POLYMORPHISMS IN THE MATRIX METALLOPROTEINASE-2 AND TISSUE INHIBITOR OF METALLOPROTEINASE-2 GENE PROMOTERS: IMPLICATION IN SUSCEPTIBILITY AND AGGRESSIVENESS OF HEAD AND NECK CANCER

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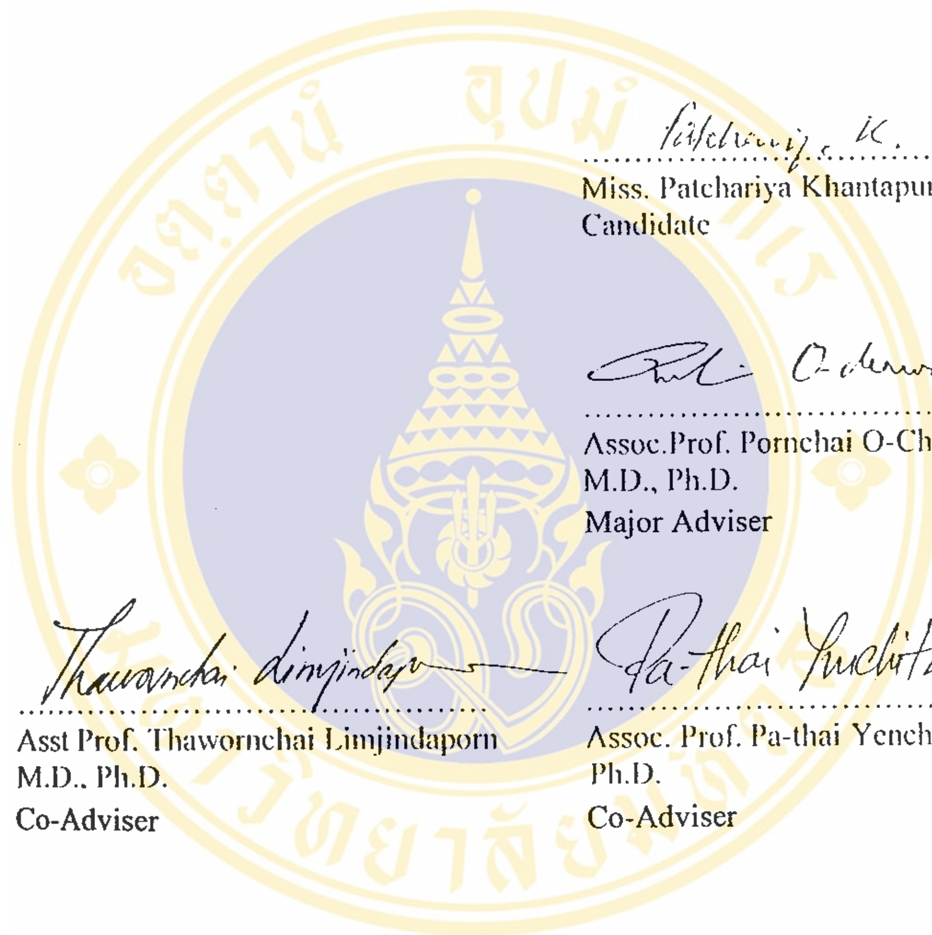

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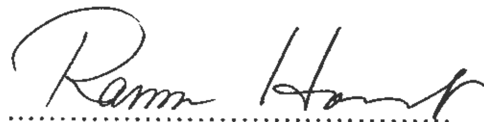

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Patchariya Khantapura.

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ABSTRACT

The matrix metalloproteinases (MMPs) and tissue inhibitor of metalloproteinases (TIMPs) have been shown to play important roles in all stages of cancer initiation and development. Single nucleotide polymorphisms identified in the promoters of *MMP-2* (-1306C/T) and *TIMP-2* (-418G/C) abolish the Sp1-binding site and thus down-regulate expression of the genes. This study aimed to evaluate the contribution of these polymorphisms on susceptibility and aggressiveness of head and neck squamous cell carcinoma (HNSCC).

A panel of HNSCC cell lines was examined for the *MMP-2* and *TIMP-2* genotypes and expression levels. Genomic DNA was extracted from peripheral blood of patients with newly diagnosed HNSCC and from age- and gender-matched cancer-free controls. *MMP-2* genotypes were determined by PCR-based allele-specific refractory mutation analysis in 239 HNSCC patients and 188 control subjects and *TIMP-2* genotypes identified by a PCR-restriction fragment length polymorphism in 194 HNSCC patients and 158 frequency matched control population. The levels of *MMP-2* mRNA expression in cell lines and cancer tissue specimens were evaluated by the semi-quantitative reverse transcription-PCR and the correlation with different genotype was determined. The MMP-2 activity was determined by gelatin zymography.

Fourteen out of 17 HNSCC cell lines showed the *C/C MMP-2* genotype (82%) and the majority of the cell lines had the *G/G TIMP-2* genotype (94.1%). Cell lines with the *MMP-2 C/C* genotype expressed significantly higher mean *MMP-2* mRNA level than those with other genotypes. We found that the variant *MMP2* genotypes (-1306C/T) were associated with substantially reduced risk of HNSCC [odds ratio (OR), 0.557; 95% confidence interval (0.337-0.921), compared with the C/C genotype. For *TIMP2*, a moderately reduced risk of the cancer (OR, 0.083; 95% CI, 0.052-1.327) was also associated with the variant genotypes (-418G/C or C/C), compared with the G/G common genotype. Furthermore, it seemed that the polymorphisms in the two genes had some additive effect and the reduced risk related to the polymorphisms appeared to be more pronounced in younger subjects. A correlation between promoter polymorphisms and the levels of *MMP-2* expression in cancer tissues was found, and C/C genotype from MMP-2 was correlated with the adverse clinicopathological parameters.

These findings suggest that the presence of the variant genotypes in the promoters of *MMP-2* or *TIMP-2* may be a protective factor for the development of HNSCC.

KEY WORDS: HEAD AND NECK CANCER/SQUAMOUS CELL CARCINOMA/MATRIX METALLOPROTEINASE/SINGLE NUCLEOTIDE POLYMORPHISM.

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ความสัมพันธ์ของ POLYMORPHISMS ใน MATRIX METALLOPROTEINASE-2 และ TISSUE INHIBITOR OF METALLOPROTEINASE-2 GENE PROMOTER กับความเสี่ยง และความรุนแรงของมะเร็งศรีษะและลำคอ (POLYMORPHISMS IN THE MATRIX METALLOPROTEINASE-2 AND TISSUE INHIBITOR OF METALLOPROTEINASE-2 GENE PROMOTER: IMPLICATION IN SUSCEPTIBILITY AND AGGRESSIVENESS OF HEAD AND NECK CANCER)

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บทคัดย่อ

Matrix metalloproteinase (MMPs) และ tissue inhibitor of metalloproteinases (TIMPs) มีบทบาทสำคัญในการเริ่มต้นและการลุกลามของมะเร็ง โพลีมอร์ฟิซึมในส่วนโปรโมเตอร์ของ *MMP-2* (-1360C/T) และ *TIMP-2* (-418G/C) ทำให้ SP-1ไม่สามารถจับกับโปรโมเตอร์ได้ การแสดงออกของยีนจึงลดลง การศึกษานี้มีจุดประสงค์เพื่อประเมินผลของโพลีมอร์ฟิซึมนี้ต่อความเสี่ยงและความรุนแรงของมะเร็งที่เกิดกับเซลล์แบบสแควมัสบริเวณศีรษะและลำคอ (HNSCC)

จีโนไทป์และการแสดงออกของยีน *MMP-2* และ *TIMP-2* ใน HNSCC cell lines ได้ถูกประเมิน จีโนมิกดีเอ็นเอถูกสกัดจากเลือดของผู้ป่วยที่ได้รับการวินิจฉัยว่าเป็น HNSCC และจากกลุ่มควบคุมที่มีอายุ เพศ เหมือนกับกลุ่มผู้ป่วย จีโนไทป์ของ *MMP-2* ถูกตรวจสอบโดย PCR-based Refractory Mutation System (ARMS) ในผู้ป่วย 239 ราย และในกลุ่มควบคุม 188 ราย จีโนไทป์ของ *TIMP-2* ถูกตรวจสอบโดย PCR-RFLP ในผู้ป่วย 194 ราย และในกลุ่มควบคุม 158 ราย ระดับการแสดงออกของ mRNA ของยีน *MMP-2* ใน cell lines และ ในชิ้นเนื้อมะเร็งถูกตรวจสอบโดย semi-quantitative reverse transcription-PCR และนำมาหาความสัมพันธ์กับจีโนไทป์ ความสามารถของ MMP-2 ถูกวัดโดย zymography

HNSCC 14 cell lines มี *MMP2* -1306C/C จีโนไทป์ (82%) และ 1 cell line มี *TIMP-2* -418C/C จีโนไทป์ (5.9%) cell line ที่มี C/C จีโนไทป์มี MMP-2 mRNA แสดงออกสูงกว่าจีโนไทป์อื่นอย่างมีนัยสำคัญ นอกจากนี้ *MMP-2* -1306C/T สัมพันธ์กับการลดความเสี่ยงต่อการเกิด HNSCC OR=0.557 [0.337-0.921] สำหรับ *TIMP-2* -418GC หรือ C/C สัมพันธ์กับการลดความเสี่ยงต่อการเกิด HNSCC OR= 0.264 [0.052-1.327] โพลีมอร์ฟิซึมใน2ยีนนี้มีผลเสริมกันและการลดความเสี่ยงต่อการเกิดมะเร็งจะมีผลมากในผู้ที่มีอายุน้อย ในทางคลินิก ผู้ที่มีจีโนไทป์นี้จะมีความเสี่ยงในการเกิดมะเร็งเป็น 2.4 เท่า (OR=0.4 โพลีมอร์ฟิซึมในส่วนโปรโมเตอร์ของ *MMP-2* มีความสัมพันธ์กับระดับของ mRNA ในเนื้อเยื่อมะเร็งและสัมพันธ์กับการพยากรณ์โรค

การศึกษานี้แสดงให้เห็นว่า การมีโพลีมอร์ฟิซึมในส่วนโปรโมเตอร์ของ *MMP-2* และ *TIMP-2* อาจเป็นปัจจัยป้องกันการเกิด HNSCC

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LIST OF ABBREVIATIONS

aa	Amino acid
ASA	Allele specific amplification
bp	Base pair
°C	Degree Celsius
cDNA	Complementary deoxynucleic acid
dGTP	Deoxyguanosine-5’-triphosphate
dATP	Deoxyadenosine-5’-triphosphate
dTTP	Deoxythymidine-5’-triphosphate
dCTP	Deoxycytidine-5’-triphosphate
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
DW	Distilled water
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
g	Gram
GPI	Glycosylphosphatidylinositol
hr	Hour
HNSCC	Head and neck squamous cell carcinoma
Ig	Immunoglobulin
kb	Kilobase
kDa	Kilodalton
l	Liter
µg	Microgram
µl	Microliter
M	Molar
MAb	Monoclonal antibodies
mg	Milligram
min	Minute

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LIST OF ABBREVIATIONS (Cont.)

ml	Milliliter
mM	Millimolar
MMP	Matrix metalloproteinase
nt	Nucleotide
OD	Optical density
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide
SNP	Single nucleotide polymorphism
TAE	Tris-acetate EDTA buffer
Taq	*Thermus aquaticus*
TE	Tris-EDTA buffer
TEMED	N, N, N', N' tetramethylenediamine
TIMP	Tissue inhibitor of metalloproteinase
Tm	Melting temperature
U	Unit
UV	Ultraviolet
%	Percent

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CHAPTER I

INTRODUCTION

Squamous cell carcinoma of the head and neck region (HNSCC) is a major problem world-wide. HNSCC is an epithelial cancer arising in the mucosa of the upper aerodigestive track; potential anatomic sites affected by this cancer include area in the oral caving, aeropharyns, hypopharynx and larynx. In the Western world HNSCCs represent 5% of newly diagnosed cancers, but the incidence accounts for up to 40% of all malignancies in Thailand and South East Asia (1). While the management of HNSCC has improved, there is no evidence to suggest that therapeutic advances have resulted in increased survival rates. Indeed, improvements in local control have led to an increase in presentation of distant metastases.

Cancer invasion and metastasis is a complex, multistep process involving active interactions between the invading cell, the extracellular matrix (ECM) and other stromal elements (2). The cooperation of multiple proteolytic enzymes which are secreted by tumor cells and/or host cells is required for cancer cells to invade the ECM and penetrate the lymphatic or blood vessel walls and metastasise to distant sites. ECM proteolysis is also required for the neoangiogenesis necessary for continued growth of solid tumors. The matrix metalloproteinases (MMPs), a family of proteolytic enzymes, are believed to play an important role in the process of tumor invasion and metastasis (3, 4). MMPs are low or absent in normal tissues and overexpressed in almost all type of human cancers (3, 5-8). These endopeptidases are capable of degrading both ECM and basement membrane, two physical barriers that play important roles in preventing expanding growth and migration of cancer cells. The MMPs are a family of highly homologous extracellular zinc- and calcium-dependant endopeptidases with enzymatic activity against virtually all protein components of the ECM (3, 9, 10). Most MMPs are secreted as latent proenzymes (zymogens) which undergo proteolytic cleavage of an amino terminal domain during

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activation. A multigene family of proteins named tissue inhibitors of MMPs (TIMPs) has been demonstrated to inhibit fully activated MMPs. TIMPs comprise at least 4 structurally related members, with TIMP-1 & -3 being glycoprotein whilst TIMP-2 & -4 are unglycosylated (11-15). The TIMPs provide a tightly regulated mechanism for control of MMP activation and function by forming high affinity, non-covalent, irreversible complexes with the active forms of the proteinases. The expression of multiple MMPs and TIMPs has been demonstrated, as a characteristic of HNSCC. The correlative studies of MMP/TIMP expression suggest the potential role of MMP-2, MMP-7, MMP-9 and MMP-11 in progression and metastasis of human HNSCC (16-21). While several MMPs have been implicated in HNSCC progression, the mechanism(s) which lead to their over expression *in vivo* are largely unknown.

One member of the MMP gene family, gelatinase-A or MMP-2, has received considerable attention as a mediator of tumor angiogenesis and metastatic capability (22-24). MMP-2 (also known as 72 kDa gelatinase or type IV collagenase) is a member of the MMP family that primarily hydrolyzes gelatin and type IV collagen, the major structural component of the basement membrane. The production of this proteinase in tumors is not only by cancer cells but also by normal stromal cells and endothelial cells, suggesting that the over expression of MMP-2 is probably due to the transcriptional changes and not gene amplification or an activating mutation. Because human *MMP-2* promoter contains a number of cis-acting regulatory elements, the constitutive and induced expression of this proteinase is likely to be subject to regulation by transcription factors (25). Although somatic mutation of the *MMP-2* gene in cancer has not been reported so far, several single nucleotide polymorphisms (SNPs) in the *MMP-2* promoter region have been identified (26). Among them, a C/T transition located at nucleotide -1306 abolishes the Sp1- binding site and consequently diminishes promoter activity (27). Transient transfection experiment shows that reporter gene expression driven by the C allelic *MMP-2* promoter was significantly greater than reporter gene expression driven by the T allelic counterpart in both epithelial cells and in macrophages, indicating the functional significance of this polymorphism (27, 28).

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Of the four members in the TIMP family, TIMP-2 is particularly interesting because of its dual functions in terms of regulating MMP-2 activity (18) and its paradoxical effects on certain cancers. TIMP-2, 21 kDa proteins, binds preferentially to MMP-2 but also inhibits the activities of MMP-1, -3, -7 & -9 (12, 13). Intrinsic TIMPs are known to be inhibitors of the strong invasive activities of matrix metalloproteinases in malignant gliomas. An over expression of TIMP-2 has been shown to inhibit invasive and metastatic activity of tumor cells and decrease tumor growth in vivo. A SNP (-418G/C) has also been identified in the promoter of the *TIMP-2* gene (29-31). Although the functional significance of this germ line polymorphism is currently unknown, down regulation of the transcriptional activity due to the variant has been suggested because the G/C substitution is located within the consensus sequence for the Sp1-binding site in the promoter region of *TIMP-2*. It is, therefore, reasonable to assume that the this polymorphism may down regulate TIMP-2 expression which consequently cause an imbalance between the activities of TIMP-2 and MMP-2, which is believed to have a significance effect on cancer development and progression. Although recent molecular epidemiological studies have suggested that genetically determined matrix-degrading capacity may contribute to the risk and progression of some tumor types (30, 31), so far study in HNSCC has not yet been performed.

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CHAPTER II

OBJECTIVES

Primary Objective:

To determine whether individuals with genetic polymorphisms in promoters of the *MMP-2* and *TIMP-2* is associated with the risk and aggressiveness of HNSCC.

Secondary Objectives:

1. To determine the genotype distribution of polymorphisms in promoters of *MMP-2* and *TIMP-2* in Thai population.
2. To set up the database and the bio-bank for head and neck cancer at Siriraj hospital.

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CHAPTER III

LITERATURE REVIEW

3.1. Head and neck squamous cell carcinoma

HNSCC is the sixth most common cancer worldwide, with a lifetime risk of 2 percent for men and 0.6 percent for women. In the Western world HNSCCs represent 5% of newly diagnosed cancers, but the incidence accounts for up to 40% of all malignancies in Thailand and South East Asia. HNSCC is an epithelial cancer arising in the mucosa of the upper aerodigestive tract. Potential anatomic sites affected by this cancer include oral cavity, oropharynx, hypopharynx and larynx. While the diagnosis of HNSCC has improved, there is no evidence to suggest the increased in therapeutic advances which would result in an improvement of survival rates. Indeed, improvements in local control have led to an increase in penetration to the distant metastasis. The clinical observation that patients of HNSCC have in similar is the comparable stages of tumor leading to different clinical courses and may respond differently to similar treatment administration which is yet to be adequately explained and proven. Clearly, a more sophisticated understanding of the pathogenesis of these tumors and the identification of better biological markers could provide useful information for predicting outcome and individualizing treatment.

There are significant risk factors that contribute to HNSCC. A few are genetic which predisposes one to developing a cancer later in life. Others are environmental or behavioral based, such as the use of tobacco products, second hand smoke and alcohol. Tobacco and alcohol exposure are the most important environmental factors that contribute to the development of HNSCC. In addition to influences of exposure to carcinogenic compounds, the development of cancer may depend on individual intrinsic cancer susceptibility.

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3.2.Mechanisms of cancer invasion and metastasis

Cancer invasion and metastasis is a complex, multistep process involving active interactions between the invading cell, the ECM and other stromal elements. The capability of tumor cells to metastasise is aided by following biological events which include morphological changes and tumor cell locomotion, degradation of stroma and basement membranes through the production of matrix degradative enzymes, adhesion to microvessel endothelia and ECM and autonomous growth at distant sites. The cooperation of multiple proteolytic enzymes which are secreted by tumor cells and/or host cells is required for cancer cells to invade the ECM and penetrate the lymphatic or blood vessel walls and metastasise to distant sites (32). ECM proteolysis is also required for the neoangiogenesis necessary for continued growth of solid tumors (33). HNSCC is characterized by its capacity to invade adjacent tissues and metastasize loco-regionally. The presence or absence of local and regional disease primarily determines the treatment and the outcome among these patients. Nonetheless, although the incidence of distant metastases is relatively low, as compared with that of other cancers of the upper aerodigestive tract, distant metastases will develop in almost one third of patients.

3.3. Matrix metalloproteinases (MMPs)

Accumulating evidence indicates that, among the proteolytic enzymes implicated in cancer, the MMPs may play a casual role in progression. Proteolytic enzymes are classified as either exopeptidases or endopeptideases based on whether they cleave terminal or internal peptide bonds, respectively. Most endopeptidases are classified as serine; cysteine, aspartic or metalloproteinases based on their catalytic mechanism and inhibitor sensitivities, and the MMPs are further separated into five superfamilies based on sequence considerations. Of these, the metzincin superfamily is distinguished by a highly conserved motif containing three histidines that bound zinc at the catalytic site and conserved methionine turn that sits beneath the active site Zn and Ca dependant endopeptidase can collectively degrade virtually all protein components of the ECM, thereby regulating cell behavior in several ways.

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3.4. MMP structure and function

The MMPs constitute a multigene family of over 25 secreted and cell surface enzymes that process or degrade numerous pericellular substrates. Their targets include other proteinases, proteinase inhibitors, clotting factors, chemotactic molecules, latent growth factors, growth factor–binding proteins, cell surface receptors, cell-cell adhesion molecules, and virtually all structural ECM proteins (32). Thus MMPs are able to regulate many biologic processes and are closely regulated themselves. In addition to being differentially regulated at the level of transcription, MMPs can be controlled at the protein level by their endogenous activators and inhibitors and by factors that influence their secretion, their cell surface localization, and their own degradation and clearance. Thus like the many proteins they modify, the MMPs influence diverse physiologic and pathologic processes, including aspects of embryonic development, tissue morphogenesis, wound repair, inflammatory diseases, and cancer.

The first group (collagenous) degrade type I, II, III, and fibrillar collagens and are represented by MMP-1 (intenstitial collagenous, collagenous-1), MMP-8 (nutrophil collagenous, collagenous-2) and MMP-13 (collagenous-3). They all cleave fibillar collagens at a specific site, resulting in generation of amino-terminal ¾ and carboxyl-terminal fragments, which then rapidly denature at body temperature and are further degraded by the other MMPs. Stromelysins represent the second group and have 4 members: MMP-7 (matrilysin, PUMP-1) contains the minimal number of domains, i.e. a predomain, a prodomain and a catalytic domain, MMP-3 (stromelysin-1), MMP-10 (stromelysin-2) and MMP-11 (stromelysin-3) contain an additional carboxy-terminal haemopexin-like domain. The stromelysins have broad substrate specificity and a capable of degrading many extracellular components, for example laminin, fibronectin, proteoglycans and the nonhelical portions of basement membrane collagens. Gelatinases-A and -B (MMP-2 and MMP-9, respectively) are further distinguished from other type of MMP family by the insertion of three head-to-tail cysteine-rich repeats within their catalytic domain. These inserts resemble the collagen-binding type II repeats of fibronectin and are required to bind and cleave collagen and elastin (34), (35). Finally, the membrane-type (MT) MMPs have a single-

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pass transmembrane domain and a short cytoplasmic C-terminal tail (MMPs 14, 15, 16, and 24) or a C-terminal hydrophobic region that acts as a glycophosphatidyl inositol (GPI) membrane anchoring signal (MMP- 17 and MMP- 25) (36, 37). These domains play an essential role in the localization of several important proteolytic events to specific regions of the cell surface.

Matrix metalloproteinase (MMP)-2 and -9 degrade type IV collagen, which is one of the major components of the basement membrane in normal tissue and expressed in the surroundings of the cancer nest in squamous cell carinoma. The degeneration of type IV collagen is an essential step in the metastasis to lymph nodes and distant organs. Although gelatinase-A regulatory control has been frequently characterized as “constitutive” in nature so by examining gelatinase-A patterns of expression during development or disease states is consistent with multiple levels of cellular control. Given these considerations, several groups gave attempted to define the transcriptional regulatory elements that provide the cellular- or tissue-specific levels of gelatinase-A synthesis. Studies have been performed within both the rat and human genomic contexts and have characterized several motifs operative within defined cellular types (38-41). Studies demonstrated that a proximal, overlapping binding site for transcription factors AP-2, Sp1, and Sp3 in the human gelatinase-A promoter is responsible for high level gelatinase-A transcription in glioblastoma cell lines (42). The domain structure of the MMP family are further classified according to their domain structure and shown in Figure 1.

