REGULATION OF COLLAGENASES MATRIX METALLOPROTEINASES WITH CTCF/YB-1 TRANSCRIPTION FACTORS IN HUMAN MALIGNANT MELANOMA CANCER *IN VITRO*

BY

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ABSTRACT

The invasiveness of malignant melanoma is mainly attributed to the enzymatic destruction of the extracellular matrix and basement membrane components by a group of enzymes known as matrix metalloproteinases (MMPs). The expression of these proteinases is mainly regulated at the transcriptional level; therefore, high expression of MMPs is manly attributed to different transcription factors which enhance or inhibit the promoter activity of *MMPs* genes. Among these factors, YB-1 and CTCF proteins are transcription factors in which CTCF is mainly a tumour suppressor protein; while YB-1 is an oncogenic factor and a prognostic indicator in a wide range of tumours that regulates most of the cancer processes such as proliferation, invasion and metastasis by regulating the expression of genes related to those processes. However; the expression of these transcription factors and their potential effect on the expression of collagenases MMPs in malignant melanoma cells are not yet confirmed. Therefore, this study was conducted to determine the expression of collagenases MMPs (*MMP1*, *MMP8* and *MMP13*), *YB-1* and *CTCF* transcription factors in A375 melanoma cancer cells. In addition, the stromal effect of normal skin fibroblasts on the expression of collagenases and proliferation in A375 cell was determined. The results of this experiment demonstrated an increase in the expression of *YB-1, MMP8* and *MMP13* in A375 cells. Thereafter, this was followed by the establishment of YB-1 silenced strain of A375 cells using a silencing short hairpin RNA (shRNA) construct. The effect of YB-1 knockdown on the expression of collagenases MMPs was determined using reverse transcription PCR and Western blotting. In addition, the antiproliferative effect was determined using flow-cytometry, colorimetric MTT assay and cell counting; while the anti-invasive properties were determined using wound healing assay. The results of this experiment elucidated that YB-1 protein regulates the expression of *MMP13*, cell cycle progression, cell proliferation and cell migration of A375 cancer cells *in vitro*. Therefore, the direct interaction between YB-1 protein and the AP-1 promoter sequence of *MMP13* was evaluated using Chromatin Immunoprecipitation assay (ChIP). The ChIP analysis has confirmed no interaction between YB-1 protein and the AP-1 promoter sequence. Finally, these experiments demonstrated *YB-1*, *MMP8* and *MMP13* were highly expressed in the A375 cancer cells. The stromal cells were found to promote A375 cell proliferation and enhance the expression of *MMP1*. In addition, YB-1 silencing was significantly associated with reduced expression of MMP13 enzyme, reduction in cancer cell proliferation in a cell cycle specific manner and anti-invasive properties. Therefore, YB-1, MMP13 and stromal cells are considered as promising elements that might help as a potential target in the treatment of melanoma tumour due to their roles in the processes of invasion, migration and proliferation. Further experiments are needed to demonstrate novel protein partners of YB-1 and novel binding sites within gene promoter region of *MMP13* with determination of the involved signalling pathways.

خلاصة البحث

إن سرطان الجلد الميلانيني هو أحد أشد أنواع سرطان الجلد خطورة من ناحية الانتشار، مسببا معدلات أعلى من الوفيات من بين الأنواع الأخرى من الاورام الخبيثة. ويعزى هذا السلوك السرطاني الى التحلل الانزيمي المصاحب في مكونات الغشاء القاعدي للنسيج بواسطة مجموعة من الانزيمات تعرف بانزيمات تحطيم النسيج البروتيني او الـ(ام ام بيس). هذا النوع من الانزيمات مهمة في العديد من العمليات الحيويه والفسيولوجيه كتطور الجنين والتئام الجروح، ولكن فعاليات هذه الانزيمات المفرطه تساهم في تكوين وانتشار العديد من الاورام السرطانية. مستوى فرز هذه الانزيمات وفعاليتها يعتمد بصورة رئيسيه على معدل النسخ الجيني، ولذالك فان هذه الدراسه تهدف الى قياس مستوى تكوين انزيمات الـ(ام ام بي1)، الـ(ام ام بي 8) والـ(ام ام بي13)، بالاضافة الى عوامل الاستنساخ الـ(واي بي1) والـ(سي تي سي اف) والتي تعتبر من البروتينات الفعاله في تقدم الاورام السرطانيه من خلال تنظيم الاستنساخ الجيني في الخلايا السرطانيه. لقد اثبتت الدراسه زياده في افراز انزيمات الـ(ام ام بي 8)، الـ(ام ام بي 13) وعامل الاستنساخ الـ(واي بي1) ولذالك كانت التجربه اللاحقه هي انشاء خلايا سرطان ميلانيني مع التثبيط الدائم لعامل النسخ الجيني الـ(واي بي1) باستخدام تقنيه تكوين احماض نوويه قصيره مكمله لاحماض الـ(واي بي1) النوويه حيث تم التأكد من التثبيط لاحقا بقياس مستوى الحمض النووي ومستوى بروتين الـ(واي بي1). بالاضافة الى ذالك تم دراسه تاثير التثبيط على مستوى التكوين الجيني والبروتيني للانزيمات المحطمة الثلاث. أيضا تمت دراسة تاثير التثبيط على سرعه تكاثر الخلايا السرطانيه وقدرتها على الاقتحام الموضعي. ولقد أثبتت هذه الدراسه ان تثبيط الـ(واي بي1) الدائم يسبب نقص في افراز انزيم الـ(ام ام بي13) ويسبب تدريجياً في تكاثر الخلايا السرطانيه ويحدد من قابليتها على الاقتحام ولذالك تم اجراء تجربه اخرى لتقييم التفاعل المباشر بين المنطقه المنظمه في جين انزيم الـ(ام ام بي13) التي تعرف بالـ(اي بي 1) وبين بروتين الـ(واي بي 1) من خلال استخدام تقنية الترسيب المناعي للكروماتين والذي اثبت وجود المنطقه في المكون الجيني للخلايا ولكن عدم تفاعله مع بروتين الـ(واي بي1) ولذالك اثبتت هذه الدراسه الدور الرئيسي لبروتين الـ(واي بي 1) في تنظيم افراز انزيم الـ(ام ام بي13) وفي تنظيم التكاثر والانتشار لخلايا سرطان الجلد الميلانيني. ولذالك يعتبر بروتين الـ(واي بي 1) هدف علاجي مهم قد يؤدي إلى علاج مرضى سرطان الجلد الميلانيني.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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DEDICATION

