. RT-PCR was carried out using QIAGEN OneStep^{TM}RT-PCR kit. Two hundred and fifty nanograms of RNA from each cell line

were used in a final reaction mixture of 15 µl for amplification of MMP-13 and β-Actin. The cycle numbers for amplification of MMP-13 and β-Actin to show differential expression was optimized to 36 and 16 cycles respectively. One-step RT-PCR for MMP-13 was carried out as follows: one cycle of 50 ˚C X 30 min and 95˚C X 15 min, followed by 36 cycles of denaturation at 94 ˚C X 30s, annealing at 56 ˚C X 30s and then a final extension of 72 ˚C X 5 min. The same RT-PCR protocol was carried out for MMP-13 for fifty cycles. Also, the same RT-PCR protocol was carried out for amplification of Actin for 16 cycles in the same Thermal Cycler at the same time. The RT-PCR products were analyzed on a 1% agarose gel.

RT-PCR product purification

The 324 bp RT-PCR product amplified using MMP-13 primers in MDA-MB 231 was purified using Rapid PCR Product Purification Kit (Qiagen) according to manufacturer's protocol.

Sequencing and Alignments

Sequencing by Sanger's dideoxy method was performed using AmpliCycle Sequencing Kit (Perkin Elmer) using RT-PCR products purified above. For each reaction, 50fmol of purified RT-PCR product was mixed with 4 µl of 10X sequencing buffer, 2 µl of primer (10 pmol/ µl), 0.2 µl of ^{33}P-dATP and dH_2O to a final volume of 30 µl. 6 µl of this master mixture was then added to four tubes containing 2 µl of either ddGTP, ddATP, ddTTP or ddCTP. These reactions were set up for both the forward and the reverse primers for MMP-13. The PCR reactions were carried out as follows: one cycle of 94 ˚C X 3 min, followed by 35 cycles of 94 ˚C X 30s, 58 ˚C X 30s and 72˚C X 1min. Following the sequencing reaction, 4 µl of stop solution was added to each tube.

Each sequencing reaction was heated to 94˚C for 3 min to denature the products before running them on a sequencing gel. The partial sequence obtained from sequencing the 324bp PCR product was aligned with *Homo sapiens* MMP-13 mRNA sequence form NCBI (NM_002427) using MacVector 6.5.3.

Protein extraction and Western blot

Media from the cells in the log phase of growth were removed and the cells were treated with trypsin. The cells were washed two times with PBS and lysed in Ripa buffer (250 mM Tris (pH 7.5), 120 mM sodium deoxycholate, 600 mM NaCl, 5% Triton X-100 (vol./vol.) and 0.5% SDS) with protease inhibitors. The lysate was incubated at 4˚C for 45 min. Then the lysate was spun and the supernatant was collected. This was repeated for all the cell lines. Bio-Rad protein assay kit was used to analyze the protein concentration according to manufacturer's protocol. BSA was used to generate the standard curve. Equal amounts of proteins (88 μg) from each cell line mixed with SDS containing loading buffer was denatured by boiling for 3 min. Then the samples were fractionated in a 8% SDS-PAGE gel and transferred onto a nitrocellulose membrane for 1hr at 100V at 4 ˚C. Then the blot was cut to separate the regions that expect to contain MMP-13 protein and the internal loading control GAPDH. The region to cut was predicted by the use of molecular weights of the above proteins and the distribution of the marker in the blot. The blot containing MMP-13 was blocked for 1hr in 5% non-fat dry milk in TBST and then probed overnight with monoclonal anti-MMP-13 antibody (1mg/ml diluted 1:1000 in TBST). The blot was then washed and probed with a mouse anti-rabbit antibody conjugated to AP for 2 hrs (1 μg/μl diluted 1:4000 in TBST), followed by detection with 4ml of Western Blue Substrate for AP (Promega) directly

added to the membrane. The blot containing GAPDH was also blocked for 1hr in 5% milk in TBST and then probed overnight with monoclonal anti-GAPDH antibody (4 µg/µl diluted 1:2000 in TBST). The blot was then washed and probed with an AP conjugated Anti-mouse antibody for 2 hrs (1 µg/µl diluted 1:4000 in TBST) followed by detection with 4 ml of Western Blue Substrate for AP (Promega).