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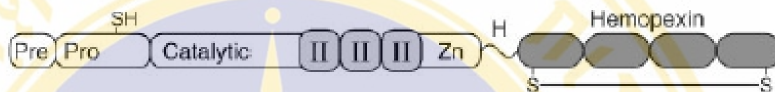
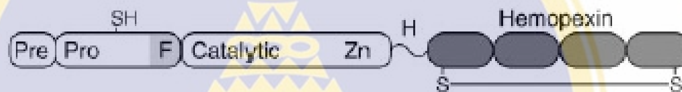
A) Minimal Domain MMPs (MMP7/matrilysin, MMP26/endometase)

B) Simple Hemopexin Domain-Containing MMPs
(MMP1/collagenase-1, MMP8/collagenase-2, MMP13/collagenase-3, MMP18/collagenase-4, MMP3/stromelysin-1, MMP10/stromelysin-2, MMP27, MMP12/metalloelastase, MMP19/RASI-1, MMP20/enamelysin, MMP22/CMMP)

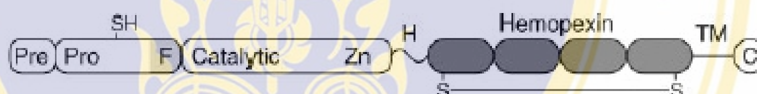
C) Gelatin-binding MMPs (MMP2/gelatinase A, MMP9/gelatinase B)



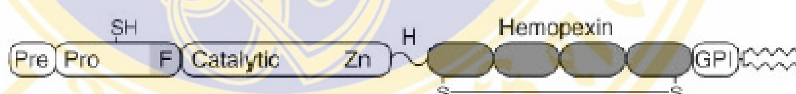
D) Furin-activated Secreted MMPs (MMP11/stromelysin-3, MMP28/epilysin)



E) Transmembrane MMPs
(MMP14/MT1-MMP, MMP15/MT2-MMP, MMP16/MT3-MMP, MMP24/MT5-MMP)



F) GPI-linked MMPs (MMP17/MT4-MMP, MMP25/MT6-MMP)



G) Vitronectin-like Insert Linker-less MMPs (MMP21/XMMP)

Figure 1. Domain structure of the MMPs. Pre, signal sequence; Pro, propeptide with a free zinc-ligating thiol (SH) group; F, furin-susceptible site; Zn, zinc-binding site; II, collagen-binding fibronectin type II inserts; H, hinge region; TM, transmembrane domain; C, cytoplasmic tail; GPI, glycophosphatidyl inositol-anchoring domain; C/P, cysteine/proline; IL-1R, interleukin-1 receptor. The hemopexin/vitronectin-like domain contains four repeats with the first and last linked by a disulfide bond.

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3.5. Regulation of MMP activity

To accomplish their normal (or pathologic) functions, MMPs must be present in the right cell type and pericellular location, at the right time, and in the right amount, and they must be activated or inhibited appropriately. Thus MMPs are tightly regulated at the transcriptional and post-transcriptional levels and are also controlled at the protein level via their activators, their inhibitors, and their cell surface localization.

3.6. Transcriptional regulation

Accordingly, most MMPs are closely regulated at the level of transcription, with the notable exception of MMP-2, which is often constitutively expressed and controlled through a unique mechanism of enzyme activation (43) and at some degree at post-transcriptional mRNA stabilization (44). Nevertheless, data indicate that the basal expression of MMP-2, MMP-14 (MT1-MMP), and TIMP-2 is co-regulated, which is consistent with their cooperation during MMP-2 activation and with specific similarities in their gene promoters (45). In addition, a functional p53-binding site has been identified in the MMP-2 gene promoter, and wild-type p53 down regulates basal and inducible MMP-1 gene expression in human fibroblasts and osteogenic sarcoma cells, whereas some mutant forms do not (46). On the other hand, the down regulation of p53 using SV40 T-antigen suppresses the expression of MMP-2, MMP-3, and MMP-9 in human placental trophoblast-like cells (47). Despite numerous advances in our understanding of MMP gene regulation, the cross-talk between the many signaling pathways, nuclear factors, and gene regulatory elements that regulate MMP expression are barely understood. Basal and inducible levels of MMP gene expression can also be influenced by genetic variations that may, in turn, influence the development or progression of several diseases. Common bi-allelic SNPs that influence the rate of transcription have been identified within several MMP gene promoters (48). A C/T SNP located -1306 bp upstream of the MMP-2 transcription start site contains either a cytidine or thymidine, such that the less common T allele disrupts an otherwise functional Sp1-binding site and diminishes promoter activity by about 50% (28, 49).

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3.7. Inhibitors and inhibition of MMP activity

In the ECM, the activity of MMP is controlled by specific inhibitors known as tissue inhibitors of MMPs (TIMPs). TIMPs are small (21-28 kDa), multi-functional proteins that regulate MMP function both at the level of their activation and in their ability to hydrolyze a particular substrate. The MMPs inhibition by TIMPs occurs in a 1:1 stoichiometry and non-covalent fashion. Four members of TIMP family have been so far described. They share 37–51% overall sequence identity, a conserved gene structure, and 12 similarly separated cysteine residues. These invariant cysteines form six intrachain disulfide bridges to yield a conserved six-loop, two-domain structure. Truncated "tiny" TIMPs 1 and 2 retain their inhibitory activity despite containing only the first three loops, thus indicating that portions of the N-terminal domain interact with the MMP catalytic site (50). Studies have revealed which TIMP residues interact directly with the MMP-3 catalytic domain and how they inhibit MMP activity. Although these studies indicate that the inhibitory activity of the TIMPs resides almost entirely in the N-terminal domain alone, both domains influence enzyme-inhibitor binding (51-53). For example, the C-terminal domain (loops 4–6) of TIMP-1 binds the hemopexin domain of MMP-9 more readily than it does the hemopexin domain of MMP-2, whereas the C-terminal domain of TIMP-2 preferentially binds the hemopexin domain of MMP-2 (50, 54). Individual TIMPs differ in their ability to inhibit various MMPs (55). There is a long history indicating that TIMPs exert growth-promoting activity independent of their metalloproteinase inhibitory activity. Indeed, TIMP-1 was first cloned for its erythroid-potentiating activity, and TIMPs 1, 2, and 3 have since been shown to act as mitogens in several other cell types (56). Although it is still unclear how TIMPs promote cell growth, this activity may explain several unexpected associations between TIMPs and cancer progression. However, TIMPs may also promote cell death or suppress mitogenic signals. TIMP-2, on the other hand, can suppress growth factor-responsiveness by interfering with the activation of tyrosine kinase-type growth factor receptors, and its ability to block mitogenic signaling is independent of its MMP-inhibitory activity. Therefore, these growth-altering activities may still reflect the ability of TIMPs to indirectly modify MMP activity. TIMPs are not the only endogenous MMP inhibitors although their activity against MMP-2 is substantially lower (57), other MMPs or metzincins may be

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their true physiologic targets. Another means of localizing MMPs to the cell surface is via cell surface docking receptors. For example, activated MMP-2 can bind to integrin αv/β3 on the surface of angiogenic endothelial cells and invasive cancer cells (58). Because the C-terminal domain of MMP-2 is required for the formation of integrin αv/β3 MMP-2 complexes in vitro, the catalytic domain probably remains exposed so that it can still carry out proteolysis. Interestingly, MT1-MMP generates only an MMP-2 activation intermediate, and another already activated MMP-2 is required to remove the residual portion of the MMP-2 propeptide and achieve full MMP-2 activation (59). Thus cell surface MMP-2 receptors may cooperate with MT1-MMP to facilitate MMP-2 maturation, and data suggest that integrin αv/β3 promotes such maturation by providing a platform for autocatalytic interactions between fully and partially activated MMP-2 (59). Moreover, colocalization data suggest that integrin αv/β3 may also corperate with MT1-MMP in the clustering of active MMP-2. Figure 2 summarizes the mechanism of the cell- surface activation of the MMP-2.

3.8. Regulation of MMP secretion

MMP-2 is refractory to activation by serine proteinases and is instead activated at the cell surface through a unique multistep pathway involving MT-MMPs and TIMP-2 (43). Indeed, MT1-MMP is a particularly efficient MMP-2 activator, whereas MT4-MMP and human (but not mouse) MT1-MMP are the only MT-MMPs that are unable to activate MMP-2. First, a cell surface MT-MMP binds and is inhibited by the N-terminal domain of TIMP-2, and the C-terminal domain of the bound TIMP-2 acts as a receptor for the hemopexin domain of ProMMP-2. Then, an adjacent, uninhibited MT-MMP cleaves and activates the tethered ProMMP-2. Following the initial cleavage of ProMMP-2 by MT1-MMP, a residual portion of the MMP-2 propeptide is removed by another MMP-2 molecule to yield a fully active, mature form of MMP-2 (59). It has been assumed that proteolytic removal of the MT-MMP prodomain by furin-like enzymes in the trans-Golgi network or by plasmin at the cell surface (60) is required for MT-MMPs to activate MMP-2. Therefore, the MT1-MMP prodomain is actually required for the cell surface activation of MMP-2 to proceed, but it does not have to remain covalently attached. Furthermore, co-expression of the MMP-1 prodomain instead of the MT1-MMP prodomain does not rescue the ability of

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prodomain-deleted MT1-MMP to activate MMP-2, thus indicating that the MT1-MMP prodomain has specific attributes that enable it to do so. Direct exogenous addition of the MT1-MMP prodomain also fails to restore the function of processed MT1-MMP, and whereas full-length MT1-MMP is prominently expressed at the cell surface, much of the prodomain-deleted MT1-MMP is retained in the secretory pathway. Therefore, the MT1-MMP propeptide may act as an intramolecular chaperone that is necessary for the efficient trafficking of MT1-MMP to the cell surface. Although processed MT1-MMP is eventually expressed at the cell surface, it lacks the ability on its own to bind TIMP-2 or activate MMP-2. MT1-MMP may also be conformationally activated through interactions with the cell membrane, and its retained propeptide domain may facilitate the binding of TIMP-2. The role of TIMP-2 in MMP-2 activation is its dominant in vivo function, as shown by targeted mutagenesis in mice (61). Nevertheless, while the C-terminal domain of TIMP-2 participates in the cell surface docking and activation of MMP-2, its N-terminal domain is an MMP inhibitor. TIMP-2 protein levels are reduced and MMP-2 activation is enhanced in the presence of the MMP-2 substrate, type IV collagen (57). Furthermore, the ability of collagen to induce MMP-2 activation on demand probably results from TIMP-2 degradation because there are no accompanying changes in MMP-2, MT1-MMP, or TIMP-2 mRNA expression or in the synthesis or activation of MT1-MMP. Therefore, local accumulation of type IV collagen may trigger its own degradation by somehow lowering local TIMP- 2 concentrations to levels that favor MMP- 2 activation. Figure 3 summarizes different levels from which the MMP are regulated.

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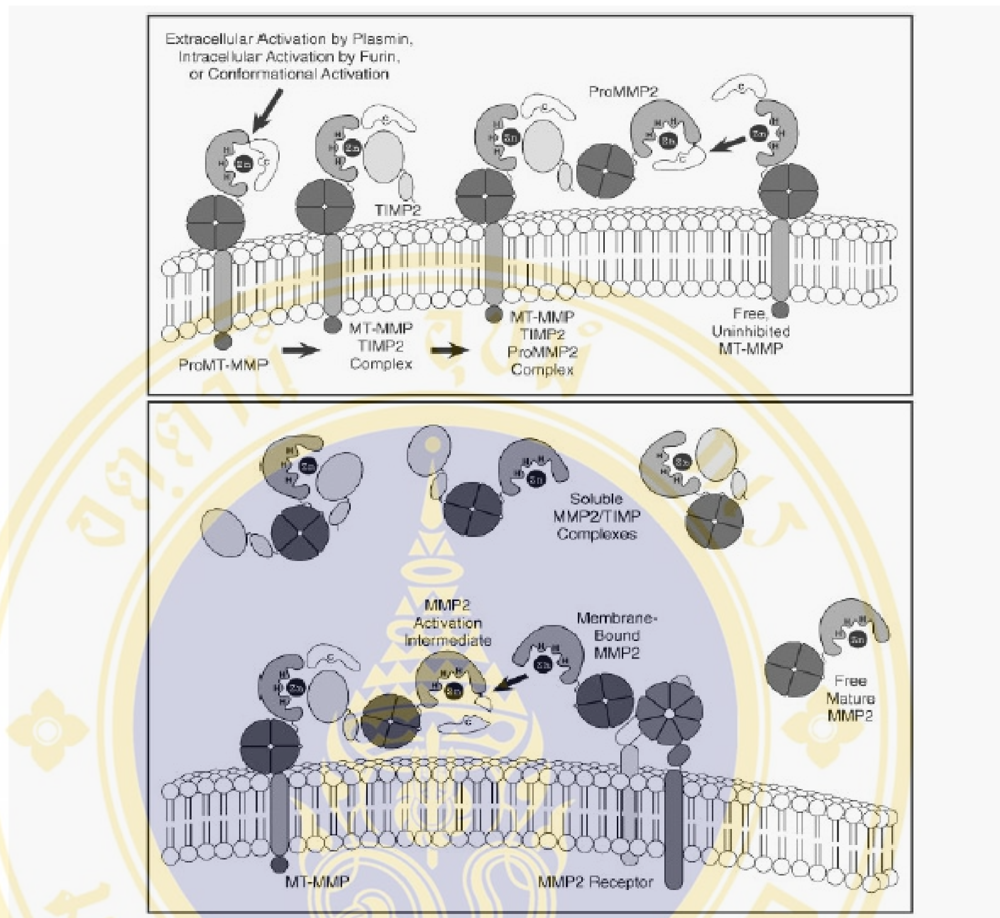

Figure 2. Cell surface activation of MMP-2. A ProMT-MMP is activated during transport to the cell surface by an intracellular furin-like serine proteinase, at the cell surface by plasmin, or by non-proteolytic conformational changes. The activated MT-MMP is then inhibited by TIMP-2 and the hemopexin domain of ProMMP-2 binds to the C Terminal portion of TIMP-2 to form a trimolecular complex. An uninhibited MT-MMP then partially activates the ProMMP-2 by removing most of the MMP-2 propeptide. The remaining portion of the propeptide is removed by a separate MMP-2 molecule at the cell surface to yield fully active mature MMP-2. Mature MMP-2 can then be released from the cell surface or bound by another cell surface MMP-2 docking protein. It can also be inhibited by another TIMP molecule or left in an uninhibited active state depending on local MMP: TIMP molar ratios.

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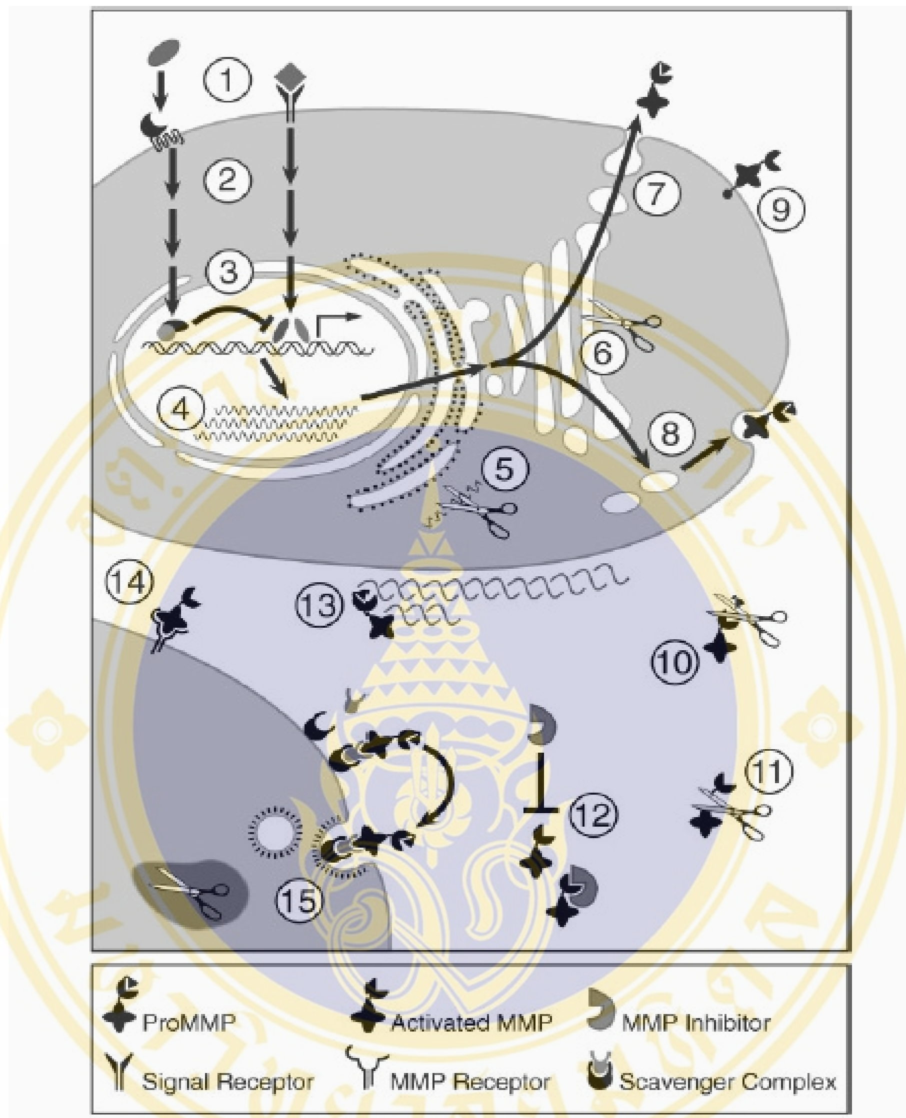

Figure 3. Regulation of the MMPs. MMP regulatory mechanisms include inductive and suppressive signaling (1), intracellular signal transduction (2), transcriptional activation and repression (3), post-transcriptional mRNA processing (4), mRNA degradation (5), intracellular activation of furin-susceptible MMPs (6), constitutive secretion (7), regulated secretion (8), cell surface expression (9), proteolytic activation (10), proteolytic processing and inactivation (11), protein inhibition (12), ECM localization (13), cell surface localization (14), and endocytosis and intracellular degradation (15).

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3.9. MMPs in cancer

MMPs are generally present in greater amounts and activated more often in and around malignant cancers than in normal, benign, or premalignant tissues, with the highest expression taking place in areas of active invasion at the tumor-stroma interface (62). Indeed, several MMPs were first cloned and have been repeatedly re-cloned as cancer-associated genes (63). Significant positive correlations have been found between MMP expression and various indicators of a poor prognosis in virtually all types of cancer, and in some instances, increased MMP levels represent an independent predictor of shortened disease-free and overall survival (64). Thus MMPs from adjacent stromal cells are often induced and commandeered by the malignant epithelial cells. In addition to the extensive correlative data linking MMP overexpression with more aggressive malignant behavior and poor clinical outcome, compelling experimental data show that MMPs actively contribute to cancer progression. Without the aid of ECM-degrading MMPs, endothelial cells would probably be unable to penetrate the ECM, and cancer cells would be unable to cross the matrix barriers that otherwise contain their spread. However, recent data indicate that MMPs do far more to influence cancer than merely remove the physical barriers to invasion and metastasis. These data support the notion that MMPs can contribute to virtually all stages of cancer evolution, both early and late, thus expanding the potential clinical utility of MMP inhibitors. Conversely, some MMPs may defy cancer progression, and others may not participate in cancer, but undoubtedly play normal physiologic roles. These possibilities must be considered and the mechanisms underlying the influence of MMPs in cancer must be understood in the design of therapeutic agents in order to optimize their efficacy and minimize their toxicity. For TIMP-2, conflicting data from clinical samples may also reflect the fact that it participates both in the activation and inhibition of MMP-2 in a dose-dependent manner. Thus increasing levels of TIMP-2 should increase MMP-2 activation to a point, after which the activation and proteolytic activity of MMP-2 would be blocked by the inhibitory activity of TIMP-2. Ultimately, the TIMPs may block some aspects of cancer by inhibiting certain MMPs, yet promote other aspects of cancer by inhibiting different MMPs and by influencing cells in a metalloproteinase-independent manner. Although it is clearly difficult to distinguish whether MMP-

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independent and -dependent TIMP activities are involved in a given process, it will be necessary to do so in order to gain a better understanding of their role in cancer.

3.10. Polymorphism in matrix metalloproteinase gene promoter

Recently, naturally occurring sequence variation has been detected in the promoter of a number of MMP genes. These genetic polymorphisms have been shown to have allele-specific effects on the transcriptional activities of MMP gene promoters, and to be associated with susceptibility to coronary heart disease, aneurysms and cancers. These findings indicate that variation in the MMP genes may contribute to inter-individual differences in susceptibility to these common, complex diseases, likely through effects on the balance between the synthesis and degradation of ECM proteins. Gene promoters are regions that control gene transcription. Recently, DNA polymorphisms have been found in the promoter region of several MMPs. Polymorphism represents natural sequence variants (alleles), which may occur with more than one form, having a frequency greater than 1% in a human population. Approximately, ninety percent of DNA polymorphisms are SNP due to single base exchange.

A SNP in the promoter region of human MMP- 1 gene has been described. An insertion/deletion of a guanine at position –1607 creates two different alleles, one having a single guanine (1G) and the other having two guanines (2G). The 2G allele together with an adjacent adenosine creates a core binding site (5‘-GGA-3‘) for Ets family of transcription factors that increases the transcriptional activity significantly. Tumors bearing the 2G allele can secrete higher levels of MMP-1, and the presence of this allele was associated with the development of ovarian cancer. A 5A/6A polymorphism has been reported in the MMP-3 (stromelysin-3) gene promoter (64).

MMP-2, among other MMPs, primarily hydrolyzes type IV collagen, the major structural component of basement membrane. MMP-2 structure and their location are shown in Figure 4. This proteinase also has high activity toward many other bioactive molecules, such as growth factor-binding proteins and growth factor receptors, e.g., MMP-2 can cleave insulin-like growth factor-binding proteins and release insulin-like

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growth factors, which are well known to have a strong effect on stimulating cell proliferation and inhibiting apoptosis. These activities of MMP-2 are believed to be linked to both cancer development and progression. In contrast to the majority of MMPs, MMP-2 is constitutively expressed by a large number of cell types and over expressed in a wide variety of human cancer, including gastro esophageal cancer (28), breast cancer (27), lung cancer (49) and other type of cancer (65-67). Although the activity of MMP-2 is known to be regulated by post-transcriptional mechanisms, including the activation of proenzyme and inhibition of enzyme activity, transcriptional regulation is also believed to be pivotally important because the human MMP- 2 promoter contains a number of cis-acting regulatory elements, and several transcription factors, including p53, Sp1, Sp3, and AP-2, participate in the control of its constitutive expression (42). Recently, Price et al. reported a single nucleotide polymorphism in the promoter of the MMP-2 gene (-1306C/T). The -1306C/T transition is located in a core recognition sequence of Sp1 (CCACC box), which abolishes the Sp1-binding site and consequently diminishes promoter activity.The effects of this SNP is shown in Figure 4. Transient transfection experiments showed that reporter gene expression driven by the C allelic MMP-2 promoter was significantly greater than reporter gene expression driven by the T allelic counterpart both in epithelial cells and macrophages, indicating the functional significance of this polymorphism.

The cancers in which an effect for MMP-2 has been established are characterized by varying individual susceptibility, implying the role of genetic factors. However, like many MMPs, MMP-2 is not up-regulated by gene amplification or activating mutations, and genetic alternations in the gene of the cancer cells are generally lacking. Therefore, germ-line polymorphisms that alter constitutive and/or induced expression and enzyme activity of MMP-2 may affects individual susceptibility to certain cancers. It has been hypothesized that the MMP-2 -1306C alleles might render increased risk for carcinogenesis because of an elevated level of MMP-2 expression over a lifetime. The molecular epidemiological study have shown that the frequency of the MMP- 2 -1306C/C genotype was significantly higher in patients with lung cancer than in the healthy population, and this genotype was

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associated with several folds of increased risk of lung cancer solely or in a manner of interaction with smoking exposure (49) .