“To my beloved parents for their support and to my beloved wife for her patience and support “

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LIST OF SYMBOLS

^{o}C	Degree Celsius
%	Percentage
-	Minus
bp	Base pair
g	Gram
mg	Milligram
mM	Millimolar
mL	Millilitre
nm	Nanometre
NaCl	Sodium chloride
µg	Microgram
µL	Microliter
µM	Micromolar
ng	Nanogram
M	Molar
V	Volts
H_2O	Water
CO_2	Carbon dioxide
$MgCl_2$	Magnesium chloride
BSC	Biosafety cabinet

LIST OF ABBREVIATIONS

cDNA	Complimentary DNA
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
dNTP	Deoxy nucleoside triphosphates
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
EGF	Epidermal growth factor
FGF	Fibroblasts growth factor
PDGF	Platelets derived growth factor
KDGF	Keratinocyte growth factor
TGF-β	Tumour growth factor -β
TNF-α	Tumour necrosis factor -α
HGF	Hepatocytes growth factor
VEGF	Vascular endothelial growth factor
NGF	Nerve growth factor
MAPK	Mitogen activated phosphokinases
FITC	Fluorescein isothiocyanate
IgG	Immunoglobulin G
IgM	Immunoglobulin M
MMPs	Matrix Metalloproteinases
CTCF	CCCTC-binding factor

LIST OF ABBREVIATIONS

YB-1	Y-box binding protein
MMP13	Matrix Metalloproteinase 13
TIMPs	Tissue inhibitors of matrix proteinases
mRNA	Messenger ribonucleic acid
shRNA	Short hairpin ribonucleic acid
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
PFA	Paraformaldehyde
RT-qPCR	Quantitative reverse transcriptase polymerase chain reaction
TBE	Tris/Borate/EDTA buffer
TAE	Tris/Acetic acid/EDTA buffer
UV	Ultraviolet
ACTB	Actin Beta
B2M	Beta-2-Microglobulin
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase.
HPRT1	Hypoxanthine Phosphoribosyltransferase 1
RPL13A	Ribosomal Protein L13a
TUBA1A	Alpha Tubulin
Abs.	Absorbance

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Malignant melanoma is the third most common malignant skin cancer and is the most serious cancer in terms of local invasiveness and mortality rate (Kumar, Abbas, Fausto, & Aster, 2014). While the incidence rate of this malignancy is constantly increasing worldwide (Yamashita et al., 2013), malignant melanoma is considerably associated with poor prognosis, with an estimated 10-year survival rate of less than 10% (Hall & Kudchadkar, 2014).

Cancer cells in general, possess a broad spectrum of migration and invasion mechanisms including the destruction of tissue basement membrane and extracellular matrix (ECM) components allowing the migration of cancer cells (Bourboulia & Stetler-Stevenson, 2010). The matrix metalloproteinases (MMPs) group of enzymes play a major role in this destruction process that participates in the remodelling of the ECM to support the growth of tumour cells (Moro, Mauch, & Zigrino, 2014). These proteinases are involved in remodelling processes of normal tissue such as in morphogenesis, angiogenesis, tissue repair and embryonic development (Kessenbrock, Plaks, & Werb, 2010). The secretion of these enzymes is tightly controlled to regulate these normal functions; in which imbalance between the inhibition and activation of MMPs results in disturbances characterized by an excessive degradation of the ECM such as in osteoarthritis, rheumatoid arthritis & different types of malignancies (Frank et al., 2012; Hadler-Olsen, Winberg, & Uhlin-Hansen, 2013; Moro et al., 2014; Nissinen & Kähäri, 2014). Within the tumour mass, MMPs are expressed by cancer cells and to a larger extent by the surrounding normal cells within the tumour environment known

as cancer stromal cells participating in the cleavage of ECM and basement membrane components (Hadler-Olsen et al., 2013; Hashimoto et al., 2016).