RESULTS

RT-PCR was performed to detect the levels of MMP-13 expression in transcript level in three different breast cancer cell lines with different invasiveness and phenotypes MCF 7, MDA-MB 231, HTB-122 compared to a normal breast cell line, MCF 10A. The cycle number was optimized to 36 to see the differential expression of MMP-13 in the above cell lines by RT-PCR at linear phase of amplification. RT-PCR was carried out for 50 cycles to see whether these cell lines express MMP-13, even at low levels. Two hundred fifty nanograms of RNA from each cell line were used for RT-PCR, and the products were analyzed on a 1% agarose gel as described in the materials and methods. As an external control to normalize the variations of RNA quantities within the samples, β-Actin was amplified in equal amounts of RNA in all the cell lines by RT-PCR using β-Actin primers for 16 cycles as described in the materials and methods. The RT-PCR 324 bp products generated using MMP-13 primers were confirmed to be from MMP-13 mRNA and not from genomic DNA, because amplification of genomic DNA would have generated a 3441 bp band. The results from RT-PCR showed that, MMP-13 is differentially expressed in these four cell lines (Fig 1b). The non invasive MCF-7 and highly invasive MDA-MB 231 breast cancer cell lines showed high levels of MMP-13

mRNA expression compared to normal breast cell line MCF 10A, however no expression was detected in HTB-122 under the same incubation conditions (Fig 1b) . The HTB 122 cell line showed a band when the cycle number was increased to 50, where the RT-PCR reaction reached saturation (Fig 1a).