Besides transcriptional regulation, the activity of MMP-2, among other MMPs, is also regulated by endogenous factors, including a family of anti-proteinases known as tissue inhibitors of metalloprotinases (TIMPs) (56). A multigene family of proteins named TIMPs has been demonstrated to inhibit fully activated MMPs. TIMPs comprises at least 4 structurally related members, with TIMP-1 & -3 being glycoproteins whilst TIMP-2 & -4 is unglycosylated. The TIMP provide a tightly regulated mechanism for control of MMP activation and function by forming high affinity, non-covalent, irreversible complexes with the active forms of the proteinases. Of the four members in the TIMP family, TIMP-2 is particularly interesting because of its dual functions in terms of regulating MMP-2 activity (61, 68) and its paradoxical effects on certain cancers (69-71). A single nucleotide polymorphism (-418G/C) has also been identified in the promoter of the *TIMP-2* gene (72). Although the functional significance of this germline polymorphism is currently unknown, down regulation of the transcriptional activity due to the variant has been suggested because the G/C substitution is located within the consensus sequence for the Sp1-binding site in the promoter region of *TIMP-2* (73) and this polymorphism has been associated with chronic obstructive pulmonary disease (72). It is, therefore, reasonable to assume that the this polymorphism may down regulate TIMP- 2 expression which consequently cause an imbalance between the activities of TIMP- 2 and MMP- 2 , which is believed to have a significance effect on cancer development and progression. TIMP-2, 21 kDa proteins, binds preferentially to MMP-2 but also inhibits the activities of MMP-1, 3, 7 & 9. Intrinsic TIMPs are known to be inhibitors of the strong invasive activities of matrix metalloproteinases in malignant gliomas. TIMP-2 is a member of the family of tissue inhibitor of metalloproteinases. An overexpression of TIMP-2 has been shown to inhibit invasive and metastatic activity of tumor cells and decrease tumor growth in vivo. TIMP-2 gene structure and chromosome location including effects of the SNP on the Sp1 transcription binding activity is shown in Figure 5.

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MMP- 2; Matrix metalloproteinase 2

(gelatinase A, 72kD gelatinase, 72kD type IV collagenase)

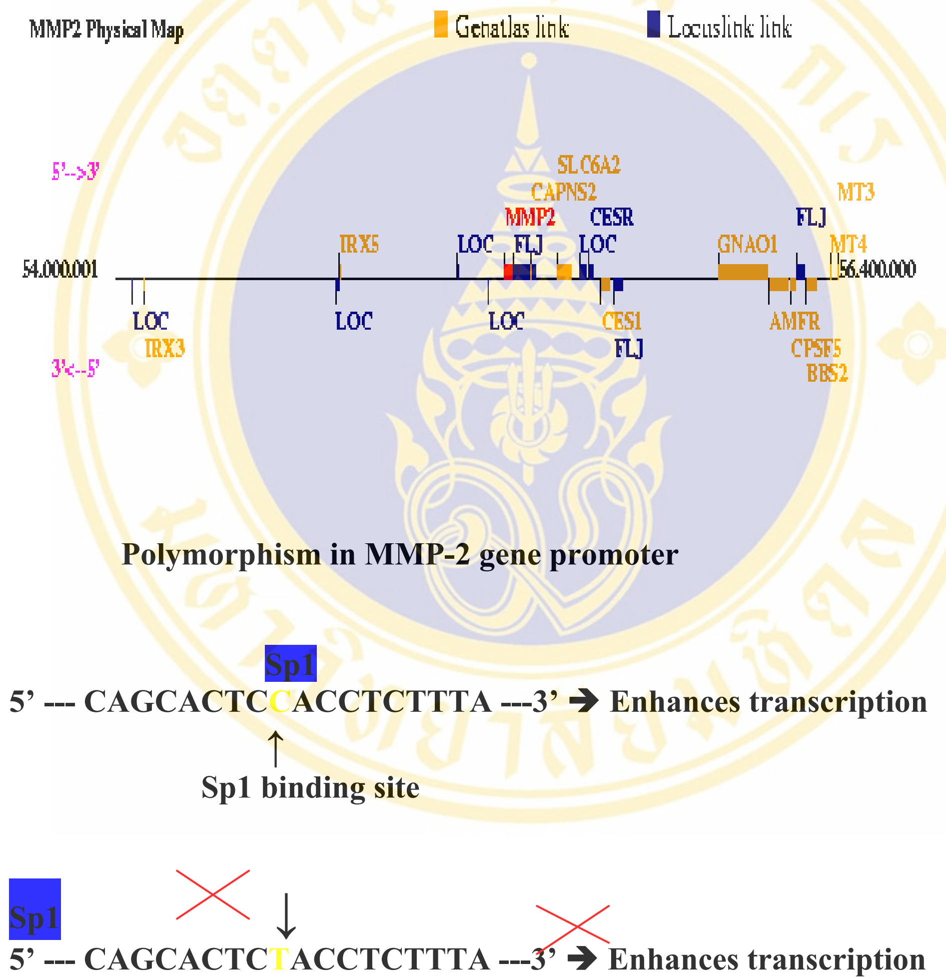
Chromosome: **16q13-q21**

Location: 16q13

Aliases: CLG4A, CLG4

Structure 2,705kb;

EXONS 13



Polymorphism in MMP-2 gene promoter

Sp1

5' --- CAGCACTCCACCTCTTTA ---3' ➔ Enhances transcription

↑

Sp1 binding site

Sp1

↓

5' --- CAGCACTCTACCTCTTTA ---3' ➔ Enhances transcription

Figure 4. MMP-2 gene structure & SNP mechanism

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TIMP-2; Tissue inhibitor of metalloproteinase-2

- **Location** 17q25..3
- **Structure** 18,37 kb; 4 Exons

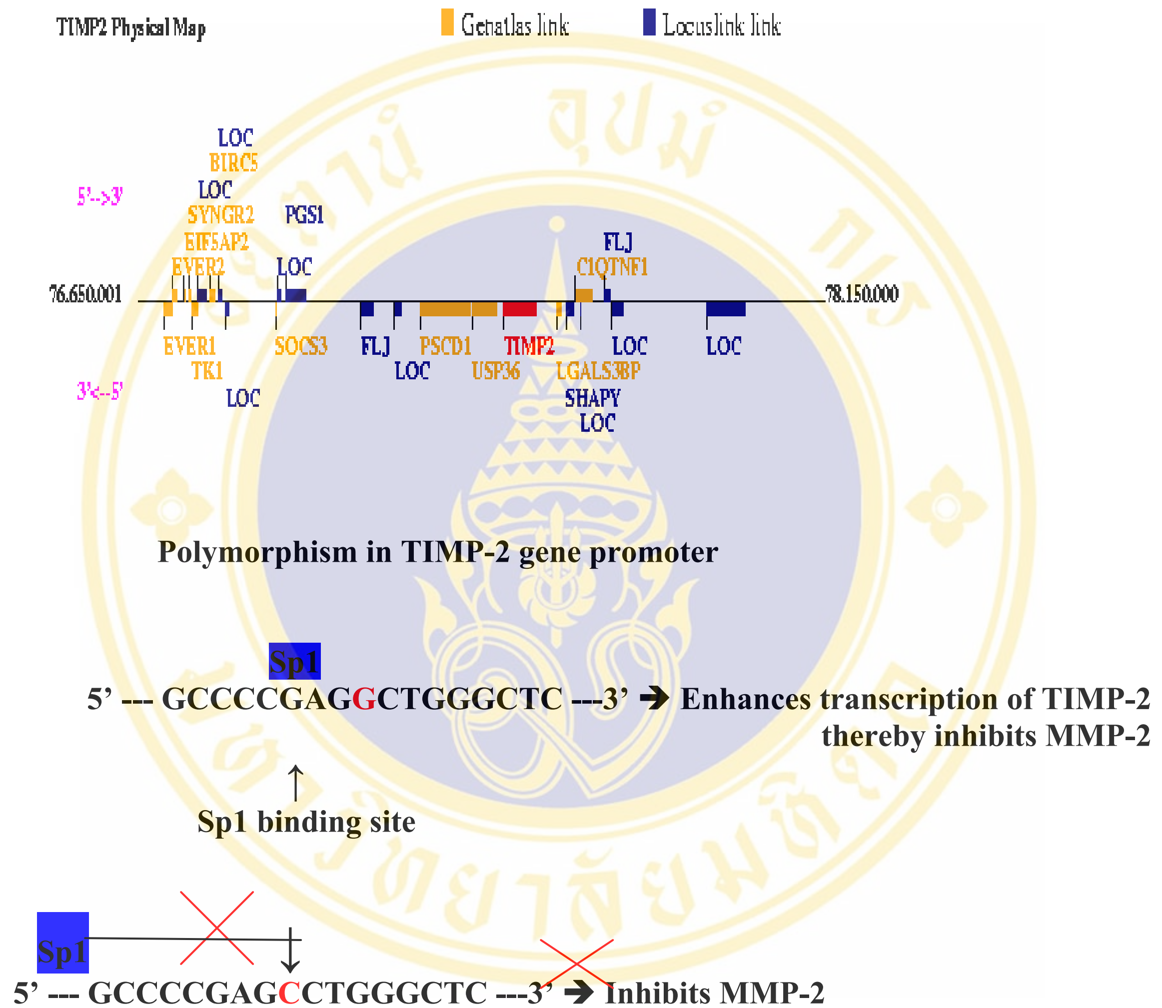

Figure 5. TIMP-2 gene structure & SNP mechanism

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3.11. Genetic polymorphism in cancer

Genetic polymorphisms provide us with the ability to predict inter-individual differences in susceptibility to clinical disease. Biomarkers of susceptibility include: polymorphisms in drug/carcinogen metabolism, in DNA repair capacity, and in genes that control cell growth. Wide variations in drug/carcinogen metabolism have been widely investigated and clearly shown to be an important determinant of individual cancer susceptibility and adverse drug reactions. Such polymorphisms in drug/carcinogen-metabolizing enzymes may be due to heritable and/or to environmental factors; and the modern application of metabolic phenotype and genotyping methods to epidemiological studies has provided new insights into such gene-environmental interactions. Polymorphisms in DNA repair or processing of DNA damage have long been evident from rare hereditary disorders involving defective DNA repair or chromosomal stability. Today, about 130 different genes have been shown to be involved in DNA excision or base repair and polymorphisms in gene-specific DNA adduct repair have been correlated with biological outcomes (mutations, drug sensitivity). Moreover, lower DNA repair proficiency has recently been associated with increased susceptibility to cancers of the skin, brain, lung, stomach, breast, bladder, HNSCC, and colon. While over 100 genes have been identified that serve as positive (proto-oncogenes) or negative (tumor suppressor genes) regulators of cell growth, as well as the cell cycle and apoptosis (e.g., cyclins, CDKs, cytokines, chemokines, their receptors, caspases, and so on), these have been largely associated with rare hereditary disorders involving greatly increased human cancer susceptibility. However, while the common polymorphisms in these genes, which include p53, p21, Her/neu, ras, APC, IL-10, and cyclin D1 (CCND1) have not yet received much attention, initial studies indicate that these may be associated with breast, endometrial, ovarian, bladder, colon, lung, thyroid, gastric, nasopharyngeal, esophageal, multiple myeloma, and HNSCC. It should be emphasized that although these common genetic polymorphisms do not in themselves confer high individual cancer risk (low penetrance), they involve a large proportion of the population (high prevalence). Thus, their attributable risk in any given population can be quite high and the importance of public health measures that result in lowering risk in the susceptible subgroup is of paramount importance because it can affect a larger number of people, as compared to

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those rare defects (low prevalence) that greatly increase disease risk (high penetrance) but only in a few individuals (low attributable risk), such as BRCA1 and BRCA2. In addition, the combination of several high risk alleles in a single individual, i.e. gene-gene interactions, can result in substantial increases in relative risk. When combined with a known carcinogen exposure (e.g., dietary heterocyclic amines or environmental aromatic amines), the probability of developing cancer in such persons becomes extremely high. Some examples of such gene-gene-environmental interactions from our ongoing molecular epidemiologic studies of breast and colon cancer will be presented. Although the majority of DNA polymorphisms are probably functionally neutral, a proportion of them can exert allele-specific effect on the regulation of gene expression or function of the coded protein, thus underlying between-individual differences in various biological traits and in susceptibility to disease. Figure 6 shows the roles of the tumor suppressor gene and oncogene in phenotype alteration by regulating the proliferation, differentiation, apoptosis and the response to genetic damages. However the roles of these genes in invasion and metastasis of the tumor is yet unknown which brings us to the proposition of the roles of the proteolytic enzymes such as the MMPs family.

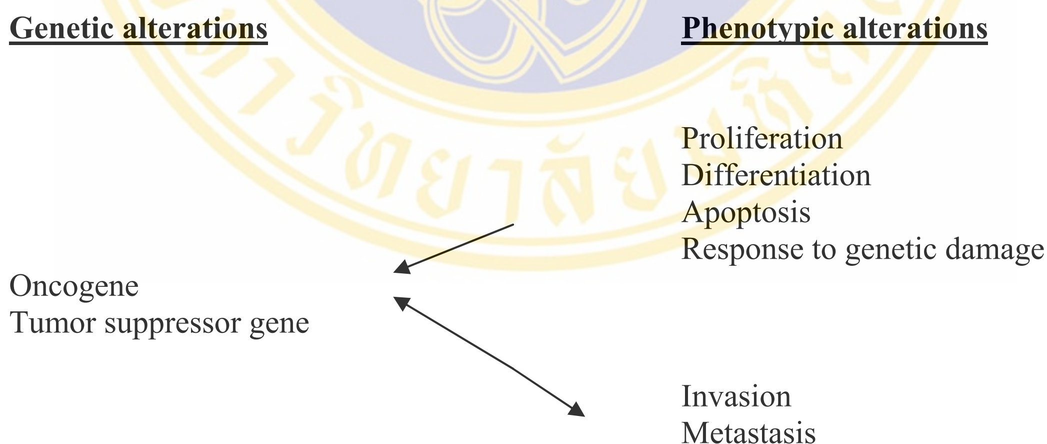

Figure 6. Crossroads between genetic and phenotype alterations in human cancer. Oncogenes and Tumor suppressor genes are known to have functions to regulate the proliferation, differentiation, apoptosis and responses to genetic damages; however, their involvement in invasion and metastasis remain unclear.

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3.12. Studies of MMPs/TIMPs polymorphisms in diseases and cancer

Over the last few years, polymorphisms have been identified in the promoters of a number of MMP genes. As summarized in Table 1 and further discussed below, these polymorphisms have been shown to influence MMP gene expression and be associated with susceptibility of coronary heart disease, abdominal aortic aneurysm, and cancers.

Table 1: Selected Polymorphisms in the promoter of matrix metalloproteinase gene

Gene	Polymorphism	Position	Polymorphism	Disease studied	Reference
MMP-1	1607del ins	1607	G	Ovarian cancer	Rutter et al., 1998 Kanamori et al., 1999
MMP-2	C-1306T	1306	C to T	Lung cancer Gastric cardia carcinoma Breast cancer Colorectal cancer Esophageal cancer	Yu et al., 2002 Miao et al., 2003 Zhou et al., 2004 Xu et al., 2004 Yu et al., 2004
MMP-3	1612del ins	1612	A (5A 6A polymorphism)	Coronary atherosclerosis Myocardial infarction Abdominal aortic aneurysm	Ye et al.,1995 de Maat et al., 1999 Humphries et al.,1998 Terashima et al., 1999 Yoon et al.,1999
MMP-9	C-1562T Microsatellite	1562 90	C to T (CA)n	Coronary atherosclerosis Intracranial aneurysm Abdominal aortic aneurysm Multiple sclerosis	Zhang et al., 1999b Shimajiri et al., 1999 Peters at al.,1999 Yoon et al.,1999 Nelissen et al., 2000
MMP-12	A-82G	82	A to G	Coronary atherosclerosis	Jormsjo et al., 2000
TIMP-2	C-418G	418	C to G	Breast cancer Chronic periodontitis	Zhou et al., 2004 de Souza et al.,2005

Although recent molecular epidemiological studies have suggested that genetically determined matrix-degrading capacity may contribute to the risk and progression of some tumor types (30, 31), so far study in HNSCC has not yet been performed.

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CHAPTER IV

MATERIALS AND METHODS

4.1 Cell culture

4.1.1 Cell lines, culture media and reagents

The following human HNSCC lines were used in these studies: LICR-HN-5, LICR-HN-6, and LICR-HN-15 cells which were gifts from Dr. Mike O'Hare, University College London, United Kingdom. The cell lines SIHN-005A, SIHN-005B, SIHN-006, SIHN-008, SIHN-011A, SIHN-011B, SIHN-013, SIHN-13/2, SIHN-015B, and SIHN-034B (74) were kindly provided from Dr. Suzanne Eccles, Institute of Cancer Research, Sutton, United Kingdom. Detroit-562 human was purchased from the American Type Culture Collection (Rockville, MD). A panel of seven new HNSCC cell lines (SITH-HN-series) was recently established in our laboratory from Thai patients and was used during early passage (from 5 to 10). Origins, characteristics and culture conditions will be described elsewhere. All cell lines were cultured at 37°C in a humidified atmosphere of 5% CO2 and 95% air. All HNSCC lines were maintained in DMEM containing 2 mM of L-glutamine, 100 units/ml of penicillin and 100 µg/ml of streptomycin supplemented with FCS to be referred to as 'DMEM/FCS'. FCS was de-complemented by heating at 56°C for 45 minutes before use. All cell culture reagents were purchased from GibcoBRL™ and all chemical reagents of the highest purity available were obtained from Sigma® (Sigma-Aldrich Company Ltd., Dorset, England) unless otherwise stated.

4.1.2 Collection of clinical materials

Numbers of consecutive tumor samples, where material in excess of diagnostic requirements was available, the establishment of tumor cell lines were carried out as previously described with some modifications (75). Briefly, surgical

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specimens were transported to the laboratory in cold 'establishment media' consisting of DMEM/20 % FCS with 2.5 µg/ml amphotericin-B. Tissues were reduced to 0.5-1 mm^3 fragments with a scalpel under aseptic conditions. After three washes with PBS containing 2.5 µg/ml amphotericin-B, about 8-10 fragments were transferred to a 25-cm^2 plastic culture flask containing not more than 3 ml of 'establishment media'. Cultures were maintained in humidified incubators at 37^oC in an atmosphere of 5% CO_2 and 95% air for at least four days without disturbance. Medium was renewed twice weekly once the tissue fragments had become firmly attached. Within 7-10 days, epithelial and fibroblast cells migrated from the tumor explants. Fibroblast outgrowth was controlled by selective detachment via mechanical removal using a cell-scraper or exposure to 0.05 % trypsin/ 0.04% Na_2EDTA. After the first passage, cultures were maintained in DMEM/10 % FCS without anti-fungal agent. Cells were subculture at 95% confluence with a dilution of 1:6 to 1:8 and were stored frozen at –130^oC. New HNSCC lines (designated SIHN- series) were used during passages 5-15.

4.1.3 Subculturing, maintenance and expansion of established cell cultures

All cell lines were cultured routinely at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were maintained in monolayer culture and passaged weekly by incubation with 0.01% bovine pancreatic trypsin in PBS containing 0.04% Na_2 EDTA for about 3-5 minutes at 37^oC. Detached cells were collected in DMEM/10 % FCS, mixed into a single-cell suspension in a universal and pelleted by centrifugation in an IEC Centra-7 centrifuge for 3 min at 800g. Following resuspension in fresh, pre-warmed medium, cell counts and viability estimation were carried out by making a dilution of cell suspension in trypan blue and counting using a Haemocytometer Counting Chamber. Cells were diluted to the required density (1-5 x 10^4/ml) before plating into culture flasks (5 ml/25 cm^2, 25 ml/80 cm^2, 50 ml/170 cm^2) or plates (200 µl/well of 96-well plates, 2 ml/well of 24-well plates, 5 ml/well of 6-well plates). Medium was replaced two or three times per week.

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4.1.4 Long term storage of cells

Cells of early passages and high viability (> 80 %) were stored at –135°C in liquid nitrogen. Between 10^6-10^7 cells were pelleted by centrifugation and the supernatant was discarded. The cells were resuspending in 1 ml of an ice-cold freezing mixture containing 7% DMSO and 93% FCS and transferred to 1.5 ml cryotubes. After controlled freezing, the samples were immersed in liquid nitrogen. When required aliquots of cells were thawed quickly at 37°C and diluted into 20 ml DMEM/10 % FCS in a 30 ml universal tube. After centrifugation, the cells were washed once more with 20 ml of DMEM/10% FCS and then plated out into 25 cm^2 flasks.

4.2 Patient materials

Two hundred and thirty-nine newly diagnosed, previously untreated patients with HNSCC (primaries of the oral cavity, oropharynx, hypopharynx and larynx) at the Department of Head and Neck Surgery, Siriraj Hospital, Thailand were recruited between September 2002 - August 2004. Those with second primary HNSCC, primaries of the nasopharynx or sinonasal tract or primaries outside the upper aerodigestive tract, cervical metastasis of unknown origin and histopathological diagnoses other than HNSCC were excluded. One hundred and eighty-eight control subjects were selected from people who came for routine physical checkups or had non-neoplastic operations in the same hospital, and frequency-matched to the cases by age (± 5 years), sex, ethnicity and tobacco/alcohol use status. The frequency matching was used to evaluate the main effect of the polymorphism. Those with previous diagnosis of any cancer type, autoimmune disorders, and blood diseases were excluded from the control group. Epidemiological data were collected by personal interview. This study was approved by an ethics reviewing committee. After written informed consent was obtained, data were collected on socio-demographic characteristics, recent and prior use of tobacco/alcohol, other life-style habits, and family history of cancer. Smoking status was defined as follows: (a) an ever smoker, smoked at least 100 cigarettes in lifetime; (b) former smoker, ever smoker who had stopped smoking at least 1 year prior to diagnosis for the cases and for the controls 1 year before the interview; the other as current smoker. Study subjects who smoked 100

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or fewer cigarettes in their lifetime were categorized as never-smokers. Pack-years were defined as the number of cigarettes/day divided by 20 and then multiplied by the number of years smoked. Alcohol status was defined as follows: (a) ever drinker, has drank alcoholic beverages at least once a week for more than one year previously, (b) former drinker, ever drinker who had quit drinking at least 1 year previously; the other as current drinker. Subjects were classified as never-drinkers if they consumed fewer than once a week. 'Node-positive cases' in this study included cases in which positive cervical nodes were identified based on a histological diagnosis after a neck dissection. Patients who experienced no metastasis for at least 12 months post-operatively are scored as 'node-negative cases'. The clinical and pathological characteristics of patients are summarized in Table 5. Tumors were staged according to the AJCCUICC TNM classification 6th edition.

4.3 Molecular biology techniques

4.3.1 Materials for molecular biology for studies

All chemicals used for nucleic acid preparation were of molecular biology grade with no detectable RNase or DNase activities and purchased from Sigma unless stated otherwise. Agarose and low melting point (LMP) agarose of ultrapure quality were obtained form Kramel Biotech. Ethidium bromide solution at 10 mg/ml was formed. DNA ladder (1 kb), guanidine thiocyanate, Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase, and proteinase K were purchased from Promega (Promega Corporation, Madison, WI, USA). Human placenta DNA, 10 x PCR buffer and RED Taq DNA polymerase were from Sigma. Oligonucleotide primers were ordered from Sigma-Genosys (Cambridgeshire, UK). All buffers and reagents were thawed quickly in 37°C water bath and then centrifuged briefly to collect all liquid to the bottom of the tube. A bulk reaction master mix was prepared which contained all components except the template.

4.3.2 DNA preparation

The extraction of DNA from cultured cells or tissue samples was performed as described previously (76). In brief, 5 x 10^{6} cells or 100 mg of tissue were washed and

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suspended in 300 µl of digestion buffer [100 µg/ml proteinases K, 100 mM NaCl, 50 mM Tris-HCl, 1% SDS, and 50 mM EDTA, pH 8.0]. After incubation for 2 h at 50°C and overnight at 37°C, 300 µl of 5 M LiCl was added and mixed thoroughly by inversion for 1 min, then 600 µl of chloroform was added and the samples placed on a rotating wheel for 30 min. The sample was centrifuged for 15 min at 13 000 g and the supernatant was carefully collected to a new microfuge tube. Exactly 2 volumes of room temperature absolute ethanol were added and the tube inverted several times until the DNA precipitated. The DNA was recovered by high speed centrifugation, the supernatant decanted, and the DNA pellet washed briefly with 70% ethanol. Excess ethanol was removed and the sample allowed to air dry for 10 min. The DNA sample was then resuspended in 100-200 µl of TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and allowed to dissolve overnight at 4 °C. DNA concentration was measured by absorbance at 260 nm. Spectrophotometric absorbance ratios (260/280 nm) were consistently around 1.8.

4.3.3 Polymorphism analysis

Genomic DNA was isolated from the peripheral blood lymphocytes of the study subjects. Ten mL of venous blood samples were drawn into EDTA containing tubes and processed within 30 minutes. A leukocyte cell pellet obtained from the buffy coat was used for genomic DNA extraction with a standard phenol-chloroform method. Fresh tissue samples were obtained from patients undergoing therapeutic surgical resection for HNSCC. In each case, the portion of tumor was resected near the advancing edge of the tumor, avoiding its necrotic center and immediately snap-frozen and stored in liquid nitrogen until use. The adjacent tissues were submitted for histopathological study to confirm the presence of $\geq$ 80% cancer cells in the procured sample. Histologically normal mucosae of the upper aerodigestive tract, resected 5 cm distant from the tumor area, was obtained in all cases and used as controls.