This family of enzymes is classified by their structure and substrate specificity into collagenases, gelatinases, stromelysins, and matrilysins (Gialeli, Theocharis, & Karamanos, 2011a). Collagenases MMPs include collagenases-1 (MMP1), collagenase-2 (MMP8) and collagenase-3 (MMP13) which are capable of initiating degradation of native fibrillary collagens (type I, II, III, V and IX) and obviously play a crucial role in degradation of collagenous extracellular matrix (ECM) in various tumours (Moro et al., 2014). Both MMP1 and MMP13 are associated with accelerated invasive processes by cancer cells and are either secreted by the cancer cells or the tumour surrounding stroma (Gialeli et al., 2011a; Kim et al., 2011; Martinet, Hirsch, Mulshine, & Vignaud, 2012). Meanwhile, MMP8 is associated with some controversial effects within cancer microenvironment ranging from a promoter to an inhibitor in different malignancies (Schröpfer et al., 2010a; Thirkettle et al., 2013).

Generally, the expression of these collagenases is regulated at different levels and mechanisms; however, the most influential mechanism of control is at the transcriptional level at the noncoding promoter sequence via the specific binding of different transcription factors (Brown & Murray, 2015). The AP-1 DNA binding sites among the regulatory sites are present in the gene promoters of *MMP1* and *MMP13* gene and are mainly responsible for the transcriptional regulation of collagenases expression (Samuel, Beifuss, & Bernstein, 2007; Xia et al., 2015; Lu et al., 2016). The transcriptional regulation at this gene site is believed to participate actively in the progression of cancer cells by its binding with different oncogenic transcription factors expressed in cancer cells causing an increase in the expression of collagenases (Kessenbrock, Wang, & Werb, 2015).

CTCF and YB-1 transcription factors are among the many transcription factors that are involved with different regulatory roles in cancer cell development and progression. These factors are capable of specific binding with different gene promoter sequences and thereby can transcriptionally control the expression of different genes responsible for cancer cell proliferation and growth (H. Wang et al., 2015; Chen et al., 2016). CTCF is a well-defined tumour suppressor protein mainly regulating the epigenetic changes in cancer cells (Kemp et al., 2014). While, the YB-1 transcription factor is an oncogenic factor that is involved in stimulating different hallmarks of cancer cells (Lasham, Woolley, Dunn, & Braithwaite, 2013; Kemp et al., 2014).

Although both transcription factors might work in the opposite manner; however, some studies reported their interaction with each other in a way that modifies their binding specificity with gene promoter sequences (Samuel et al., 2007; Wallace, 2007). Both factors were reported in the transcriptional regulation of MMPs through interaction with different promoter sequences including the AP-1 site (Abdull Rasad et al., 2008). However, the influence of these transcription factors on the expression of collagenases MMPs is not well elucidated in melanoma cells or other types of cancer cells. Therefore, this study would elucidate the possible mechanism regulating the expression of collagenases MMPs in A375 malignant melanoma cell line *in vitro* including YB-1, CTCF transcription factors and stromal cells in melanoma microenvironment *in vitro*.

1.2 SUMMARY OF THESIS

The aim of this study was to evaluate and discuss the expression of collagenases MMPs in malignant melanoma cancer cells and to determine novel mechanisms or transcription factors capable of regulating the expression of these enzymes.

In Chapter 1 and Chapter 2, all the theoretical background was provided to introduce the reader to cancer cells biology and to comprehensively describe the different regulatory aspects of the expression of MMPs from transcription to translation.

In Chapter 3, all the methods used in this study were described including the reagents, equipment and the basic protocols with their optimizations. The basic techniques of cell culture maintenance were fully described. In addition, the full description of reverse transcription polymerase chain reaction (RT-PCR) protocol and analysis were described in details. The Western blotting protocol with optimizations and the analysis were also fully described in this thesis. The description and explanation of plasmid transfection, validation, and maintenance of the cells was also described and finally, the protocol of chromatin immunoprecipitation (ChIP) assay was also described.

In Chapter 4, the expression of all *MMPs* and *TIMPs* were screened in the melanoma cancer cells followed by evaluating the expression of collagenases MMPs, YB-1 and CTCF proteins in melanoma cells and in the stromal- cancer cells model by the use of the human skin fibroblasts cells. The results were fully analysed and discussed in the Chapter which provided the preliminary data for the implementation of the next Chapter.

In Chapter 5, a stable strain of melanoma cells with constant knockdown of YB-1 was established, validated and tested for its influence on the expression of collagenases MMPs, cancer cell proliferation and invasion *in vitro*.

In Chapter 6, the potential interaction of YB-1 and CTCF with the AP-1 promoter sequence of MMP13 was determined *in ex vivo* using ChIP assay to elucidate the possible protein – DNA interactions and their possible regulation of collagenases expression.