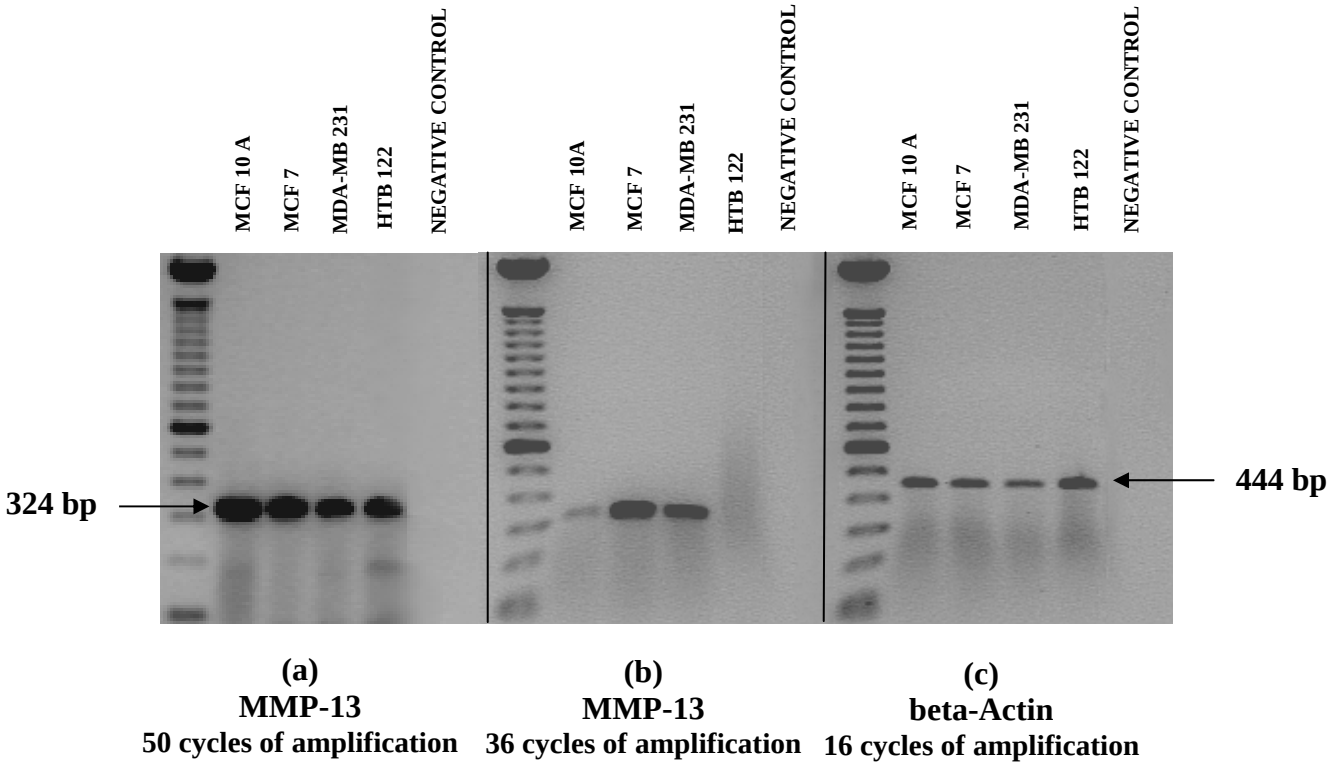

Figure 1. RT-PCR analysis of MMP-13 transcript levels in MCF 10A, MCF 7, MDA-MB 231 and HTB 122 cell lines. (a) RT-PCR products generated from RNA isolated from each of the cell lines, amplified for 50 cycles by primers recognizing nucleotides 959-977 and 1264-1283 of the MMP-13 mRNA sequence from NCBI (NM_002427) **(b)** RT-PCR products generated from RNA isolated from each of the cell lines, amplified for 36 cycles by primers recognizing nucleotides 959-977 and 1264-1283 of the MMP-13 mRNA sequence from NCBI (NM_002427) **(C).** RT-PCR products generated from RNA isolated from each of the cell lines, amplified for 16 cycles by primers recognizing nucleotides 637-657 and 1061-1081of the beta-Actin mRNA sequence from NCBI (AK 223055).

In order to confirm the identity of the 324 bp RT-PCR product amplified using MMP-13 primers in MDA-MB 231 cells, the products were sequenced by Sanger

Dideoxy method as described in materials and methods. The partial sequence obtained from sequencing was aligned with *Homo sapiens* MMP-13 mRNA sequence form NCBI (NM_002427) using MacVector 6.5.3. The ClustalW alignment confirmed the identity of the PCR product with the mRNA of MMP-13 in *Homo sapiens* (NM_002427) (Fig. 2).

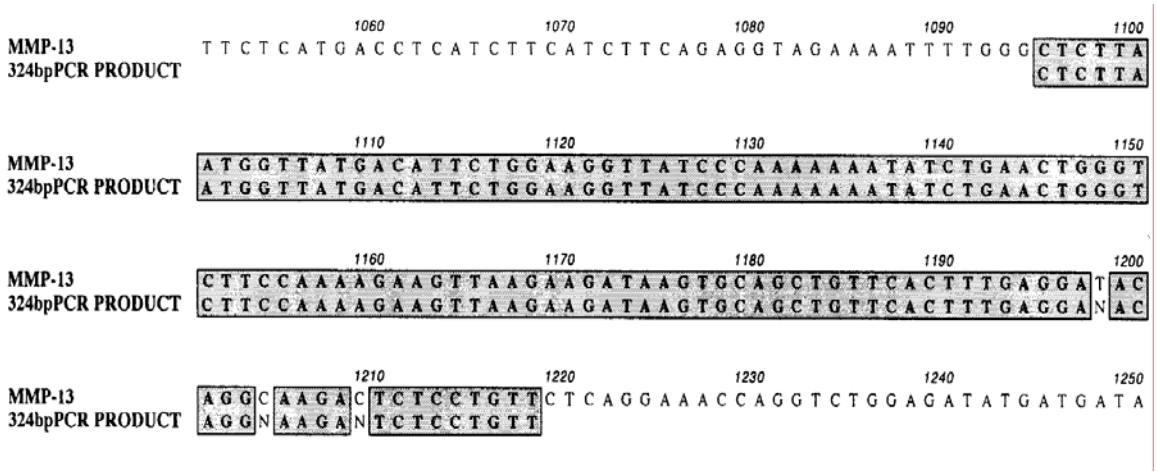

Figure 2. The ClustalW alignment of the partial sequence of the 324 bp RT-PCR product amplified using MMP-13 primers in MDA-MB 231 cells with a segment of *Homo sapiens* MMP-13 mRNA sequence from NCBI (NM_002427).