Genotypes were analyzed using PCR-based methods as described below. Genotyping was performed without knowledge of subjects' case/control status. The SNP of *MMP-2* promoter at –1,306 C/T were determined by polymerase chain reaction (PCR) - amplification refractory mutation system (ARMS) analysis. The

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amplification refractory mutation system (ARMS), which has also been described as allele-specific PCR (ASP) and PCR amplification of specific alleles (PASA), is a PCR-based method of detecting single base mutations (77). A PCR primer is designed so that it can discriminate between templates which differ at a specific single nucleotide residue; an ARMS primer can specifically amplify one allele of a multiallelic system. It is based on the principle that *Taq* polymerase has no 3' to 5' exonuclease activity; therefore a mismatch between the 3' end of the primer and the template will result in its inability to function as a primer under appropriate conditions. A standard ARMS PCR consists of two complementary reactions (two tubes) and utilizes 3 primers. One primer is constant and complementary to the template in both reactions, the other primers differ at their 3' terminal residues and are specific to either the wild type DNA sequence or the mutated sequence at a given base - only one of these primers is used per tube. If the sample is homozygous mutant or homozygous wild type amplification will only occur in only one of the tubes, if the sample is heterozygous amplification will be seen in both tubes. The primers used to amplify at 292 bp fragment of the *MMP- 2* promoter containing the -1306 C/T site were: MMP-2F, 50- TAATCGTGACCTCCAATGCC; MMP-2R, 50-SNPT: CCTGAAGAGCTCAAGCGTTA. and SNP C: CCTGAAGAGCTCAAGCGTTG PCR was accomplished with a 25 ml reaction mixture containing ~50 ng DNA, 1 mM each primer, 0.2 mM dNTP, 2 mM $MgCl_2$,1.0 U *Taq* DNA polymerase with 1x reaction buffer (Promega, Madison, WI) and 2% dimethylsulfoxide. The reaction was carried out under the following conditions: an initial melting step of 2 min at 94 ^{0}C, followed by 35 cycles of 30 s at 94 ^{0}C, 30 s at 64 ^{0}C and 45 s at 72 ^{0}C, with a final elongation of 7 min at 72 ^{0}C.

The genotypes of *TIMP- 2* at the -418 G/C site were analyzed by a PCR-RFLP method on the basis of that reported previously (72). Restriction fragment length polymorphism (RFLP) is a technique in which nucleotide may be differentiated by analysis of patterns derived from cleavage of their DNA. If nucleoside differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme. The similarity of the patterns generated can be used to differentiate

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nucleotide from one another. Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 base pairs in length. Generally, the shorter the recognition sequence, the greater the number of fragments generated. If molecules differ in nucleotide sequence, fragments of different sizes may be generated. The fragments can be separated by gel electrophoresis. The primers used, which produced a 304 bp fragment containing the -418 G/C site, were: TIMP- 2 F, 50-CGT CTC TTG TTG GCT GGT CA; TIMP-2R, 50-CCT TCA GCT CGA CTC TGG AG. Amplification was accomplished with a 25 ml reaction mixture containing ~50 ng template DNA, 1.25 mM each primer, 0.5 mM each dNTP, 1.5 mM MgCl2 and 0.24 U *Taq* DNA polymerase with 2.5 x reaction buffer (Promega). The PCR profile consisted of an initial melting step of 2 min at 94 ^{0}C, followed by 35 cycles of 30 s at 94 ^{0}C, 30 s at 62 ^{0}C and 30 s at 72 ^{0}C, with a final elongation step of 7 min at 72 ^{0}C. The 304 bp PCR products were then subject to the digestion with BsoBI (New England Biolabs, Beverly, MA) and separated on a 2.5% agarose gel. The G allele had two BsoBI restriction sites and resulted in three bands (230, 51 and 23 bp) whereas the C allele lacked one BsoBI restriction site and thus produced two fragments of 253 and 51 bp. The genotypes identified by BsoBI digestion were confirmed by DNA sequencing. Three different allelic PCR products were directly analyzed with an ABI PRISM 377 autosequencer using a dye terminator sequencing kit and sequences were compared with the published TIMP- 2 sequence (78).

4.3.4. Agarose gel electrophoresis

Agarose gel electrophoresis was performed for examining PCR products, restriction enzyme-digested DNA fragments, and to estimate the amount of DNA. Five µl of the PCR products was mixed with 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30%glycerol in deionized water) and loaded into each well of 1% agarose gel (see Appendix). Electrophoresis was achieved at constant 100 Volts for approximately 30 min in 1X TAE buffer (see Appendix) by using a horizontal electrophoresis apparatus (Mupid-2, Tokyo, Japan). Thereafter, the agarose gel was stained with ethidium bromide, visualized by a UV trans-illuminator, and photographed with a polaroid camera (Fotodyne, Hartland, USA). The DNA

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molecular weight marker used in the electrophoresis was *Hind* III-digested λ DNA and *Hae* III-digested ϕX174 RF DNA fragments (New England BioLabs, Inc., Beverly, MA, USA.

4.3.5. DNA purification

Following amplification, the amplicons were purified by QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) acccording to the manufacturer's protocol. Briefly, DNA fragment with the estimated size for the PCR segment, which was separated from the PCR reaction mixture by electrophoresis, was excised from the agarose gel with a clean and sharp blade. The gel slice was mixed with 3 volume of Buffer QG (solubilization and binding buffer) and incubated at 50ºC for 10 min. prior to the application onto a QIAquick column. After 1-min centrifugation at 13,000 rpm (~17,900 x g) in a fixed angle rotor centrifuge (Beckman microfuge E), flow through was discarded and the column was washed once with 0.75 ml of ethanol-containing Buffer PE followed by centrifugation for 1 min at 13,000 rpm. In order to elute DNA from the column, 30 µl of Buffer EB (10 mM Tris-Cl, pH 8.5) was added to the center of the QIAquick membrane and incubated for 1 min. Thereafter, the column was centrifuged at 13,000 rpm for 1 min to collect the purified products. The purified DNA was analyzed and estimated for DNA concentration by agarose gel electrophoresis as described above.

4.3.6. DNA sequencing analysis

The genotypes identified by the PCR-ARMS and PCR-RFLP assay were confirmed by sequencing. Direct sequencing of gel-purified amplicons (Qiaex II Gel Extraction Kit; Qiagen) was performed using a 377-18 DNA sequencer. The three allelic PCR products were directly analyzed with an ABI PRISM 377 autosequencer using a dye terminator sequencing kit and sequences were compared with the published TIMP-2 sequence.

Sequences of PCR primer sets of MMP-2 (in 5'-3' direction) sequencing was as follow: forward: CCTAGGCTGGTCCTTACTG, backward:

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GAAGTGACTTCTGAGCTGAG and sequences of PCR primer sets of TIMP-2 (in 5'-3' direction) sequencing was as follow: forward – CGTCTGTTGTTGGCTGGTCA, backward – CCTTCAGCTCGACTCTGGAG. To confirm the genotypic results, representative gel-purified PCR products (Qiagen PCR purification kit) were purified and directly sequenced by a 377-18 DNA sequencer (Applied Biosystem).

4.4. RNA treatment techniques

4.4.1. RNA preparation

All glassware used for RNA solution preparation was first treated with 0.02 % DEPC in water, rinsed with sterile DDW and autoclaved. All solutions were autoclaved and prepared in DEPC-treated DDW. DEPC water was prepared by mixing DDW with 0.02 % DEPC and autoclaving.

The following procedures were performed on ice or at 4°C to prevent the degradation of RNA. The purification of total RNA was performed according to the procedures described previously (79) with minor modifications. In brief, monolayer cells were rinsed twice with ice-cold PBS, lysed by the addition of 1.2 ml RNA lyses buffer 'solution A'{[water-saturated phenol, 'solution B' (4 M guanidinium thiocyanate, 25 mM sodium citrate, 0.8% sarcosyl, pH 7.0) and 2 M NaOAc (pH 4.0) in a ratio of 1:1:0.1] and 0.34% 2-mercaptoethanol} and scraped into pre-chilled Eppendorf tubes. For tissue samples, snap-frozen tissues were pulverized with 'solution A' (1.2 ml per gram of tissue) in a liquid nitrogen-bathed mortar. After adding 120 µL of chloroform/isoamyl alcohol (49:1), lysates were vortexes at high speed for 10 sec and left on ice for 15-30 min. The cellular debris and nuclei were removed by centrifuge at 12 000 x g for 20 min. The upper aqueous phase (about 600 µl) was transferred to a new Eppendorf tube containing an equal volume (~600 µl) of ice-cold isopropanol. Samples were mixed, placed at -20^{0}C for 1-3 h or overnight and centrifuged at 12 000 g for 20 min. The RNA pellets were rinsed twice with ice-cold 70% ethanol and once with absolute ethanol. The RNA pellets were air-dried for 10 min and dissolved in DEPC-treated H_2O.

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Alternatively, poly A+ mRNA was isolated using a QuickPrep Micro kit (Pharmacia Biotech) according to the manufacturer's instructions. The extraction method was based on the guanidine thiocyanate, oligo (dT)-cellulose and microspin column. The mRNA was precipitated with glycogen and 95% ethanol under acid pH.

Estimations of RNA concentration and quality were performed on 10 µl of the RNA sample by measuring the OD_{260} and OD_{280} in a GeneQuant (Pharmacia). Spectrophotometric absorbance ratios (260/280 nm) were consistent from 1.8 to 2.0. In some cases, the integrity of RNA was checked by ethidium bromide (1 µg/ml) staining of 0.8% agarose/TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA, pH 8.0) minigel electrophoresis. The RNA samples were stored at -70°C until used.

4.4.2. DNase treatment of RNA

In order to eliminate DNA contamination in RNA samples, DNAase treatment was routinely employed (77). In brief, 10-100 µg of total RNA (in 300 µl DEPC-water) was mixed with 1 in 10 volumes of 10 x PCR buffer, 10 units of human placental ribonuclease inhibitor and 10 units of DNase I. After incubation for 30 min at 37°C, the DNase I was inactivated by adding an equal volume of phenol: chloroform (3:1). Samples were vortexes 5 sec, left on ice for 10 min, and centrifuged at 12 000 g for 5 min at 4°C. The supernatant (aqueous phase) was carefully transferred into a new microfuge tube containing 3 volumes of ice-cold isopropanol and 0.3 M Na Acetate (pH 5.2) and incubated at -20°C for 30 min. The RNA pellet was collected by cold centrifugation at 12 000g for 10 min, rinsed once with ice-cold 70% ethanol and 95% ethanol. The RNA was dissolved in DEPC-water with the appropriate amount.

4.4.3. Reverse transcription of RNA (cDNA synthesis)

The RNA sample (2 µg of total RNA or 150 ng of poly A+ mRNA) was made up to 16 µl of DDW and denatured by heating for 5 min at 90°C then quickly freeze on dry ice. Five µl of 5x RT buffer and 4 µl (2 µg or 3 pmol) of oligo($dT_{12\text{-}18}$) or pd(N_6) random hexamer were added and incubated for 15 min at 4°C or at room temperature for oligo $dT_{12\text{-}18}$ or pd(N_6) respectively. The random hexamer was preferred for the

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RNA extracted from tissue samples. To this, 5 μl of 5 x RT buffer, 5 μl of BSA solution (1 mg/ml), 1 μl of RNAguard RNase inhibitor (5 U/μl in DDW), 2 μl of 350 mM β-mercaptoethanol, 2 μL of 25 mM dNTPs, 1 μl of 200 mM Na pyrophosphate, 8 μl of DDW and 1 μl of Moloney murine leukemia virus reverse transcriptase (100 units/μg RNA). This reaction mixture was incubated at 42^0C for 60 min, denatured at 95^0C for 5 min, quickly chilled on ice and then used immediately for PCR or stored at -20^0C. The conditions for RT were the same for each of the comparison samples and β-actin control including all the components, volume, temperature, and reaction time.

4.4.4. Quantification of mRNA levels using RT-PCR analysis

Total RNA was extracted from cell monolayer or homogenized snap-frozen tissue samples with TRIzol® reagent (Gibco/BRL) following the manufacturer's protocol and re-purified with the RNeasy® Mini spin column (Qiagen, Valencia, CA). RNA integrity was electrophoretically verified by ethidium bromide staining and by OD_{260}/OD_{280} nm absorption ratio >1.95. Two μg of total RNA was reversed transcribed with 4 U of Omniscriptt^M Reverse Transcriptase (Qiagen, Valencia, CA) in 20 μl of a reaction mixture with 100 μM of random hexamer primers (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's protocol. Data regarding gene sequences were obtained from GenBank. Highly purified salt-free gene specific primers were designed based on strict criteria using the Primer3 programmed (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi) and were purchased from Proligo Singapore Pty. Ltd. Sequences of PCR primer sets for MMP-2, TIMP-2 were as described previously (80). Data on gene sequences were obtained from GenBank. Primers for PCR were designed based on strict criteria using the Primer Designer program version 2.0 (S&E Software, PA, USA).

Sequences of PCR primer sets of MMP-2 (in 5'-3' direction) were as follow: forward - AGCTAGCTCAGGATGACATTGATG, backward - CCGATGGGCTGG ACAGG, products size: 580 bps and sequences of PCR primer sets of TIMP-2 (in 5'-3' direction) were as follow: forward – AAACGACATTTATGGCAACCCTATC, backward - ACAGGAGCCGTCACTTCTCTTGATG, products size: 405 bps.

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Sequences of PCR primer sets for 18S rRNA were described previously (81). Conditions for all PCRs were optimized with regard to $MgCl_2$ concentrations, forward and reverse primers and various annealing temperatures (50-60°C). Specificity of the RT-PCR amplification products were documented with a 4% high resolution NuSieve agarose gel electrophoresis and resulted in single products with the desired length.

Table 2. Semi-quantitative RT-PCR protocol

Component	**Volume/reaction**	**Final concentration**
Master mix		
10x Buffer RT	2.0 µl	1x
dNTP Mix (5 mM each dNTP)	2.0 µl	0.5 mM each dNTP
Oligo-dT primer (10 µM)	2.0 µl	1 µM
RNase inhibitor (10 units/µl)	1.0 µl	10 units (per 20 µl reaction)
Omniscript Reverse Transcriptase	1.0 µl	4 units (per 20 µl reaction)
RNase-free water	Variable	
Template RNA		
Template RNA	Variable	Up to 2 µg (per 20 µl reaction)
Total Volume	**20.0 µl**	

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β-actin was used to check RNA integrity and as an internal control. Typical PCR reaction mixtures contained 5 µl of 10x PCR buffer, 0.25 mM of dNTPs mix, 2.0 units of *Taq* DNA polymerase, 200 pM of each upstream and downstream primer, and DDW up to an end-volume of 45 µl. Finally 5 µl of cDNA were added and the reaction mix was overlaid with mineral oil. The PCR reaction was carried out in a TRIO-Thermoblock thermal cycler. Amplification cycles consisted of denaturing the cDNA for 90 sec at 94 0C, primer annealing for 1-2 min at 50-600C and primer extension during incubation for 1-2 min at 720C with the last extension step for 10 min. The optimal condition for each primer pair was achieved by adjusting the annealing/extension temperature and time. A negative control reaction was always prepared which contained water or non-RT RNA in place of the DNA template. In order to quantify the level of mRNA expression between different samples, preliminary experiments were performed which established the range of PCR cycles in which exponential amplification occurred for each primer pair studied. A PCR cycle was chosen in the middle of the linear range of amplification. Ten-µl aliquots of the RT-PCR products were electrophoresed in 2% agarose gels containing 1 x TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) and 1 µg/ml of ethidium bromide. Molecular weight markers (1 kb Ladder) were loaded into the end well of each gel for estimation of the size of PCR products. Electrophoresis was carried out at a constant voltage of 80 (small gel) or 120 V (large gel). Bands were visualized by examining the gel under UV light and captured using a Mitsubishi UVP video copy processor connecting to the Gel-Pro Analyzer™ Software version 3.1.0 (Media Cybernetics, Silver Spring, MD, USA). The intensity of bands with background subtraction was measured using Quantiscan Image Analysis software (Cambridge, UK). In addition, a series of PCR templates (0.5 µg, 1 µg, 2 µg and 4 µg of reverse transcribed-tRNA) were prepared and underwent the PCR reaction with appropriate number of cycles to confirm the linear correlation between the amount of starting mRNA and the signal intensity of PCR products. Relative density was calculated by dividing specific gene signals by the β-actin signal. In order to control gel-to-gel variability, each PCR products from the HNSCC tissue HN-12T was also electrophoresed as a control. The level of mRNA

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was calculated as the ratio of tissue sample to HN-12T on the same photograph and was then corrected as a ratio to the corresponding β-actin level.

β-actin was used to check RNA integrity and as an internal control. Sequences of PCR primer sets of β-actin (in 5'-3' direction) were as follow: forward - TCGACAACGGCTCCGGCAT, backward - AAGGTGGTGCCAGATTTTC, Products size: 239 bps. Typical PCR reaction mixtures contained 5 µl of 10x PCR buffer, 0.25 mM of dNTPs mix, 2.0 units of Red *Taq* DNA polymerase, 200 pM of each upstream and downstream primer, and DDW up to an end-volume of 45 µl. Finally 5 µl of cDNA were added and the reaction mix was overlaid with mineral oil. The PCR reaction was carried out in a TRIO-Thermoblock thermal cycler. Amplification cycles consisted of denaturing the cDNA for 90 sec at 94 ^{0}C, primer annealing for 1-2 min at 50-60^{0}C and primer extension during incubation for 1-2 min at 72^{0}C with the last extension step for 10 min. The optimal condition for each primer pair was achieved by adjusting the annealing/extension temperature and time. A negative control reaction was always prepared which contained water or non-RT RNA in place of the DNA template.

In order to quantify the level of mRNA expression between different samples, preliminary experiments were performed which established the range of PCR cycles in which exponential amplification occurred for each primer pair studied. A PCR cycle was chosen in the middle of the linear range of amplification. Ten-µl aliquots of the RT-PCR products were electrophoresed in 2% agarose gels containing 1 x TAE (mM Tris, glacial acetic acid, 1 mM EDTA, pH 8.0) and 1 µg/ml of ethidium bromide. Molecular weight markers (1 Kb Ladder) were loaded into the end well of each gel for estimation of the size of PCR products. Electrophoresis was carried out at a constant voltage of 80 (small gel) or 120 V (large gel). Bands were visualized by examining the gel under UV light and captured using a Mitsubishi UVP video copy processor connecting to the ImagePro Software. The intensity of bands with background subtraction was measured using Quantiscan Image Analysis software (Cambridge, UK). Pilot studies had shown the similar results compared with the conventional

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radioisotopic (^{32}P) technique. In addition, a series of PCR templates (0.5 µg, 1 µg, 2 µg and 4 µg of reverse transcribed-tRNA) were prepared and underwent the PCR reaction with appropriate number of cycles to confirm the linear correlation between the amount of starting mRNA and the signal intensity of PCR products. Relative density was calculated by dividing specific gene signals by the β-actin signal. In order to control gel-to-gel variability, each PCR products from the HNSCC cell line SIHN-006 was also electrophoresed as a control. The level of mRNA was calculated as the ratio of tissue sample to SIHN-006 on the same photograph and was then corrected as a ratio to the corresponding β-actin level.

4.5 Substrate zymography

4.5.1. Materials for substrate zymography

Gelatin zymography method is used to determine the levels of MMP-2 and MMP-9 expression/activities in fresh frozen tissues. Gelatin zymography were performed as previously described (82). Duplicate gels were incubated as controls in buffer containing 20 mM EDTA to inhibit MMP activity. MMP activity was detected as clear zones of lyses against a blue background. The amount of each MMP activity band was measured by determining the area of the cleared band on dried gels. The extent of digestion was quantified using a scanner and the Quantiscan Image Analysis Software. Conditioned medium from TPA-treated HT-1080 fibrosarcoma cell line served as a positive control and a standard for inter-experimental variation. HT-1080, human fibrosarcoma cells are aggressively invasive and will form tumors in nude mice. They grow rapidly and should be spilt 1:10 or even 1:15 twice a week.

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Table 3. Composition of resolving and stacking gels for SDS-zymogel (in ml.)

	Type of gel			Stacking gel
	Gelatin	**Casein**	**Reverse**	**4.5%**
Acrylamide stock[a]	11.0	16.0	15.0	1.2
Gel buffer	7.5	10.0	7.5	2.5
Sterilized water	8.5	13.6	-	6.25
Gelatin (bovine skin type III)	3.0	-	6.75	-
β-casein	-	4.5	-	-
pro-MMP-2 or pro MMP-9	-	-	4.8 μg	-
Ammonium persulfate[b]	0.3	0.3	0.3	0.1
TEMED	0.03	0.03	0.03	0.01

[a] Stock is 30% (w/v) acrylamide (37.5:1) bisacrylamide (Northumbria Biological Ltd.)

[b] Of a freshly made 10% (w/v) stock in DDW.

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4.5.2 Preparation of gels

Samples (conditioned media, cell or tissue lysates) were analyzed under non-reducing conditions and separated in SDS-PAGE co-polymerized with gelatin, β-casein or gelatin and pro gelatinases in order to demonstrate gelatinolytic activity (MMP-2 and MMP-9), caseinolytic activity (MMP-3 and MMP-7) or MMP inhibitory activity (TIMP-1 and TIMP-2) as previously described (82), (83), (84) with minor modification. Resolving gels (1 mm thickness), containing the appropriate concentration of polyacrylamide for substrate zymography were prepared according to the values given in Table 3 and were overlaid with water saturated butanol-3-ol. When polymerization was completed (30-60 minutes), the butanol was washed off and the gels wee overlaid with a standard 4.5% acrylamide stacking gel.

4.4.3 Sample preparation

The protein containing samples were mixed with a one-fifth volume of 5 x non-reducing sample buffer for 30 min at room temperature. Samples of up to 25 µl (containing 10-40 µg protein) were subjected without boiling to SDS-PAGE containing appropriate substrate. Conditioned medium from TPA-treated HT-1080 fibrosarcoma cell line and TPA-treated MDA-MB 231 mammary carcinoma cells served as a positive control and a standard for inter-experimental variation for gelatin zymography/reverse zymography and casein zymography, respectively.

4.4.4 Electrophoresis

Electrophoresis was carried out at a constant current of 15-20 mA/gel at 4°C for 12-14 h. Electrophoresis was stopped when the bromophenol blue marker ran out approximately 30 min from the bottom of the gel. To increase the sensitivity for casein zymogram, the gel was pre-run as described previously (83). Briefly, sample buffer was loaded into one lane and the gel was run constantly at 50 mA at room temperature until 10 min after blue dye reached the bottom and allowed to cool for 1 h before ready to use.

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4.4.5 Staining of gels with coomassie blue

Proteins separated by SDS-PAGE were fixed and stained simultaneously with a solution containing 0.25% Coomassie Brilliant Blue R250 in a mixture (1:1) of absolute methanol and 10% acetic acid. After incubation overnight on a rocking platform, the gels were destained by incubation, for 2 h each time, with 40 % ethanol/5 % acetic acid, 20 % ethanol / 5 % acetic acid, and finally for 2 h in 10 % ethanol/5 % acetic acid. To make a permanent record, the gel was incubated with water containing 2 % glycerol and dried under vacuum on a heated-bed gel dryer.

4.4.6 Gels processing

Following electrophoresis, SDS was removed from the gel by washing twice for 30 min in 100 ml of 2.5% (v/v) Triton X-100 in DDW and then twice for 5 min in 50 mM Tris (pH 7.6). Gels were incubated in 100 ml of 'low salt buffer' (section 2.9.1) at 37^0C for 15 h. For visualization of MMP/TIMP activities, the gels were incubated in 30% (v/v) methanol/10% (v/v) acetic acid containing 0.5% (w/v) Coomassie brilliant blue R-250 for 4 h at room temperature. The gels were de-stained with four changes of 30% methanol/10% acetic acid (for 1, 15, 30 and 60 min of destain time respectively, for each change). The gels were incubated for 15 min with shaking in 30% (v/v) methanol/0.5% (v/v) glycerol prior to drying between sheets of cellophane. Prestained Seeblue marker protein was used to estimate the molecular weight.

4.4.7 Quantification

Regions of proteolytic activity were visualized as clear zones against a blue background whereas areas of inhibition were visualized as blue staining regions of the reverse zymogram on a clear background. The amount of each MMP activity band was measured by determining the area of the cleared band on dried gels. The extent of digestion or inhibition of digestion (for reverse zymograms) was quantified using an Arcus scanner and the Quantiscan Image Analysis Software (Cambridge, UK). Pilot studies using serial dilutions of standard HT1080 showed a good linear correlation between the amount of loading MMPs and the measured intensity.

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4.6. Statistical analysis

The distributions of selected demographic variables, smoking, alcohol consumption and genotype frequencies between cases and controls were tested by using the Pearson's χ^2 test. The Odd Ratios (ORs) and 95% confidence intervals (CIs) were also calculated as estimates of the relative risk. The adjusted ORs were calculated by logistic regression to control for age, gender, smoking and drinking status. Tests for an interaction between the *MMP-2* -1306C/T and *TIMP-2*C/G -418 polymorphisms were performed using the likelihood ratio test. The association between MMP-2/TIMP-2 genotype and clinicopathological parameters was evaluated by Fisher's exact T-test. A Mann-Whitney U test was used to analyze differences in MMP-2 and TIMP-2 expression according to the genotypes for the polymorphism. A P - value of $<0.05/N$ was considered as significant to correct for multiple comparisons using Bonferroni correction when patients were stratified into 2 groups by clinical factors (N, the number of factors). All statistical analysis was performed using the JMP statistical software package version 4.0.0 (SAS Institute Inc., Cary, NC).

All experiments were performed in triplicate and repeated on at least 2 separate occasions. Zymograms were performed at least 6 times. Values are given as means + s.e.m. For evaluation of correlation, the non-parametric test was employed using the GraphPad Prism program. The unpaired two-tailed Student's t-test was used to determine the difference between 2 groups. A p-value < 0.05 was considered to be statistically significant. For comparison between the levels of mRNA or protein expression between groups, the two-tailed Mann-Whitney (rank sum) test and the Kruskal-Wallis test with Dunn's multiple comparison tests were employed when comparing two groups and three groups respectively. Correlations between the mRNA and protein levels were computed using the two-tailed Spearman nonparametric correlation. Primary tumors were grouped into low and high MMP-2/TIMP-2 expression according to the RT-PCR results (below and above the median value respectively). The chi-square test with Fisher's exact test was used to assess the association of cervical nodal metastasis with mRNA expression of MMP-2/TIMP-2 and clinicopatholigical parameters. The prognostic significance of MMP-2/TIMP-2 expression and clinicopathologic parameters in cervical nodal metastasis was assessed

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with univariate and multivariate logistic regression analysis. Results were expressed as mean ± sem. A P-value of less than 0.05 was considered statistically significant.

The levels of mRNA and protein were measured from three parts of the same tissue in triplicate. To determine the significance of increased expression of MMP-2/TIMP-2 with various clinical parameters (age, sex, duration of symptoms, histological abnormal versus adjacent normal epithelium, histological grade, site and size of primary tumor, status of cervical lymph node metastasis, degree of lymphatic or vascular invasion and stage of disease), the Wilcoxon rank sum non-parametric test was employed. A P-value of less than 0.05 was considered statistically significant. Unless otherwise stated, each experiment was repeated twice with virtually identical results.

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CHAPTER V

RESULTS

5.1. MMP-2 and TIMP-2 genotypes of cancer cell lines

The SNP *C/C* amplified by the PCR –ASA is known to be present in majority of the cell-line sample and with only three samples containing the SNP *C/T* which is taken for preparation of the sample to determine the protein activity as described earlier in the paper. Fourteen out of seventeen HNSCC cell lines showed the *C/C* MMP-2 genotype (82%) and the majority of the cell lines had the *G/G TIMP-2* genotype (94.1%). Cell lines with the *C/C* genotype expressed significantly higher mean *MMP-2* mRNA level than those with other genotypes. Samples with different SNP are then sending for direct sequencing and the results from both SNP that is from MMP-2 and TIMP-2 are shown below in Figure 11 and 12 respectively. Gel electrophoresis result of MMP-2 ASA. This gel photographs consists of the sample from the cell-line in which the sample name is labeled above the photograph and the conclusive result is put into the table 4. The upper band at the 388bp is the internal control of each reaction and the SNP band at the 292bp is for detection of the respective SNP as labeled above the band as well and shown in Figure 7. Gel electrophoresis result of TIMP-2 PCR-RFLP in Figure 8: Gel photograph from electrophoresis result with first lane as a marker , follow with the second lane containing un-cut sample(no enzyme activity) used for control and the result of the lane is the result from sample of cell- line PCR-RFLP detection of -418 SNP and the sample contain all the G/G genotype that is the present of only one band at the 230 bp and another two position which is invisible at 51 and 23 bp and the only sample that is HN-9 which has C/C genotype are cut into 2 band at 253bp the one shown on the last lane of the first picture and another one which is also an invisible one at 51bp. The G/C genotype which will contain 4 bands but unfortunately the sample is not from the cell-line so the lane number 9 is an example taken from the blood sample.

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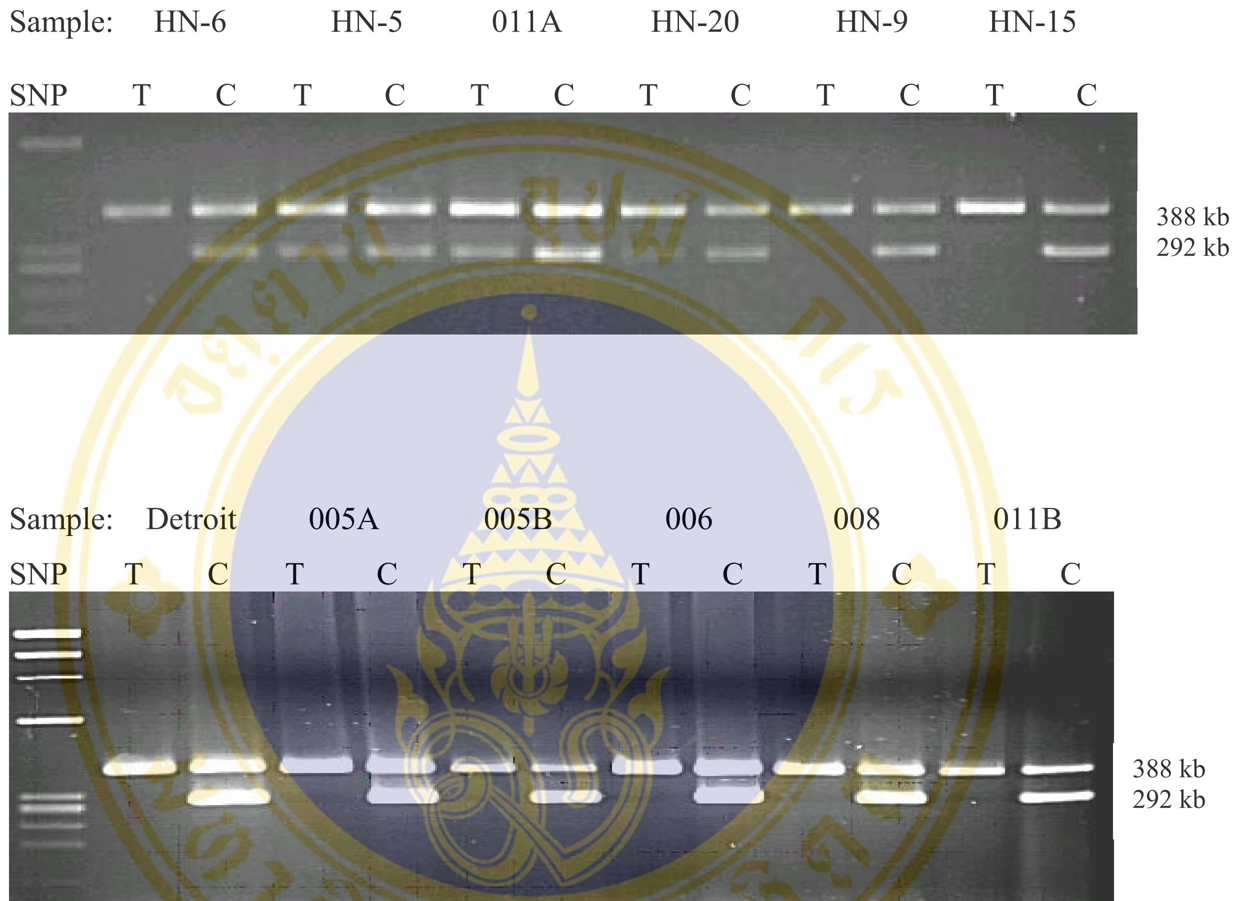

Figure 7. Gel electrophoresis result of MMP-2 ASA. This gel photographs consists of the sample from the cell-line in which the sample name is labeled above the photograph and the conclusive result is put into the excel table 5 below. The upper band at the 388bp is the internal control of each reaction and the SNP band at the 292bp is for detection of the respective SNP as labeled above the band as well. The first band however is the 5X marker used for control as described in the appendix.

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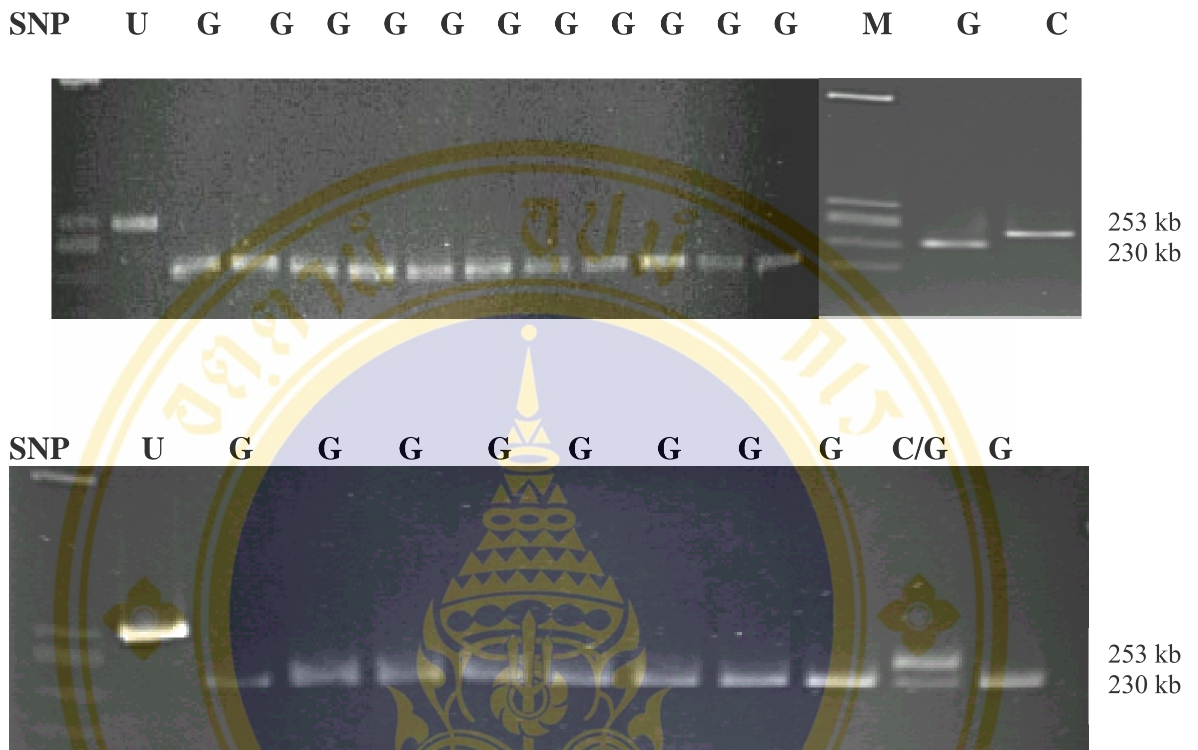

Figure 8. Gel electrophoresis result of TIMP-2 PCR-RFLP: Gel photograph from electrophoresis result with first lane as a marker , follow with the second lane containing un-cut sample(no enzyme activity) used for control and the result of the lane is the result from sample of cell- line PCR-RFLP detection of -418 SNP and the sample contain all the G/G genotype that is the present of only one band at the 230 bp and another two position which is invisible at 51 and 23 bp and the only sample that is HN-9 which has C/C genotype are cut into 2 band at 253bp the one shown on the last lane of the first picture and another one which is also an invisible one at 51bp. The G/C genotype which will contain 4 bands but unfortunately the sample is not from the cell-line so the lane number 9. is an example taken from the blood sample.

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Table 4. MMP-2 and TIMP-2 SNP distribution from cell-line sample

Cell Line	**MMP- 2 SNP**			**TIMP-2 SNP**		
	T/T	**C/T**	**C/C**	**C/C**	**C/G**	**G/G**
HN5		C/T				G/G
HN6			C/C			G/G
HN15			C/C			G/G
Detroit 562			C/C			G/G
SIHN- 005A			C/C			G/G
SIHN- 005B			C/C			G/G
SIHN- 006			C/C			G/G
SIHN- 008			C/C			G/G
SIHN- 011A		C/T				G/G
SIHN- 011B			C/C			G/G
SIHN- 013			C/C			G/G
SIHN- 013/2			C/C			G/G
SIHN- 015B			C/C			G/G
SIHN- 034B			C/C			G/G
SITH- HN8			C/C			G/G
SITH- HN9			C/C	C/C		
SITH- HN20		C/T				G/G

The SNP C/C amplified by the PCR –ASA is known to be present in majority of the cell-line sample and with only three samples containing the SNP C/T which is taken for preparation of the sample to determine the protein activity as described earlier in the paper. Fourteen out of seventeen HNSCC cell lines showed the *C/C* MMP-2 genotype (82%) and the majority of the cell lines had the *G/G TIMP-2* genotype (94.1%). Cell lines with the *C/C* genotype expressed significantly higher mean *MMP-2* mRNA level than those with other genotypes. Samples with different SNP are then send for direct sequencing and the results from both SNP that is from MMP-2 and TIMP-2 are shown below.

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5.2. MMP-2 and TIMP-2 genotypes in cases and controls

Table 4 displays the selected characteristics of 239 HNSCC cases and 188 controls from our Thai population screened for MMP-2/TIMP-2 genotypes. By study design, there were no statistically significant differences between the cases and controls by age, gender, smoking or drinking status.

Table 5. Distributions of select characteristics by case-control status

Variable	Cases (*n* = 239) *n* (%)	Controls (*n* = 188) *n* (%)	*P*
Gender			
Male	134 (56)	103 (54.8)	
Female	105 (44)	85 (45.2)	0.845
Age (yrs), mean (SD)	62.0 (9.3)	62.1 (9.2)	0.912
Smoking status			
Never	29 (12.1)	25 (13.3)	
Former	126 (52.7)	102 (54.3)	
Current	84 (35.2)	61 (32.4)	0.825
Pack-years, mean (SD)	52.4 (30.3)	50.5 (30.9)	0.524
Drinking status			
Never	38 (15.9)	28 (14.9)	
Former	107 (44.8)	86 (45.7)	
Current	94 (39.3)	74 (39.4)	0.956

[a] Pack-years are for ever-smokers.

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Table 6 and Table 7 show the genotype distribution of MMP-2 and TIMP-2 respectively. Allele frequencies for the MMP- 2 -1306C and -1306T were 88.6 and 11.4% in controls, compared with 93.1 and 6.9% in patients. The MMP-2 genotype distributions in patients were 86.2 % (C/C), 12.8% (C/T) and 0 % (T/T) and in controls were 77.7 % (C/C), 22.3 % (C/T) and 0 % (T/T), neither of which deviated from those expected from the Hardy Weinberg equilibrium (P = 0.337 and 0.921, respectively). Although the T/T homozygote patients failed to demonstrate a difference from controls due to the extreme rarity of this genotype in both patients and controls, the frequency of the heterozygote (C/T) was significantly lower in patients than in controls (P = 0.02143). The frequencies of the alleles TIMP-2 -418G and -418C were 83.5 and 16.5% in patients and 82.6 and 17.4% in controls, a difference which was only borderline significant (P = 0.063). The distributions of three TIMP-2 genotypes in patients and controls also fitted the Hardy Weinberg equilibrium law (P = 0.834 and 0.466, respectively). The frequencies of the three TIMP-2 genotypes in patients were 68.1 % (G/G), 30.9 % (G/C) and 1 % (C/C), which were not significantly different from those in controls (P = 0.083).

By using logistic regression analysis, I evaluated the association between the MMP-2 -1306C/T or TIMP-2 -418G/C polymorphism and risk of HNSCC. I found that subjects carrying the MMP-2 -1306C/T genotype had a significantly reduced risk of cancer (adjusted OR, 0.557; 95% CI, 0.337-0.921) compared with those carrying the C/C genotype, suggesting that the variant allele is the protective allele. Similarly, a moderately reduced risk of HNSCC was observed among subjects carrying the TIMP-2 -418C/C genotype, with an adjusted OR of 0.264 (95% CI, 0.052-1.327), compared with subjects carrying the G/G and G/C genotypes (Table 6 and 7).

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Table 6. Numbers (percentage) of subjects with different MMP-2 genotype in HNSCC and control groups

	Cases	**Controls**	**Trend test**	**Adjusted OR***
MMP-2 genotype	***n* (%)**	***n* (%)**	***P***	**[95% CI]**
C/C	206 (86.2)	146 (77.7)		
C/T	33 (13.8)	42 (22.3)		
T/T	0 (0)	0 (0)	0.0214	0.557 (0.337-0.921)

*Odd ratio and 95% CI were calculated by logistic regression, with C/C genotype as the reference group and adjusted for age, gender, smoking and drinking status.

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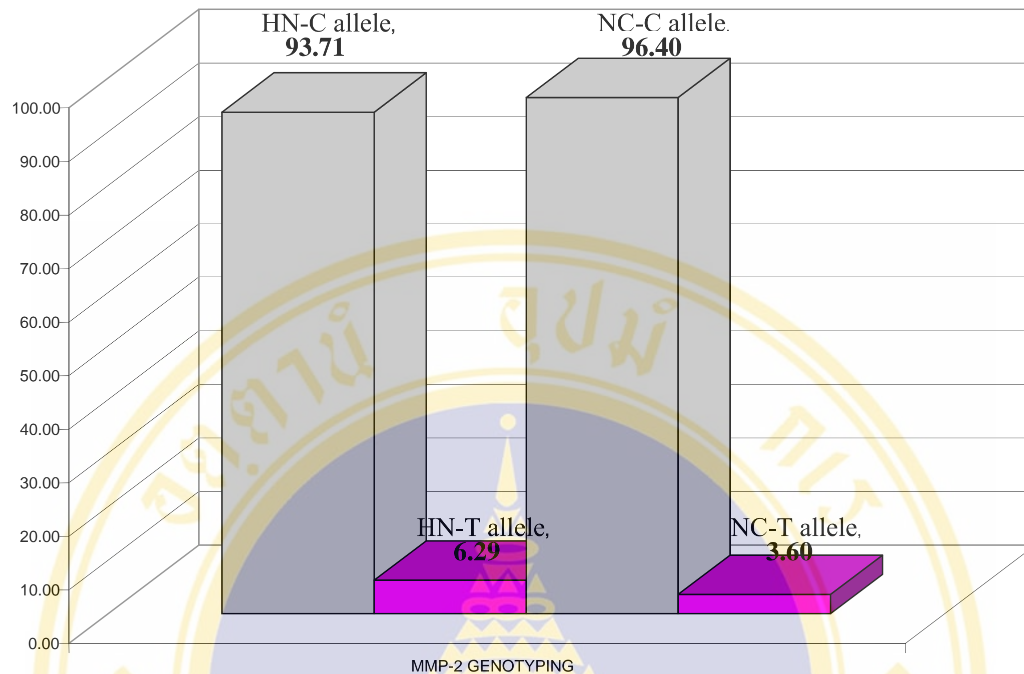

Figure 9. MMP-2 genotype distributions. This graph represents the distribution of the genotype of Matrix Metalloteinase- 2 (MMP- 2) as classifies by the difference between the normal and patients. Among 239 patients, the number patients were found to have the C/C genotype were 206 and other was from the C/T genotype. Neither patients nor control were found to have the T/T genotype due to the rare case to be found among them. Among the 188 normal, 146 of them are under the C/C genotype and 42 are found to have C/T genotype. The allele frequency for the normal is c- 313 and t- 21 and patients c- 428 and t- 16. The percentage differences are shown in the graph above.

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Table 7. Numbers (percentage) of subjects with different TIMP-2 genotype in HNSCC and control groups

TIMP-2 genotype	Cases *n* (%)	Controls *n* (%)	Trend test *P*	Adjusted OR* [95% CI]
C/C	2 (1.0)	6 (3.8)		
C/G	60 (30.9)	42 (26.6)		
G/G	132 (68.1)	110 (69.6)	0.083	0.264 (0.052-1.327)

*Odd ratio and 95% CI were calculated by logistic regression, with G/G and G/C genotypes as the reference group and adjusted for age, gender, smoking and drinking status.

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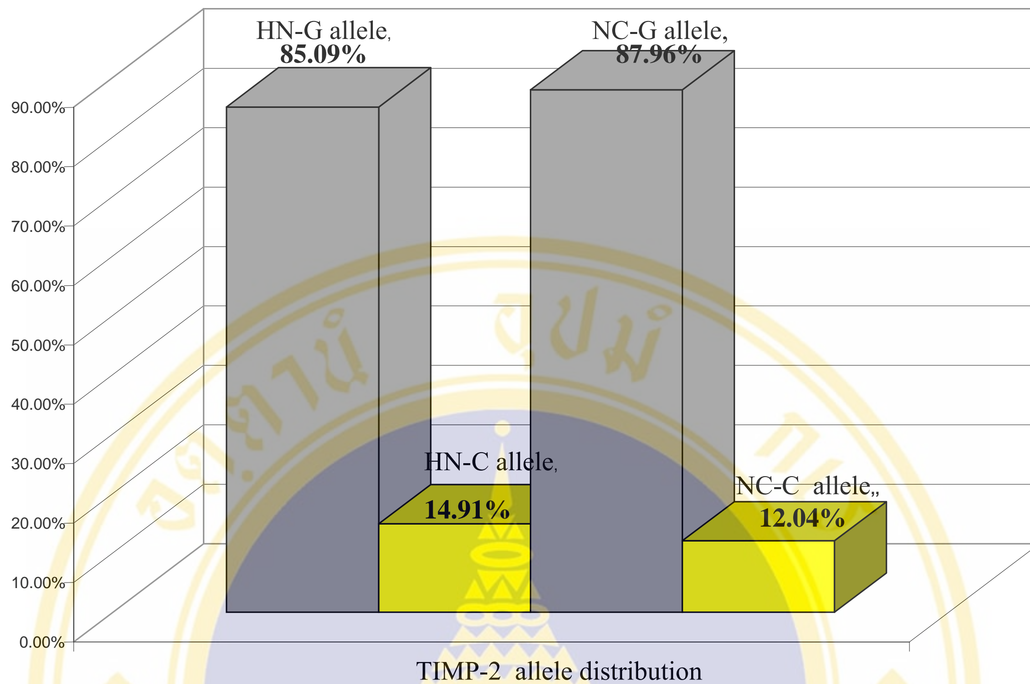

Figure 10. TIMP-2 allele distribution. This graph represents the distribution of the C and G allele of Tissue Inhibitor of Matrix Metalloteinase- 2 (TIMP- 2) as classifies by the difference between the normal and patients. Among 194 patients, the number patients were found to have the G allele were 132 and 60 was from the C allele with only 2 patients have the C allele. Among the 158 normal, 110 of them are under the G/G genotype and 42 are found to have C/G genotype with 6 C/C genotype. The allele frequency for the patients is G- 194 and C- 34 and normal G- 241 and C- 33. The percentage differences are shown in the graph above.