To investigate the expression of MMP-13 in protein level, Western blot analysis was performed for total proteins extracted from MCF 10A, MCF 7, MDA-MB 231 and HTB-122 cells as described in the materials and methods. It is known that MMP-13 is synthesized as a proenzyme which becomes activated at the cell surface by a proteotytic cascade involving MMP-14 and MMP-2 (5), and Plasmin (6). MMP-13 secreted out of the cell is known to be tightly associated with collagens in ECM. The MMP-13 present in cell extracts corresponds to enzyme present inside the cells and/or associated with the extra cellular enzyme produced by the cells (11). Under the cell culture conditions used

without the use of inhibitors of collagen cross-links the MMP-13 protein is deposited and remains as a part of the cell layer (12). These observations lead to detect the MMP-13 in cell extracts in this study. Equal amounts of proteins from the cell extracts were used for Western Blot Analysis. The proteolyzed MMP-13 enzyme (48 kDa) detected in HTB-122 cell line was low compared to MCF 10A, MCF 7 and MDA-MB 231 cell lines (Fig 3). The expression of MMP-13 enzyme in MCF 10A, MCF 7 and MDA-MB 231 cell lines is nearly equal (Fig. 3), even though these cell lines showed differential expression at mRNA level in RT-PCR (Fig. 1b).The expression of GAPDH (35kDa) was detected as an internal control to normalize the variations of protein quantities in cell lines used for Western blot analysis.

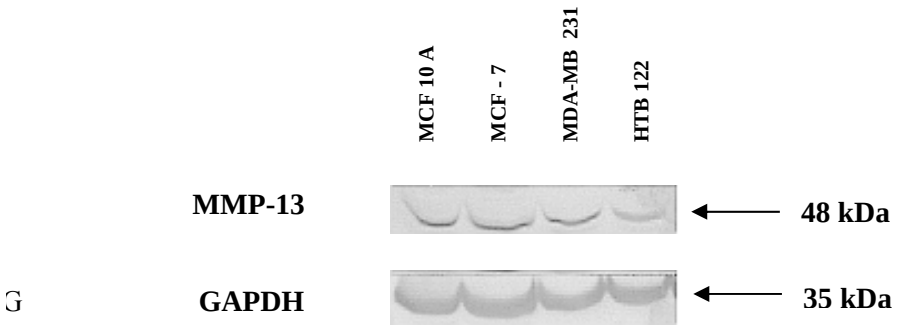

Figure 3. Western blot analysis of MMP-13 protein in MCF 10 A, MCF 7, MDA-MB 231 and HTB 122 cell lines. The top panel shows proteolyzed MMP-13 (48 kDa) detected in MCF 10 A, MCF 7, MDA-MB 231 and HTB 122 cell lines. The bottom panel shows the expression of GAPDH (35 kDa) in MCF 10 A, MCF 7, MDA-MB 231 and HTB 122 cell lines.

DISCUSSION

This study involved, investigation of expression of MMP-13 in a normal human mammary cell line (MCF 10A), a non invasive breast cancer cell line (MCF 7) and two highly invasive breast cancer cell lines (MDA-MB 231 and HTB 122). The RT-PCR

analysis of transcript levels showed high levels of expression in MCF-7 and MDA-MB 231 compared to MCF 10A, whereas HTB-122 did not show expression under identical incubation conditions. Amplification of MMP-13 was however detected in HTB-122 when amplification was performed for 50 cycles. Western blot analysis of the proteolyzed MMP-13 in cell extracts of the MCF 10A, MCF 7 and MDA-MB 231 cell lines showed higher expression levels than the levels detected in HTB 122.