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Table 8. Association distribution of MMP-2 and TIMP-2 genotype

Risk of Head and Neck Cancer Associated with the MMP-2 genotype by TIMP-2 genotypes

Genotype		**Patients**		**Control(N=)**		
MMP-2-1306 C/T	**TIMP2- 418G/C**	**N**	**%**	**N**	**%**	**OR**
CC	GG	120	60.9	73	52.5	Reference
CC	GC+CC	53	26.9	37	26.6	0.87
CC+TT	GG	16	8.1	24	17.3	0.4
CT+TT	GC+CC	8	4.1	5	3.6	0.98

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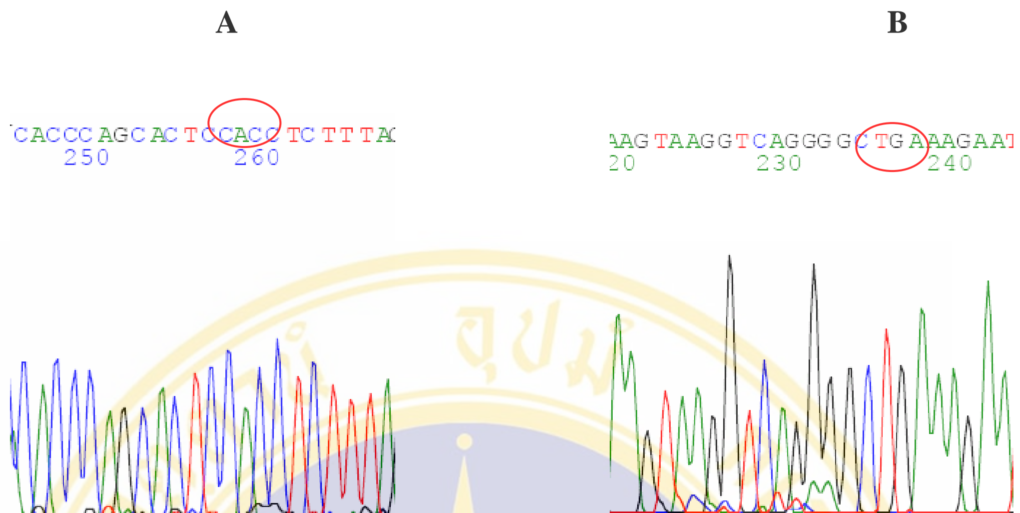

Figure 11.1 MMP-2 C/C sequencing result. MMP-2 -1306 C/C genotype , one sample with C/C genotyping by the PCR are taken for sequencing to confirm the present of the SNP in the MMP-2 gene.

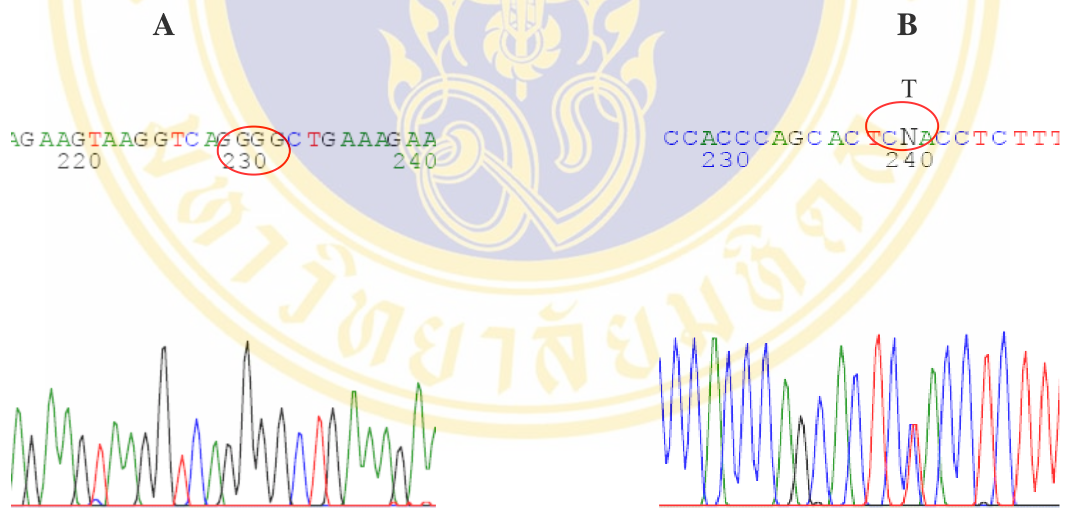

Figure 11.2 MMP-2 C/T sequencing result. MMP-2 -1306 C/T genotype of sample from the patient genotype are sent for sequencing and the result confirms the finding of the present of SNP at the -1306 in MMP-2 gene.

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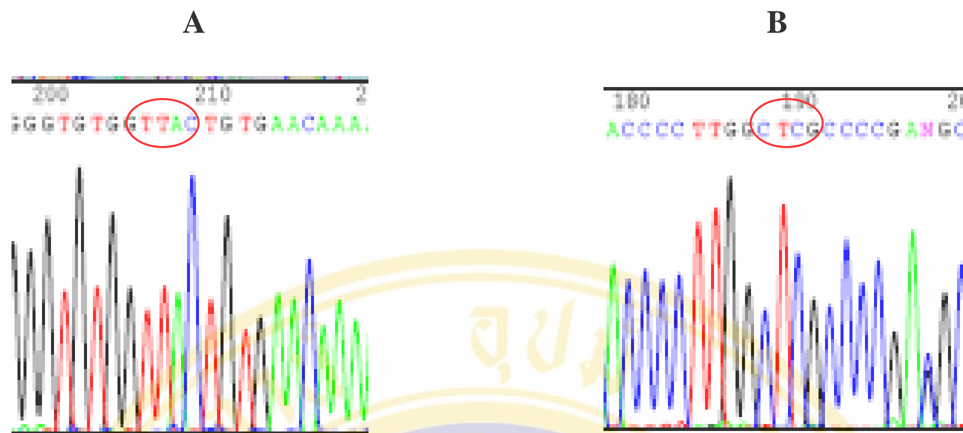

Figure 12.1 TIMP-2 C/G sequencing result. TIMP- 2 – 418 C/G Genotypes confirm by sequencing. Panel A represent the backward sequence of C which complementary to G Genotype and Panel B represents the Forward G sequence which is circle in red.

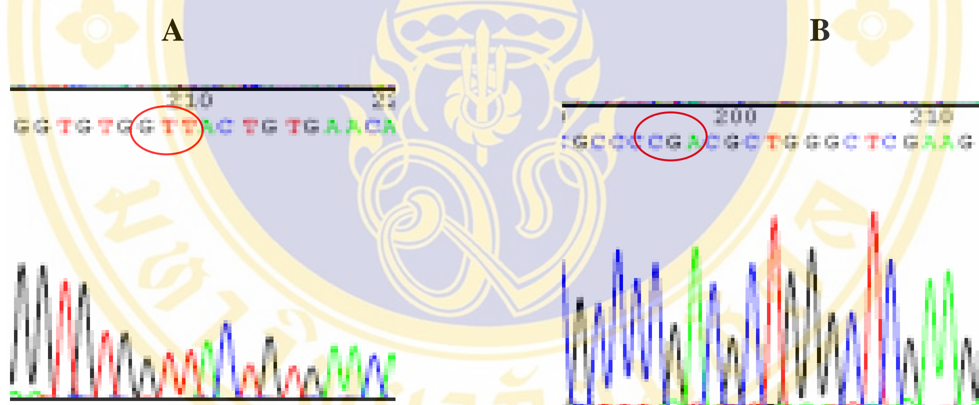

Figure 12.2 TIMP-2 C/C sequencing result. TIMP- 2 – 418 C/C Genotypes confirm by sequencing. Panel A represent the backward sequence of C Genotype and Panel B represents the Forward C which is complementary to the G which is circle in red.

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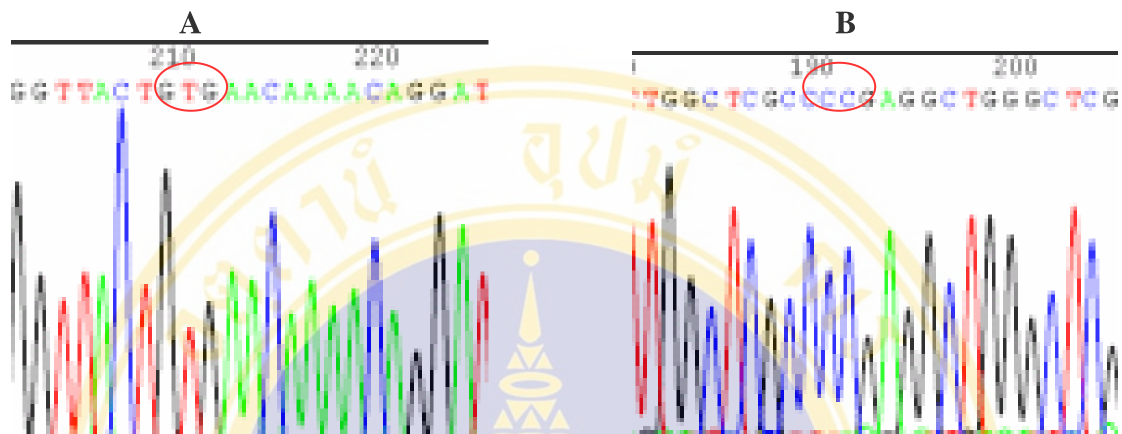

Figure 12.3 TIMP-2 G/G sequencing result. TIMP- 2 – 418 G/G Genotypes confirm by sequencing. Panel A represent the backward sequence of G Genotype and Panel B represents the Forward G sequence which is circle in red.

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5.3. Expression of MMP-2 and TIMP-2 genes according to genotypes in HNSCC tumor cells

To investigate whether the MMP-2 SNP influences expression of the MMP-2 gene in tumor cells (n = 17), quantitative RT-PCR analysis was performed. The levels of MMP-2 expression in tumor cells containing *C/C* allele were significantly higher compared with cells with *C/T* and *T/T* genotypes (Fig. 13) although the result are shown to be in reverse because of the limited number of samples from the *C/T* genotype which seems to have an outlying value. These results are in accordance with previous reports in other tumor cell types (27, 28, 31, 67, 85). Similar finding was observed in tumor with SNP of TIMP-2 at (-418) whereby the levels of TIMP-2 expression containing *G/G* homozygote were significantly higher in cancer patients than those with genotype of *C/G* and *C/C* respectively (Fig. 15) (18, 70, 86). The result of mRNA on the expression level arrived from the RT-PCR can be categorized by the genotypes of the cell line. The genotype distribution of the cell line used for the study is summarized into the Table 4 and their expression level is grouped and shown in the graph. This bar graph confirmed that the *C/C* genotypes have a more obvious intensify band on the gel comparing with those of the *C/T* genotypes in MMP-2 gene and in TIMP-2 gene, more intense band is clearly seen in sample with *G/G* genotype in Figure 15. This obvious understanding is shown in the TIMP-2 result as well. From the bar graph, the genotype distribution of the TIMP-2 is *G/*G genotype in all sample of the cell-line except for HN-9 so the distribution of the expression is biased as we have only one sample from the *C/C* TIMP-2 genotype to compare with. Thus the more obvious and yet accountable data is from the expression measure from the tissue specimen. From the Figure 13 the expression level of the MMP-2 is shown and grouped according to their genotype although we have a limited number of samples from the C/T genotype (3 samples as shown in the Table 4) which can deviate our expression result to the average of the expression in the C/C is 55.51 and C/T genotype is 91.08. However if we eliminate the outlier from both C/C and C/T genotype that is by removing the value of expression that is below 1 in the C/C and above 3 in the C/T genotype. The results are in accordance with the expectations as similar to those of the tissue specimen. If we eliminate the outlier from both C/C and C/T genotype that is by removing the value of expression that is below 1 in the C/C

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and above 3 in the C/T genotype. The results are therefore inconclusive in terms of the bias number of samples which could be improved with the increase in number of cases to be in accordance with the expectations that are the average expression for the C/C is higher and for the C/T genotype to follow with lower expression as the clear understanding can be observe in evaluation of the expression of MMP-2 from the tissue specimen.

5.4. Expression of MMP-2 and TIMP-2 genes according to genotypes in HNSCC tissue samples

To investigate whether this insertion/deletion polymorphism influences expression of the MMP-2 gene in HNSCC, we performed quantitative RT-PCR analysis using representative tumor tissues (*n* = 48) for which genotypes of the insertion/deletion polymorphism were determined (7 *C/T* heterozygote, and 41 *C/C* homozygote). In tumors carrying *C/C* homozygote, the mean expression levels of MMP-2 were significantly higher than the levels of tumors with *C/T* and *T/T* genotypes respectively (Fig. 14). The average of the expression in the C/C is 1.5225 and *C/T* genotype is 2.136667. Thus from this result the expression of the MMP-2 is in accordance with the expected result after eliminating the outlier presenting the average expression for the *C/C* is 1.9064 and for the *C/T* genotype is 1.71 as shown in comparison in Figure 14.

The expression level as shown in the graph for MMP-2 is not in accordance with the expectation as the *C/C* genotype should contain the higher expression level than the *C/T* genotype. This is due to one of the setback of having limited number of sample in the *C/T* genotype and also having distribution skewed to the right. According to the data from both the *C/C* and *C/T* genotype, the distribution of the *C/T* genotype will consists of the “leptokurtic” or experiencing the leptokurtosis in the right tails of the distribution. The expression level for the TIMP- 2 is in accordance with the expectation as the *G/G* genotype has the highest level of expression at the average of 0.875333, following by the *C/G* genotype which consists of a lesser number of sample sizes and shows the average of 0.773. The least number of expressions is in the *C/C* as the average shown is 0.4. Although the results shown are

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in accordance with the expectation, the problem of different sample size arises. The *C/C* genotype consists of only one sample and *C/G* consists of 10 sample sizes. Although it seems difficult to clarify the sample size issues, it does not raises big issues as the results are in accordance with the expectation.

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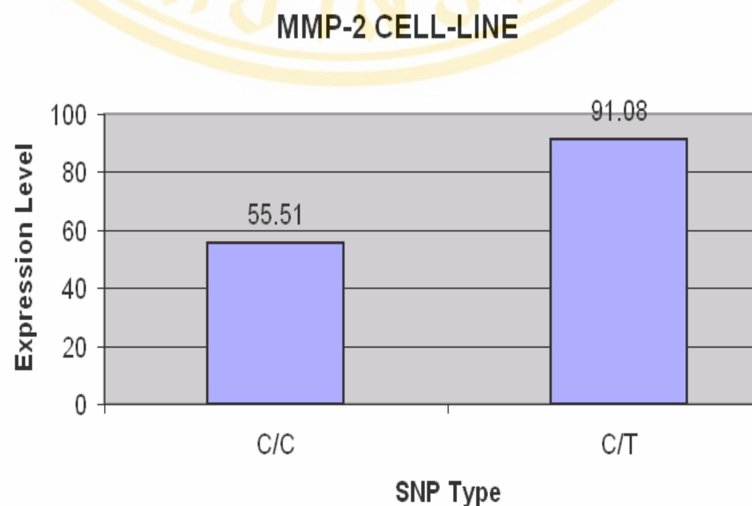
Figure 13. MMP-2 expression level by RT-PCR. Using semi-quantitative RT-PCR method to quantitative the level of mRNA from the extracted level of cDNA, this gel-electrophoresis photograph was taken and quantitate using image J soft ware and plot into the graph (by grouping them according to their genotype distribution which is observe using PCR-ASA) in Excel program shown below. The sample loaded into the gel is as follows: from left to right , upper lane , T-12, HN-5, HN-6, HN-15, Detroit-562, 005A, 005B, 006/1, 008 and the bottom lane with T-12, 011A, 011B, 013, 013/2, 015B, 034B, HN-8 HN-9, HN-20. The different SNP specifically *C/T* genotype is box in red for the direct observation in the different band intensity.



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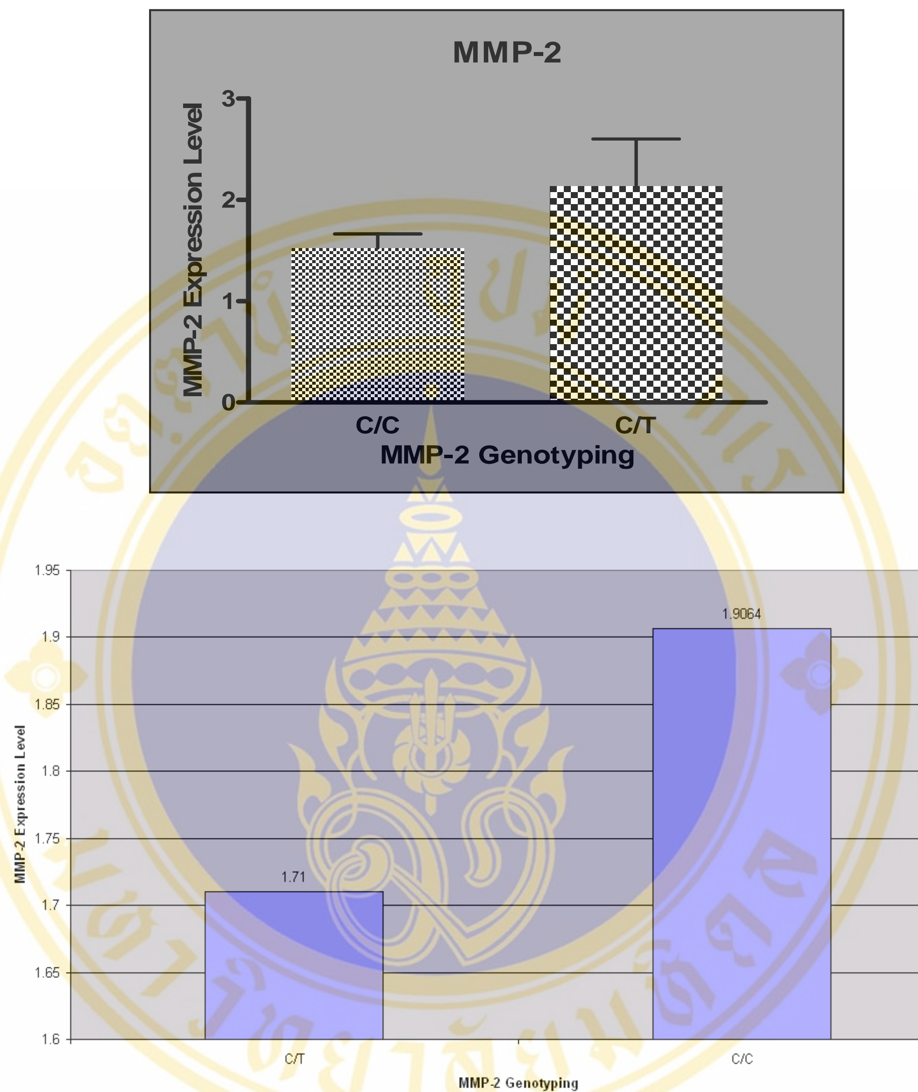

Figure 14. MMP-2 expression level from tissue samples. This graph represents the level of MMP- 2 expression of sample of cell lines that are genotype earlier and grouped into the category of C/C and C/T genotype. With the knowledge of the genomic distribution, the sample of cell line are treated to extract the cDNA to obtain the level efficient for the RT-PCR which will be later capture on the gel and quantified using program image J and imported the data in the quantity analyze in the graph pad Prism 4. The result of the expression is greater in the C/T genotype from the raw data evaluation but after eliminating the outlier, the result is in accordance with C/C genotype greater as shown in the bottom graph.

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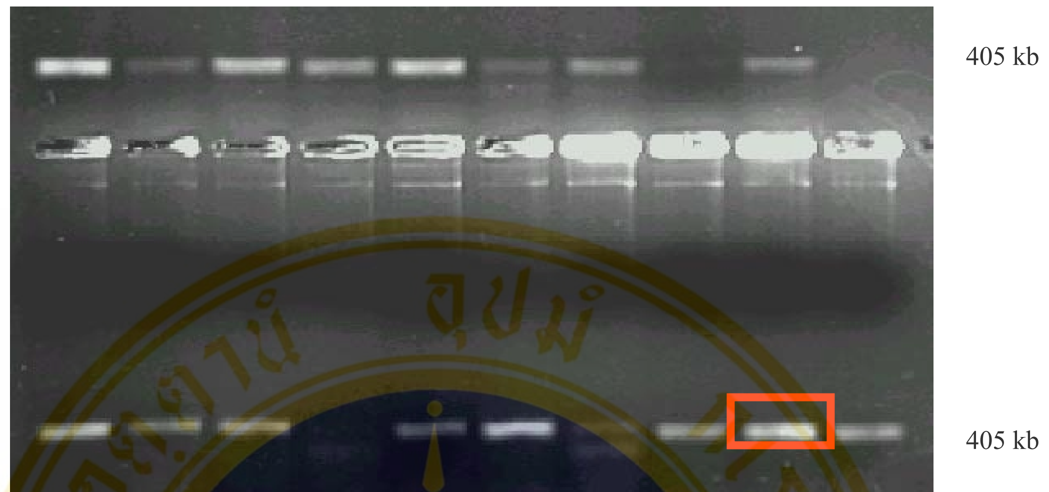
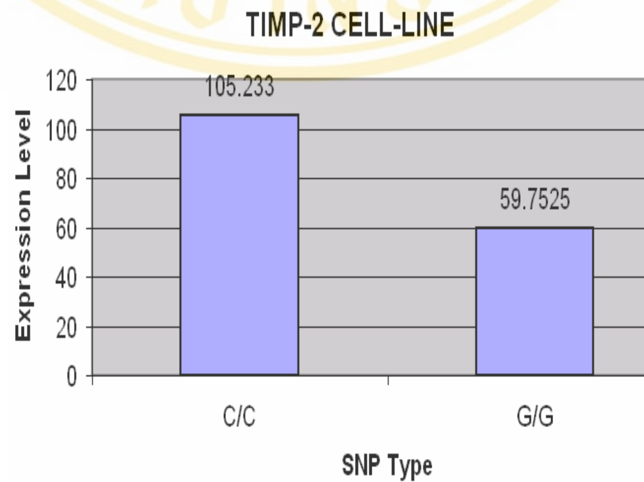


Figure 15. TIMP-2 expression level by RT-PCR. Using semi-quantitative RT-PCR method to quantitative the level of mRNA from the extracted level of cDNA, this gel-electrophoresis photograph was taken and quantitate using image J soft ware and plot into the graph (by grouping them according to their genotype distribution which is observe using PCR-RFLP) in Excel program shown below. The sample loaded into the gel is as follows: from left to right , upper lane , T-12, HN-5, HN-6, HN-15, Detroit-562, 005A, 005B, 006/1, 008 and the bottom lane with T-12, 011A, 011B, 013, 013/2, 015B, 034B, HN-8 HN-9, HN-20. The different SNP specifically *C/C* genotype is box in red for the direct observation in the different band intensity.



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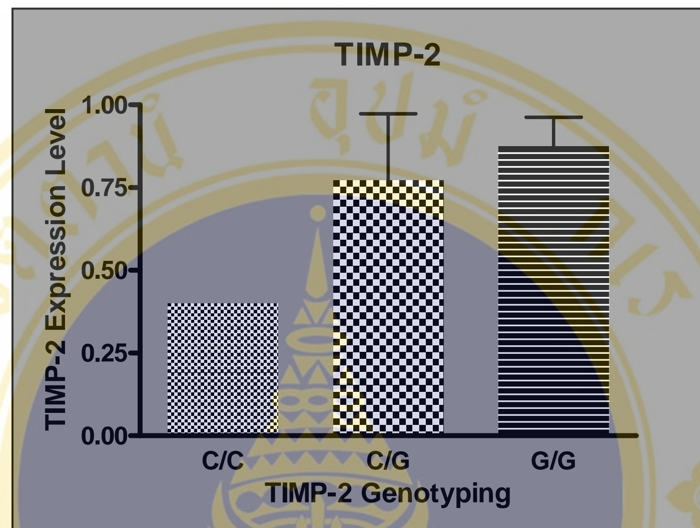

Figure 16. TIMP-2 expression level from tissue samples. This graph represents the level of TIMP- 2 expression of sample of cell lines that are genotype earlier and with the knowledge of the genomic distribution, the sample of cell line are treated to extract the cDNA to obtain the level efficient for the RT-PCR which will be later capture on the gel and quantified using program image J and imported the data in the quantity analyze in the graph pad Prism 4.

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5.5. Activity of MMP-2 gene in HNSCC cell lines

Previous report suggested of a biological trend in which the average basal activity is higher in cells homozygous for the *C/C* allele. In addition, there is a greater potential for HNSCC cell lines with a *C/C at -1306* polymorphism to have higher MMP-2 activity especially in response to (87). With the result of the genotypes of the MMP-2 and expression level from the RT-PCR, the cell-line samples are chosen based on the genotype distribution for zymography analysis in order to determine the activity level of MMP-2. In order to elucidate the role of direct interactions between HNSCC cells and peritumoral fibroblasts with different MMP-2 genotypes in the regulation of MMP-2, HNSCC cell lines and fibroblasts were seeded (5×10^4 cells per cm^2) as monocultures and cultured for 24 h before harvest. Representative HNSCC cell lines of two different MMP-2 genotypes C/C and C/T were cultured as described in the protocol above. The highest level of *MMP-2* enhancement was demonstrated in the tumor cells of C/C homozygotes whereas there was significance reduction in the level of activity of *MMP-2* level in tumor cell line sample with C/T heterozygote (Fig. 3) and as expected there was no tumor cell line found consisting with the T/T genotype due to rare case. The cell lines are chosen and prepare to determine the activity of the MMP- 2 protein based on the results from the genotype that are obtained from PCR-ASA based method as discussed earlier. The HT1080 cell line is cultured and used as a control of the experiment by varying the concentration of the HT1080 proteins shown in Figure 17. We obtain the optimized value to get the optical density which will be used as a database for the sample proteins density evaluation. By varying the concentration and observing the linear correlation as graph in Figure 17, the positive correlation confirmed that as the concentration of the HT1080 increases, the density also increases. With this linear correlation confirmed, the activity of the MMP-2 and MMP-9 can be confirmed this way by associating the lytic band to the activity of MMP-2 ability to digest and degrade the gelatin. With HT1080, the well-known and most aggressive fibrosarcoma which has been used in various papers and research projects as a control for the MMP-2 and MMP-9 activity. From the genotype table 5 of the cell lines, there are only two types of SNP that is the C/C and C/T genotypes. With these selections, 5 samples of the C/C are taken namely LICR - HN15, SIHN005B, SIHN 013/2, SITH-HN8 and SITH-HN9, along with 3 samples of C/T genotypes were

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taken namely HN5, SIHN011A, SITH-HN20 are prepared as described in the material and methods section. The results are captured and quantify as shown and plot into the graph. From the graph, it can be assumed that the results conformed the findings that the samples with C/C genotypes shows MMP-2 activity double to the samples found with the C/T genotypes with the average of 135.9442 C/C to 71.90059 found respectively. This confirms findings that the activity level of the MMP- 2 proteins is highly affected by the SNP at -1306C/T which is the Sp1 binding which is transcriptional regulated. The SNP at -1306C has confirmed to have over transcribed MMP- 2 genes which results in over expression of MMP- 2 proteins and higher active MMP- 2 activity. In contrast the SNP at the -1306T has regulated or lower level of MMP- 2 transcribe genes specifically the Sp1 binding site, this further results in the lower expression level and activity level in this findings.

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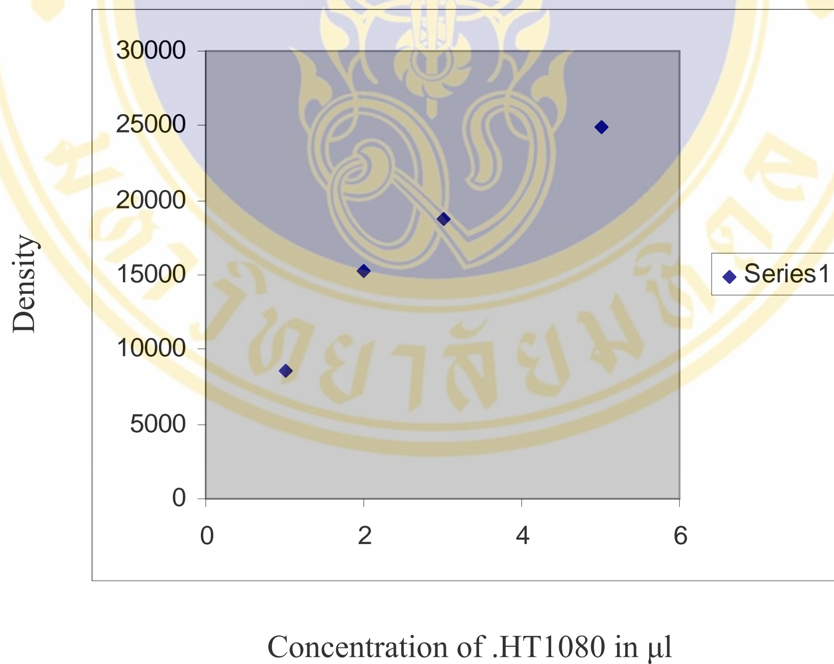
Figure 17. Varying concentration of HT1080. The HT1080 cell line is cultured and used as a control of the experiment by varying the concentration of the HT1080 proteins. We obtain the optimized value to get the optical density which will be used as a database for the sample proteins.



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Sample Name: 013/1 HN6 HN8 HN9 HN15 HT1080 µl

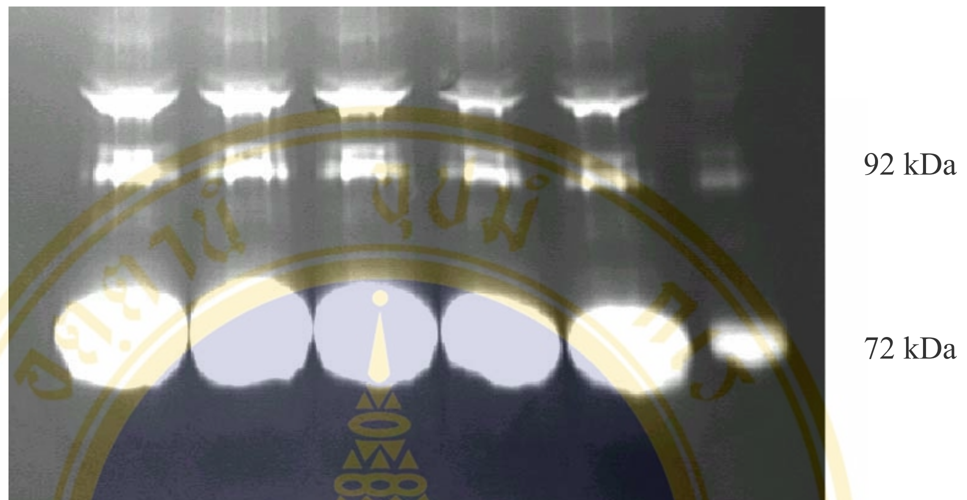

Figure 18. Zymography of C/C MMP-2 genotype. From the genotype table of the cell lines, there are only two types of SNP that is the C/C and C/T genotypes. With these selections, 5 samples of the C/C are taken namely LICR - HN15, SIHN005B, SIHN 013/2, SITH-HN8 and SITH-HN9, are prepared as described in the material and methods section. The results are captured and quantify as shown and plot into the graph. The photograph of the gel from the SNP C/T are captured and shown here with the sample concentration of 20µl and with the addition of the 5µl of the loading dyes prepared by following the protocol described in the Material and Method section.

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Sample Name: HN-20 HN-5 001A HT1080 μl

Figure 19. Zymography of C/T MMP-2 genotype. From the genotype table of the cell lines, there are only two types of SNP that is the C/C and C/T genotypes. With these selections, 5 samples of the C/C are taken namely LICR - HN15, SIHN005B, SIHN 013/2, SITH-HN8 and SITH-HN9, along with 3 samples of C/T genotypes were taken namely HN5, SIHN011A, SITH-HN20 are prepared as described in the material and methods section. The results are captured and quantify as shown and plot into the graph. The photograph of the gel from the SNP C/T are captured and shown here with the sample concentration of 20μl and with the addition of the 5μl of the loading dyes prepared by following the protocol described in the Material and Method section.

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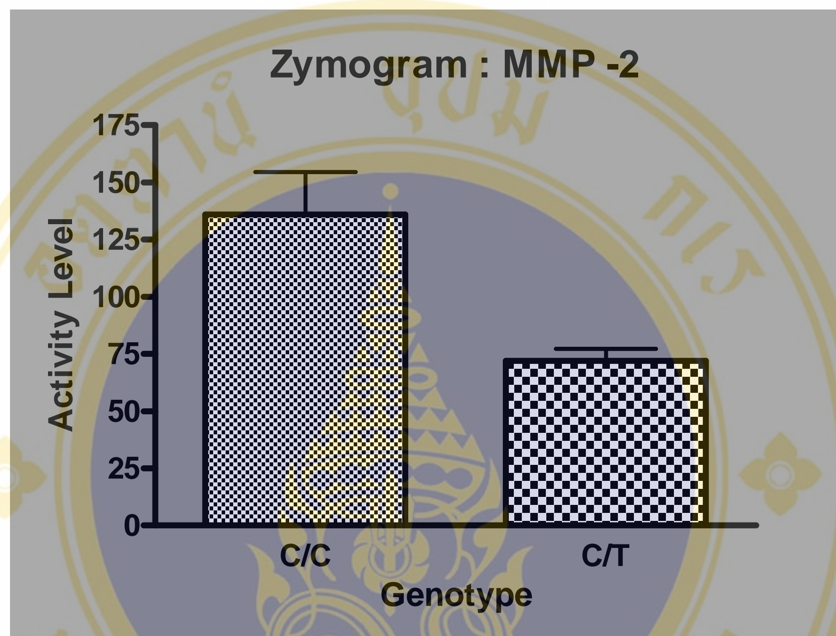

Figure 20. MMP-2 activity by zymography. From the genotype table of the cell lines, there are only two types of SNP that is the C/C and C/T genotypes. With these selections, 5 samples of the C/C are taken namely HN15, SIHN005B, SIHN013/2, SITH-HN8 and SITH-HN9. And 3 samples of C/T genotypes were taken namely HN5, SIHN011A, SITH-HN20 are prepared as described in the material and methods section. The results are captured and quantify as shown and plot into the graph.

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5.6. Correlation between MMP-2 genotype and clinicopathological parameters

HNSCC patients (*n* = 239) were stratified by clinicopathological parameters including age, gender, tumor location, histological grade, tumor thickness (depth of invasion), T category (primary tumor), N category (regional lymph node metastasis), overall stage, and their relations with the MMP-2 polymorphism were evaluated by Fisher's exact test (Table 9). I observed a significant correlation between C/C genotype and several established prognostic variables, including tumor thickness > 10 mm. (P = 0.0203), presence of nodal metastasis (P = 0.0008), and advanced pathological stages (P = 0.0043). The correlation between the *TIMP-2* polymorphisms and various clinicopathological parameters was not observed (data not shown).

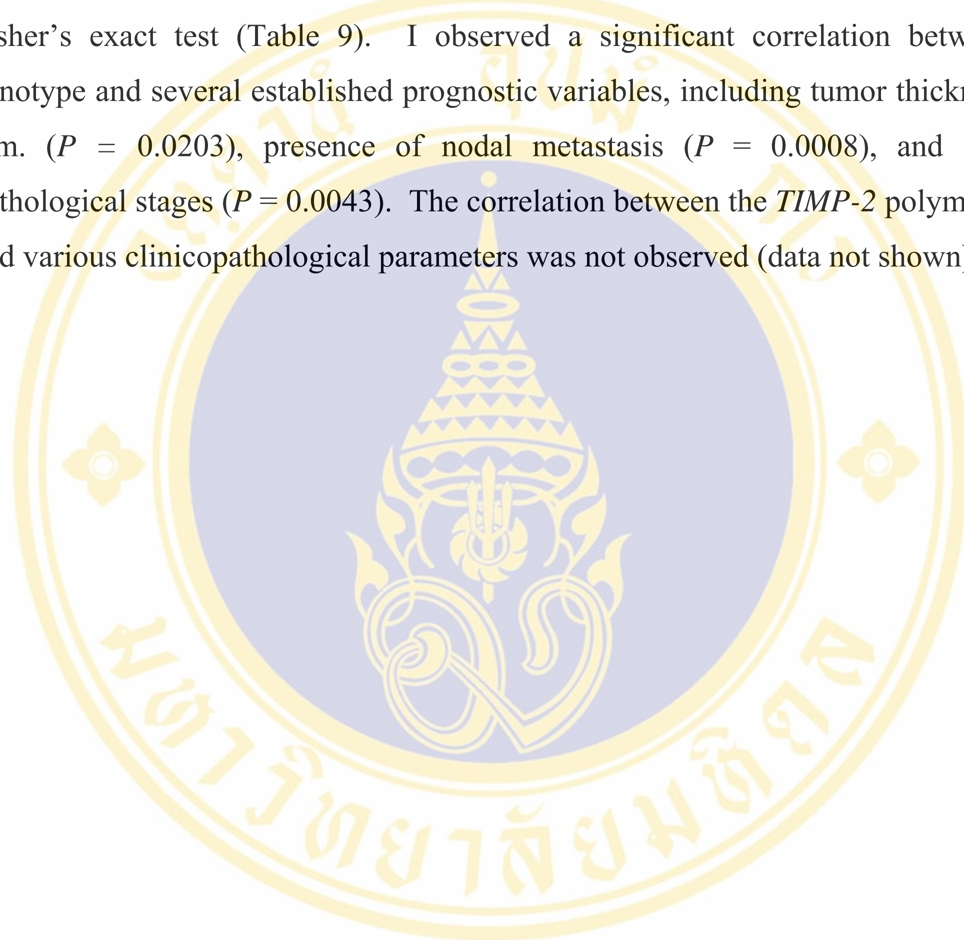

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Table 9. Relation between MMP-2 genotypes and clinicopathological characteristics of HNSCC

	C/C[a]	C/T[a]	P^b
Age[c]			
≤ 60 yr	100	15	0.8516
> 60 yr	106	18	
Gender			
Males	128	22	0.7004
Females	78	11	
Cancer Sites			
Oral cavity	98	13	0.8853
Oropharynx	46	10	
Hypopharynx	12	4	
Larynx	50	6	
Histological grading			
Well & moderate	116	17	0.7065
Poor	90	16	
Tumor thickness			
≤ 10 mm	73	19	0.0203
> 10 mm	133	14	
Perineural invasion			
No	98	15	0.8531
Yes	108	18	
Perivascular invasion			
No	83	19	0.0870
Yes	123	14	
T classification			
T1-2	92	20	0.0947
T3-4	114	13	
Lymph node involvement			
Negative	66	21	0.0008
Positive	140	12	
Pathological staging			
I-II	58	18	0.0043
III-IV	148	15	

[a] The number of patients with this genotype.
[b] Fisher's exact test with Bonferroni correction.
[c] Patients were divided into 2 groups by mean age.

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CHAPTER VI

DISCUSSION

In the previous study, there have been reported that the -1306C/C polymorphism in the promoter of *MMP- 2* is a strong genetic susceptibility factor for lung cancer, and the *C/C* genotype confers several-fold increased risk for developing the cancer (31). Because *MMP-2* has been shown to be over expressed in gastro esophageal cancer and appears to be a feature of the malignant phenotype (88-90), we therefore examined whether the functional polymorphism in the *MMP-2* and *TIMP-2* promoter could have an implication in susceptibility and aggressiveness of HNSCC. In this present study, the relationship between the polymorphisms in the promoter of *MMP-2 and TIMP-2* and levels of gene expression, activity in cancer susceptibility, and cancer progression using both *in vitro* and clinical models of HNSCC are studied.

From this study, the result found that majority of HNSCC cell lines carrying the *C/C* allele and the levels of MMP-2 expression correlated with the *C/C* genotype. Similarly, tumor tissues with *C/C* allele expressed higher MMP-2 expression that those with *C/T* allele. With this confirmation, the demonstration that sowns a significant association between the *C/C* genotype and increased head and neck cancer risk was then performed. The results are the positive correlation between the MMP-2 genotype with advanced tumor stage and adverse prognostic parameters. Furthermore, results of the zymograms experiments implies that in patients with *C/C* genotype, tumor cells are likely to express higher levels of MMP-2 due to the lack of control of MMP-2 gene at the transcriptional level of the Sp1 binding site which may be because of the effects of the TIMP-2 polymorphisms with the combination of other effected genes leading to more aggressive biological behavior and hence poorer prognosis.

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These molecular epidemiological results are consistent with the previous findings showing that the C/C polymorphism of MMP-2 is functionally important. The C/C allele binds substantially more Sp1 transcription factor and has significantly higher transcriptional activities than the C/T allele (31). This polymorphism, together with an adjacent Sp1 binding site, significantly affects the induction level of MMP-2 with loss of control at the transcriptional level of this gene (91). In addition, MMP-2 production was higher in human head and neck cell lines from *C/C* homozygote than in those from *C/T* heterozygote when stimulated with growth factors, hormones or cytokines (92-94). Therefore, the MMP-2 expression would be higher in individuals who carry the *C/C* genotype than those who carry the *C/T* genotype as supported by the present data. Various studies (66, 67, 95) examined MMP-2 mRNA levels in the tumor tissue of cancer patients using semi-quantitative, found higher expression of MMP-2 mRNA in tumor tissues of patients that contained *C/C* allele in their genome than in those with the *C/T* genotype. In HNSCC, MMP-2 is expressed in stromal cells as well as in cancer cells of tissue specimens (96-99) and is associated with its progression (40, 96, 100). Because MMP-2 and other MMPs may contribute in various ways to all stages of carcinogenesis, the increased frequency of *C/C* homozygote in HNSCC patients which leads to the increased level of this MMP-2 enzyme over a lifetime may render from hosts and their target tissues at increased susceptibility to cancer development. Furthermore, the present data demonstrated the significant correlation between the *C/C* genotype in HNSCC and various adverse features including tumor thickness, presence of nodal metastasis and advanced stages. Recent studies showed that the majority of metastasis melanomas retained the *C/C* allele of MMP-2 (101) and the *C/C* polymorphism was associated with increased invasiveness of melanoma and metastasis potential of colorectal cancer (66). These epidemiological data together with the present data establish a potential role for the *C/C* polymorphism in the MMP-2 promoter sequence as a facilitating factor for carcinogenesis and tumor aggressiveness in HNSCC patients. Although the design of hospital-based case-control study has potential drawbacks such as selection bias, the results in this study, which had large sample size and included 90% of the eligible cases, solid and reproducible genotyping procedures, and significantly increased ORs with very small *P* values, are unlikely to be attributable to selection bias. The fact that

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genotype frequencies among the control population fit the Hardy-Weinberg law further supports the randomness of our control selection. Moreover, the observed effect of MMP-2 *-1306C/T* polymorphism was not fluctuated by other potential predictive factors of HNSCC such as age, sex, and smoking. Hence, these results are unlikely to be biased by subject selection or unknown confounding factors. These molecular epidemiological results are consistent with the previous findings showing that the C/T transition at –1306, which disrupts an Sp1-type promoter site (CCACC box) and results in a strikingly lower promoter activity with the *T* allele of the MMP-2 gene (102). The Sp1 site, among other promoter elements such as AP-2, has been shown to be necessary for regulating constitutive expression of MMP-2 (103). Therefore, the presence of the Sp1 promoter site in the MMP-2 –1306*C* allele may enhance transcription, which has in fact been demonstrated *in vitro* in transient transfection experiments (102), so MMP-2 protein expression would be higher in individuals who carry the *C/C* genotype than those who carry the *T/T* or *C/T* genotype. Because MMP-2 and other forms of MMPs may contribute in multiple ways to all stages of carcinogenesis (88), the increased level of this enzyme over a lifetime may render the hosts and their target tissues at increased susceptibility to cancer development. This postulation is strongly supported by experimental cancer models. It has been shown that when induced by carcinogenic stimulus, wild-type mice developed more cancers than mice that lack the MMP- 2, -7, -9, or -11 gene (104-106), and the development of HNSCC in mice that lack MMP- 9, another form of gelatinases family, could be restored by transplanting MMP- 9 expressing bone marrow cells (105). In another experiment, cancer cells injected via vein were found to be more capable of colonizing the lungs of wild-type mice than the lungs of MMP- 2 deficient mice (31). It is also documented that overexpression of MMPs in transgenic mice results in elevated cancer susceptibility (107, 108). The involvement of MMPs in carcinogenesis is biologically plausible because they can alter the cellular microenvironment and consequently affects the process of neoplastic transformation and cancer development. It has been shown that by cleaving IGF-binding proteins, MMPs can release IGFs (109). IGFs such as IGF-1 are well known to have a strong effect on stimulating cell proliferation and inhibiting apoptosis. High levels of circulating IGF-1 and low levels of IGF-binding protein 3 are associated with

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increased risk of several common cancers, including lung cancer (110). MMPs may also release the cell membrane-bound precursor of transforming growth factor (111), another important growth factor involved in neoplastic transformation and cancer development. Moreover, MMPs are also involved in cleavage of a number of molecules on the cell surface, which may alter cell cycle checkpoint controls and conceivably promote genomic instability by affecting cell adhesion (112), may disrupt cell signaling, and may foster cancer cells to escape immunosurveillance (113). Taken together, these data provide very plausible molecular mechanisms through which the genetic polymorphism resulting in high expression of MMP-2 over a lifetime could increase cancer risk. In our study, we found that the MMP-2 (-1306C) genotype was significantly associated with HNSCC risk; however, an additive interaction between the MMP-2 polymorphism and TIMP-2 polymorphism. Several possibilities exist to explain these findings because MMPs expression can be induced and regulated by TIMP-2 (18, 61), one hypothesis is that, in addition to higher constitutive expression because of gain of an Sp1 promoter site, the inducibility by lack of TIMP-2 transcript gene and the overly expressed gene due to the present of the *C* allele of MMP-2 may also be higher than that of the *T* allele, which loses an Sp1 site. Given these conditions, it would be expected that subjects with G/G allele in TIMP-2 and carried the *C/C* genotype were more susceptible to developing HNSCC. Alternatively, a higher risk of HNSCC are found in individuals with the *C/C* genotype from MMP-2 and G/G genotype in TIMP-2 may attribute to the occurrence of larger numbers of transformed cells caused by smoking in the target tissue, which, in turn, increases the possibility that one of these cells will become malignant under the condition of higher expression of MMP-2. Another interesting finding in this study was that the increased risk related to the MMP-2 polymorphism and many publications supported this finding as was evenly observed in different subtypes of lung cancers that is adenocarcinoma, and other histological types of lung cancer. This result suggests that the MMP-2 polymorphism might be a general, but not a specific, risk factor for common cancers, further supporting the likelihood that MMPs profoundly influence early tumor initiation and development. In summary, this study demonstrated a significant association between the MMP-2 (-1306C/T) polymorphism and the risk of developing head and neck cancer solely or in a manner of interaction in a Thai population.

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Because this is the first report demonstrating the contribution of the MMP-2 polymorphism to HNSCC risk and because MMP-2 is expressed in many types of cancer and normal stromal cells, additional studies on lung cancer and also other types of common cancers would be warranted. Moreover, the possible role of the MMP-2 (-1306C/T) polymorphism in cancer invasiveness and metastasis should also be addressed.

Although MMPs are not oncogenic or mutagenic, there are several mechanisms whereby they can alter the cellular microenvironment and consequently affects the process of neoplastic transformation and tumor progression. By degrading ECM, MMPs can release active growth factors, angiogenic factors, and angiogenic inhibitors from the cell surface and ECM (114, 115). Some of these growth factors may influence tumor cells directly, whereas others may influence neighboring cells that are essential to form a permissive and supportive environment for tumor progression. It is only recently that tumor progression has been recognized as the products of an evolving crosstalk between different cell types within the tumor and its surrounding supporting tissue, or tumor stroma (116). Stromal cells respond to growth factors and cytokines in the tumor milieu, such as EGF, by secreting growth factors and pro-migratory ECM components, as well as upregulating the expression of serine proteases and several MMPs including MMP-2 that degrade and remodel the ECM. Given the fact that the microenvironment can influence tumor formation and MMPs can alter this environment, MMPs may contribute to the initial stages of cancer development, and overexpression of MMPs may be associated with progression of disease. The present study demonstrated that the presence of a C/C in the MMP-2 promoter significantly affects mean MMP-2 expression levels in cancer cells. In addition, when cell types were cultured in for zymography experiment there was a substantial increase in the MMP-2 levels of activity further. These finding supports the role of tumor-stromal interaction in cancer progression. Hence, the genetic polymorphism resulting in high expression of MMP-2 over a lifetime may contribute in multiple ways to all stages of cancer development and progression. To the best of our knowledge, this is the first study to examine the relationship between the MMP-2 and TIMP-2 polymorphisms and HNSCC risk. These results are consistent with

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previous findings for the MMP-2 polymorphism in breast, lung and gastric cardia cancer studies (28, 31).