The MMP-13 expression is known to be primarily regulated at transcriptional level by growth factors, cytokines and contact with ECM (13). The expression of MMP-13 is known to be induced by IL-α, IL-β (7) and TGF-β (14) and inhibited by a variety of compounds including thyroid hormones, Glucocorticoids, Progesterone and Androgens (8). Therefore, the expression of MMP-13 in breast cell lines is dependant on the responsiveness of the cells to these factors. The responsiveness of the cell lines can vary depending on the cell phenotype and their origin. Consistent with results from RT-PCR (Fig 1b) of this study, several other investigators reported increased expression of MMP-13 mRNA in highly invasive MDA-MB 231 cells (7, 13). The other highly invasive HTB 122 cell line did not show any expression at the same conditions, even though MMP-13 expression is known to be related to invasiveness of the tumor cells. The non invasive MCF 7 showed high level of MMP-13 expression; however it was not detected in other previous studies (7, 14). Even though MMP-13 transcripts were not expressed in another normal breast cell line (7), MCF 10A showed low levels of expression under the culture conditions of this study. The differences observed in the expression of MMP-13 in MCF 7 with previous studies could be due to different cell culture conditions, such as differences in growth factors that were present in the serums added to the culture medias.

Based on these observations and previous studies showing the regulated expression of MMP-13 by various external stimuli, it is difficult to come to a conclusion relating increased levels of MMP-13 expression to the invasiveness of the cell lines, because there are other variables such as cell phenotype and cell culture conditions that could affect MMP-13 expression. This leads to the need of further studies directed to understand signaling pathways and regulatory mechanisms involved in expression of MMP-13 in cell lines with different phenotypes and origins. Outcomes of these researches could be used to develop therapeutic drugs specifically targeted to the tumor, depending on its origin, to prevent cancer metastasis.

The ClustalW alignment, of the partial sequence of the 324 bp product from RT-PCR amplified using MMP-13 primers with the mRNA of MMP-13 in *Homo sapiens* (NM_002427), confirmed that these primers amplified the desired MMP-13 transcript investigated in this study.

The proteolyzed MMP-13 (48 kDa) was detected in MCF 10A, MCF 7 and MDA MB 231 in higher intensities compared to HTB-122 (fig 3). But the RT-PCR results showed high levels of MMP-13 transcripts in MDA–MB 231 and MCF 7 compared to MCF 10A (fig 1b). Even though, it is difficult to make conclusions based on one set of data for MMP-13 protein analysis with out further studies, the reason for MDA-MB 231 and MCF 7 cells to show equal amounts of MMP-13 protein as MCF 10A, even though MDA-MB 231 and MCF 7 cells had much higher amounts of transcripts than MCF 10A, could be due to different post transcriptional regulation mechanisms present in these cell lines. It has been reported that TIMPs (Tissue Inhibitors of Metalloproteases) inhibit the activation of other MMPs, such as MMP-2, by forming a complex with proMMP and the

protease involved in activation of the enzyme(1). It is possible that a TIMP is involved in the activation of MMP-13, and the MDA-MB 231 and MCE-7 cells have high levels of TIMP compared to MCF 10A under these conditions inhibiting the proteolysis of proMMP-13 in those two cell lines. One of the other possibilities is the proteolytic enzymes, such as MMP-2, MMP-9 and Plasimin could be present in lower levels due to some regulation under these conditions in MDA-MB 231 and MCF 7 cells compared to MCF 10A, making MMP-13 less available. Apart from those reasons, MDA-MB 231 and MCF 7 cells could have less amount of MMP-13, because of some regulatory mechanism involved in inhibiting the processing of the MMP-13 transcripts, lowering the rate of MMP-13 transcripts being translated or inhibiting the secretion of proMMP-13 out of the cell under these conditions. The presence of low levels of active MMP-13 in HTB 122 is not surprising because these cells showed RT-PCR products when the cycle number was increased to 50 (fig 1a), even though it did not show any RT-PCR product when the cycle number was 36 (fig 1b). Further studies are needed to understand the mechanisms involved in post transcriptional regulation of MMP-13.

In conclusion, from this study it was difficult to see a correlation of invasiveness and the expression of MMP-13 because its expression could also be dependant on the phenotype of the cells and the cell culture conditions. Further studies needed to be carried out to investigate the transcriptional and post transcriptional regulation mechanisms of MMP-13, to see how these affect expression of MMP-13 in cell lines with different phenotypes and invasiveness.

Acknowledgements

I would like to thank Dr. Berish Rubin for providing the opportunity to do this project and for his guidance throughout the course of this study. I would also like to thank Mr. Jinsong Qiu and Mr. Brian Fox for their help and assistance with the various molecular methods and techniques employed throughout this project. I also thank Dr. R. Kandpal for his advice on this project.

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