On the other hand, a recent study demonstrated that a reduction in Sp1 DNA binding activity or phosphorylation by nonsteroidal anti-inflammatory drugs suppresses MMP-2 expression (117). These data clearly suggest that absence of the Sp1 consensus sequence in the MMP-2 (-1306T) allele would produce a lower level of MMP-2 protein in individuals carrying the C/T or T/T genotype than those carrying the C/C genotype. Secondly, several studies with genetically modified animals have associated a low level of constitutive expression of MMP-2 with reduced risk of tumor formation. It was found that when induced by carcinogenic stimulus, mice that lack the MMP-2 or MMP-9 gene developed fewer tumors than wild-type mice (62). Cancer cells injected via a vein were found to be less capable of colonizing the lungs of MMP-2 knockout mice than the lungs of wild-type mice (118). Of particular interest, transgenic mice that over express MT/MMP-1, a known activator of pro-MMP-2, were at increased risk of mammary tumor formation and metastasis (103). In addition, functional polymorphisms in some other MMP genes have also been linked to varying susceptibility to certain cancer and a single adenosine insertion polymorphism in the MMP-3 promoter (6A allele), which has half the transcriptional activity of the 5A allele, has been associated with a reduced risk of breast cancer (119). A provocative finding from this molecular epidemiological study was the observation that a moderately decreased risk of breast cancer was associated with the TIMP-2 (-418G/C) polymorphism, which is located within the Sp1-binding site in the promoter of the gene and presumably affects transcriptional activity (72, 73). Because TIMP-2 is considered an endogenous inhibitor of MMP-2, the reason why the variant alleles are less susceptible to breast cancer may not be immediately evident. However, accumulating evidence indicates that, in addition to the inhibitory effect on MMP-2, TIMP-2 may act as a multifunctional molecule, which promotes tumor cell growth and tumor angiogenesis and inhibits tumor cell apoptosis (113, 120). These effects of TIMP-2 through MMP-2 dependent or independent pathways make it paradoxical in carcinogenesis. In fact, several clinical investigations have correlated high levels of TIMP-2 with proliferation and/or progression of breast cancer and other cancers

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including oral carcinoma (70, 86). Furthermore, it is worth noting that other members of the TIMP family, such as TIMP-1 and TIMP-4, also have promotive effects on the growth of breast cancer cells (27, 121, 122), liver cancer and HNSCC. Our results in the present study are parallel to these previous findings and suggest that lower constitutive expression of TIMP-2 might render the hosts less susceptible to head and neck cancer, probably through the MMP-independent pathway. Despite the existence of conflicting results, it is generally believed that local over expression of MMP-2 promotes and TIMP-2 inhibits cancer invasion and metastasis. Several studies have suggested that genetic polymorphisms in the promoter of MMP-1 (1G/2G) or MMP-3 (5A/6A), which alter the transcription activity of the genes as in this case at the Sp1 binding site, may influence invasiveness or metastasis of some types of cancer such as melanoma (123), colorectal cancer (105) and breast cancer (119). This activity might result from the genetically determined balance of MMP-2 and TIMP-2, which seems to have the ability to suppress HNSCC cell growth. Our molecular epidemiological results are parallel to the laboratory findings showing that the (–1306C/T) transition in the promoter region of MMP-2, which disrupts an Sp1-binding site (CCACC box), leads to a strikingly lower promoter activity with the T allele (124). Deletion or site-directed mutagenesis analysis of MMP-2 promoter has also shown that the Sp1 site, among other promoter elements such as AP-2, is critical for constitutive activity of this gene (125). On the other hand, recent study also demonstrated that reduction of Sp1 transcriptional binding activity or phosphorylation by nonsteroidal anti-inflammatory drugs suppresses MMP-2 expression (126). Taken together, these data clearly suggest that the presence of Sp1 consensus sequence in the MMP-2 (–1306C) allele may enhance transcription, which in turn would produce higher levels of MMP-2 protein in individuals carrying the C/C genotype than those carrying the T/T or C/T genotype. Because MMP-2 plays an important role in multiple ways to all stages of cancer initiation and development, one would expect that individuals who carry the C/C genotype and therefore have increased expression of this enzyme over a lifetime may be more susceptible to cancer. The association between high levels of constitutive expression of MMP-2 and susceptibility to tumor formation has been tested in several studies with genetically modified animals. It was found that when induced by carcinogenic stimulus, mice that lack the MMP-2 or MMP- 9 gene developed fewer

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tumors than wild-type mice (127). The importance of low-penetrance susceptibility genes in the etiology of cancer is now emerging. In most cases, polymorphisms in enzymes involved in carcinogen metabolism appear to have little overall effect on cancer risk. However, a more interesting picture evolves from studies that have explored the effect of the polymorphisms within levels of environmental exposures. Indeed, in the presence of an interaction between genetic and environmental variables, failure to account for both causes may lead to a bias in estimation of disease risk (128). Many of the early molecular epidemiological studies of cancer failed to investigate gene and environment interactions. Moreover, early studies of gene and environmental interactions in relation to cancer tended to be small and potentially biased. Studies (129), the case-control and the case-only analysis require substantial sample sizes in order to get reliable estimates of the interplay of genes and environment. Given the variability in the prevalence of genetic polymorphisms across populations, selection of controls from the same source population that gave rise to the cases is absolutely necessary to avoid selection bias. Research is currently underway to identify the single nucleotide polymorphisms (SNPs) that seem to occur on the average of once every 1000 base pairs throughout human DNA. To date, over millions SNPs have been identified however, the function of the majority of these genes remains unknown, and we can imagine that many of the important genetic determinants of cancer remain undiscovered. In addition, it is not always clear whether genetic alterations affect the function of the gene products. In fact, many polymorphisms are functionally silent, meaning that the mutation does not change the amino acid sequence of the protein, nor the extent to which the protein is expressed. Future research towards understanding the etiology of cancer will certainly move in the direction of molecular epidemiology to a greater extent. Moreover, the resources required to conduct such studies will necessitate larger collaborations between epidemiologists, molecular biologists, and clinicians. Such participation will help foster a deeper understanding into the complexity of cancer.

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CHAPTER VII

CONCLUSION

In conclusion, we demonstrated for the first time the distribution and the clinical significance of MMP-2 and TIMP-2 promoter polymorphisms in HNSCC patients. These findings suggested that SNP of MMP-2 promoter might influence the ability in HNSCC invasion through the increased in the transcriptional activity of this gene and the presence of the variant allele in the promoter of MMP- 2 or TIMP- 2 may be a protective factor for the development but not metastasis of head and neck cancer in Thai population. This activity might result from the genetically determined imbalance of MMP-2 and TIMP-2 expression gene, which seem to have the ability to suppress head and neck cancer cell growth. The genotyping of MMP-2 and TIMP-2 as potential marker for susceptibility to HNSCC will allow a precise and early identification of individuals at high risk and will aid the design of therapeutic modalities and evaluation of treatment outcome.

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APPENDIX

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APPENDIX

1. Chemicals

Chemicals	Molecular Weight (g/mol)	Source
Absolute ethanol (C_2H_5OH)	46.07	BDH Laboratory Supplies, Poole, England, UK
Absolute methanol (CH_3OH)	32.04	Lab-Scan, Thailand
Acetic acid glacial (CH_3COOH)	60.05	Carlo Erba, Milan, Italy
Acetone (CH_3COCH_3)	58.08	Mallinckrodt Baker, Phillipsburg, NJ, USA
Acrylamide (C_3H_5NO)	71.08	Sigma Chemicals, St Louis, MO, USA
N,N'-Methylene bis-acrylamide	154.20	Sigma Chemicals, St Louis, MO, USA
Agarose SeaKem GTG		BMA, LE65 1NG, UK
Ammonium persulfate ($(NH_4)HCO_3$)	228.20	USB, USA
Bovine Serum Albumin		Sigma Chemicals, St Louis, MO, USA
Bromophenol blue ($C_{19}H_9Br_4O_5SNa$)	670.00	Promega, Madison, WI, USA
Carbosymethylcellulose (CMC)		Sigma Chemicals, WI, USA
3'-deoxyadenosine 5'-triphosphate or dATP ($C_{10}H_{12}N_5O_{12}P_3Na_4$)	579.20	Promega, Madison, WI, USA
3'-deoxycytosine 5'-triphosphate or dCTP ($C_9H_{12}N_3O_{13}P_3Na_4$)	555.10	Promega, Madison, WI, USA

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Chemicals	Molecular Weight (g/mol)	Source
3'-deoxyguanosine 5'-triphosphate or dGTP ($C_{10}H_{12}N_5O_{13}P_3Na_4$)	595.10	Promega, Madison, WI, USA
3'-deoxythymidine 5'-triphosphate or dTTP ($C_{10}H_{12}N_5O_{13}P_3Na_4$)	570.10	Promega, Madison, WI, USA
3,3-Diaminobenzidine tetrahydrochloride, anhydrous (55)		Sigma chemicals, St Louis, MO, USA
Diethyl pyrocarbonate (DEPC)		Sigma chemicals, St Louis, MO, USA
Ethidium bromide		Bio-Rad Laboratories Hercules, CA, USA
Ethylenediaminetetraacetic acid ($C_{10}H_{14}N_2Na_2O_8.2H_2O$)	372.24	USB, USA
Fetal bovine serum		GibcoBRL, England, UK
Formaldehyde 40%m/v (HCOH)	30.026	Carlo Erba, Milan, Italy
L-Glutamine		Sigma chemicals, St Louis, MO, USA.
Glycerol ($C_3H_8O_3$)	92.10	BDH Laboratory Supplies, Poole, England, UK
Glycine ($H_2NCH_2CO_2H$)	75.07	USB, USA
Gum tragacanth		Sigma Chemicals, St Louis, MO, USA
Hydrogen peroxide (H_2O_2)	74.015	Sahakarn-Osos (1996), Bangkok
Hydrochloric acid (HCl)	36.50	E.Merck, Darmstadt, Germany
Isopropanol ($CH_3CHOHCH_3$)	60.10	BDH Laboratory Supplies, Poole, England, UK

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Chemicals	Molecular Weight (g/mol)	Source
β-Mercaptoethanol ($HSCH_2CH_2OH$)	78.13	Fluka chemika, Milan, Italy
3-(N-morpholino) propanesulfonic acid (MOPS) ($HO_3S(CH_2)_3(C_4H_8NO)$)	209.27	USB, USA
Nickle chloride hexahydrate ($NiCl_2.6H_2O$)	237.7	Sigma Chemicals, St Louis, MO, USA
Paraformaldehyde		Sigma Chemicals, St Louis, MO, USA
Penicillin ($C_{16}H_{17}N_2O_4SNa$) 6130 U/mg	356.4	Sigma Chemicals, St Louis, MO, USA
Potassium chloride (KCl)	74.56	E.Merck, Darmstadt, Germany
Potassium dihydrogen phosphate (KH_2PO_4)	136.09	E.Merck, Darmstadt, Germany
Skim milk (Instant non fat milk powder)		Mission, Thailand
Sodium azide (NaN_3)	65.01	E.Merck, Darmstadt, Germany
Sodium chloride (NaCl)	58.44	E.Merck, Darmstadt Germany
Sodium hydroxide (NaOH)	40.00	E.Merck, Darmstadt, Germany
di-sodium hydrogen phosphateanhydrous	358.14	E.Merck, Darmstadt, Germany
Sodium dodecyl sulfate or SDS ($C_{12}H_{25}O_4SNa$)	288.38	Sigma chemicals, St Louis, MO, USA

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Chemicals	Molecular Weight (g/mol)	Source
Streptomycin sesquisulfate 750U/mg		Sigma chemicals, St Louis, MO, USA
N,N,N’,N’-Tetramethyl ethylene-Diamine or TEMED ($C_4H_{11}NO_3$)	166.21	BioRad Laboratories, Hercules, CA, USA Hong Kong
Tris (Hydroxymethyl aminomethane)	121.10	Sigma chemical,
Tris (Hydroxymethyl aminomethane) ($C_4H_{11}NO_3$)	121.10	Sigma chemical, St Louis, MO, USA
TritonX-100		Fluka chemika, Milan, Italy
Tryptone (Pancreatic Digest of Casein)		Becton, Dickinson and Company, NJ, USA
Tryptose phosphate broth		Sigma chemicals, St Louis, MO, USA
Xylene cyanol FF ($C_{25}H_{27}N_2O_6Na$)	538.60	BDH Laboratory Supplies, Poole, England, UK

2. Instruments

2.1 Autoclave, Laserclave, Mode HA-240M, Tokyo, Japan.

2.2 Automatic Pipettes, Gilson, Villiers-le-B4el, France.

2.3 Beckman Microfuge E, California, USA.

2.4 Biofreezer (-70°C), Forma Scientific, Marietta, Ohio, USA

2.5 CO_2 incubator, Forma Scientific, Marietta, Ohio, USA

2.6 Vacuum pump, Sartorius, Göttingen, Germany.

2.7 Digital camera, Nikon, coolpix 950, Japan.

2.8 Digital, refrigerated centrifuge IEC Centra-8R, International equipment company, England.

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2.9 Dotted Apparatus, Bio-Rad, USA

2.10 Electronic Analytical and Precision Balance, Sartorius, Göttingen, Germany.

2.11 Filter Sterilization Unit, Sartorius, Göttingen, Germany.

2.12 Flow cytometer, FACScan, Becton Dickinson Immunocytometry System, San Jose, CA, USA.

2.13 Fluorescent microscope, Zeiss, Oberkochen, Germany.

2.14 Freezer (-20°C), Sanyo Medical freezer model MDF 0535, Sanyo Electric Co. Ltd., Japan.

2.15 Fume Hood, TOXICAP 1000, CARTAIR LABX, USA.

2.16 GeneAmp PCR System P9700, Perkin Elmer-Applied Biosystems, Foster City, CA, USA.

2.17 Gel Electrophoresis apparatus, Mupid-2, Tokyo, Japan.

2.18 Gel Electrophoresis apparatus, BRL Life technologies, Middle Sex. England.

2.19 High speed refrigerated centrifuge MTX-150, Tomy Seiko, Tokyo, Japan.

2.20 Horizon 11.14 Gel electrophoresis apparatus, BRL Life technologies, Middle Sex. England.

2.21 Incubator, Ehret, Germany.

2.22 Inverted microscope, Olympus CK2, Tokyo, Japan.

2.23 Laboratory centrifuge, Biofuge pico Heraeus, Kendro Laboratory Product, Germany.

2.24 Laminar airflow equipment, NuAir Biological Safty Cabinets, USA.

2.25 Magnetic stirrers Hotplate, Stuart Scientific, Bibby Sterilin Ltd., UK.

2.26Microwave oven, Mitsubishi cube-C2, Japan.

2.27 Milli-Q Plus, Millipore Corporation Massachusetts, USA

2.28 Model J2-MC centrifuge, Beckman, California, USA.

2.29 Multichannel pipette, Biohit praline, Biohit Oyj, Helsinki, Finland.

2.30 pH meter, Orion 520 A, Boston, USA.

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2.31 Pipetboy acu, integra Bioscience

2.32 Power supply E-C Apparatus Corporation, St.Peterberg, Florida, USA.

2.33 Refrigerator, Sanyo New touch, Sanyo Electric Co. Ltd., Japan.

2.34 Refrigerator, Traflo Framec model Expo 310 PT/E, San Giorgio Monf.(AL), Italy.

2.35 Rocker,

2.36 Shaking water bath Julabo SW-20C, Julabo Labortechnik, Germany.

2.37 Vertical gel electrophoretic apparatus model AE-6410E, AT/TO corporation, Japan

2.38 Vertical gel electrophoretic apparatus, Bio-Rad Mini-PROTEAN® II, USA

2.39 Vortex mixer, Vortex Gene 2, Scientific Industries, Bohemia, N.Y., USA.

2.40 Transilluminator, TVC 312A, Spectronics, Florida, USA.

3. Enzyme

3.1 **Restriction enzyme**

3.1.1 BsoBI (5 U/µl), New England BioLabs, Beverly, MA, USA.

3.2 **Modified enzyme**

3.2.1 Taq DNA polymerase (Promega, Madison, WI, USA)

4. Marker

4.1 DNA marker

4.1.1 λ DNA-*Hind* III digest, New England Biolabs, Beverly, MA, USA

4.1.2 ϕX174 RF DNA/*Hae* III digest, New England Biolabs, Beverly, MA, USA

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4.2 Sample Buffer

4.2.1. 5x For media, fluids

5 ml Upper Gel Buffer + 5 ml Glycerol + 0.5 g SDS + Bromophenol Blue

4.2.2. 1x For tissues, cells etc.

1 ml Upper Gel Buffer + 1 ml Glycerol + 0.1 g SDS + 8 ml MilliQ Water + Bromophenol Blue

4.3 Non-reducing Sample Buffer (5x concentrated):

Stacking gel buffer (section 2.8.1)	5 ml
Glycerol	5 ml
SDS	0.5 g
Bromophenol blue	trace

Gelatin Solution:

Gelatin or β-casein (Sigma) 1.2 g

Dissolve with warming in 100 ml DDW. Store in 10 ml aliquots at 4°C and rewarm to ~ 50°C before use.

Low Salt Collagenase Buffer (10 x, pH 7.6):

Trisma base	60.6 g
NaCl	117.0 g
$CaCl_2$	5.5 g
Brij-35 (30 % w/v)	6.7 ml
Add DDW to	1000 ml

Adjust with HCl to pH 7.6

5. Reagents

5.1. Reagents for PCR reaction and agarose gel electrophoresis

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5.1.1 0.5 M EDTA (pH 8.0)

Add 186.1 g of EDTA.Na_2.$2H_2O$ to 800 ml of deionised water. The solution was vigorously stirred on a magnetic stirrer and then adjusted pH to 8.0 with NaOH. The solution was adjusted the final volume to 1 liter with deionised water.

5.1.2. 50X Concentrated stock solution of Tris-acetate-EDTA (TAE) electrophoresis buffer

Tris base	242.0	g
Glacial acetic acid	57.1	ml
0.5 M EDTA (pH 8.0)	100	ml

These solutions were mixed and adjusted the final volume to 1 liter with deionised water. The reagent was diluted with deionised water just before use.

5.1.3. 2.5% Agarose gel in 1X TAE buffer

Agarose gel	2.5	g
1X TAE buffer	100	ml

The mixture was heated in microwave oven until agarose gel was completely dissolved, poured into gel tray and then comb was placed to create well.

5.2. Cell culture reagent

5.2.1. Phosphate buffered-saline (PBS), pH 7.4

NaCl	8	g
KCl	0.2	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.2	g

These chemicals were mixed, well dissolved in deionised water, and adjusted the final volume to 1 liter prior to sterile by autoclave.

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5.2.2. Cell dissociation solution

5.2.2.1. 2.5 mM EDTA in PBS

EDTA.Na2.2H2O 0.4653 g was dissolved in PBS, pH 7.4, stirred, and adjusted the final volume to 500 ml in volumetric flask. The reagent was sterilized by autoclave at 121°C for 15 min.

5.2.2.2. Trypsin solution (10%Trypsin in 2.5 mM EDTA/PBS)

Trypsin 2 g was dissolved in 20 ml of 2.5 mM EDTA/PBS, stirred until completely dissolved, and sterilized by filtrated through 0.2 µM cellulose acetate filter membrane. The reagent was diluted to the desire concentration with sterile 2.5 mM EDTA/PBS before use.

5.2.3. 30.8% (w/v) Acrylamide-Bisacrylamide

Acrylamide	30.0	g
Bis-acrylamide	0.8	g

These chemicals were dissolved in deionised water, and adjust the final volume to 100 ml. The reagent was filtrated through 125 mm diameter-filter paper (Whatman No.1).

5.2.4. Resolving gel buffer pH8.8: 3 M Tris-HCl

Tris	36.3	g
1 M HCl	48	ml

Tris was added to 1 M HCl and adjusted the final volume to 100 ml with deionised water. The solution was adjusted the pH to 8.8 with 1 M HCl, and stored at 4°C.

5.2.5. 10% (w/v) Sodium dodecyl sulphate

Sodium dodecyl sulphate (SDS) 10 g was dissolved in 100 ml deionised water and stored at room temperature.

5.2.6. 10% (w/v) Ammonium persulfate

Ammonium persulfate 1.0 g was dissolved in deionised water and adjusted the final volume to 10 ml.

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5.2.7. 10% Resolving gel of SDS-PAGE (for 1 PAGE)

Lower Gel Buffer	7.5 ml
MilliQ Water	8.5 ml
Acrylamide	11.0ml
Gelatin	3.0ml
10%Ammonium persulfate	150 ml
TEMED	10 ml

5.2.8. Upper gel (pour immediately before use)

Upper Gel Buffer	2.50 ml
MilliQ Water	6.25 ml
Acrylamide	1.20 ml

Mix by gently swirling

Add ul TEMED and then 50 ul 10% Ammonium Persulphate

Mix again by gently swirling and pour gels - should be enough for 2 gels. Carefully position comb to avoid trapping air bubbles and overlay at sides with isobutanol or MilliQ Water.

5.2.9. 10X Running buffer pH 8.3 (0.25 M Tris-HCl, 1.92 M Glycine, 1% (w/v) SDS)

Tris	30.3	g
Glycine	144.0	g
SDS	10.0	g

These chemicals were dissolved and adjusted the final volume to 1 liter with deionised water. The solution was diluted to 1X with deionised water just before use.

5.2.10. Lower gel buffer

Tris	90.8

Make up in approx 450 ml MilliQ Water

Adjust pH to 8.8 with Conc.HCl

Make up to 500 ml final volume with MilliQ Water

Add 2.0 g SDS

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5.2.11. Acylamide

ICN-Flow Acrylamide/Bis Premix 29:1

Make up as per instructions on bottle.

Store at 4 degree Celcius

5.2.12. Running (Electrodes) buffer - 10x, pH 8.3

Tris	30.3 g
Glycine	144.2 g
Sodium Dodecyl Sulphate (SDS)	10.0 g

Make up to 1 Litre with MilliQ Water; do not adjust pH.

Dilute 1:9 with MilliQ Water to use; rescue dilute buffer 2-3 times

5.2.13. Sample buffer

5x for media, fluids etc.

5 ml upper gel buffer + 5 ml Glycerol + 0.5 g SDS + Bromophenol Blue

1x For tissues, cells etc.

1 ml Upper gel buffer + 1 ml Glyercol + 0.1 g SDS + 8 ml MilliQ Water + Bromophenol Blue

5.2.14. Gelatin (ICN-Flow) 1.2g/100 ml

Dissolve with warming in 100 ml MilliQ Water - until just hot to touch.

Store in 10 ml aliquots (sterile tubes) at 4 degree celcius

Rewarm to approx. 50 degree celcius to use.

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5.2.15. Processing gels

Low Salt (LS) Collagenase Buffer - 10x, pH 7.6

Tris	60.6 g
NaCl	117.0 g
$CaCl_2$	5.5 g

Make up to 900 ml with MilliQ Water

Adjust pH to 7.6 with Conc.HCl

Add 6.7 ml 30% (w/v) Brij-35

Make up to final volume of 1 Litre with MilliQ Water.

Dilute 1:9 with MilliQ Water to use

6. Consumable supplies

6.1. Microcentrifuge tubes (1.5 ml), Treff, Switzerland.

6.2. 15 ml centrifuge tube, Costar, Corning Incorporated, Corning, NY, USA.

6.3. 50 ml centrifuge tube, Costar, Corning Incorporated, Corning, NY, USA.

6.4. 24-well cell culture clusters flat bottom with lid 3524, Costar, Corning Incorporated, Corning, NY, USA.

6.5. 96-well cell culture clusters flat bottom with lid 3599, Costar, Corning Incorporated, Corning, NY, USA.

6.6. 25-cm2 cell culture flask phenolic style cap 3055, Corning Incorporated, Corning, NY, USA.

6.7. 75-cm2 cell culture flask phenolic style cap 3055, Corning Incorporated, Corning, NY, USA.

6.8. 35 mm x 10 mm style cell culture dish, Corning Incorporated, Corning, NY, USA.

6.9. 60 mm x 10 mm style cell culture dish, Corning Incorporated, Corning, NY, USA.

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