10. MATRIX METALLOPROTEINASES IN THYROID CANCER

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INTRODUCTION

Cancer is a multistage disorder in which sequential and cumulative genetic aberrations lead to malignant cell transformation (1–2). Approximately 50% of cancer mortality results from invasion and metastasis. Tumor cell invasion and metastasis is a complex multistep process that involves the degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs), an important step in the process of cancer invasion and metastasis. Correlation between MMPs overexpression and cancer metastasis have been repeatedly made by numerous studies. Malignant cells rely on these proteinases to disrupt basement membranes, invade surrounding tissues and metastasize to different organs. It is now apparent that not only tumor cells but also non-malignant stromal cells actively participate in the proteolytic degradation of ECM. Tissue inhibitors of metalloproteinases (TIMPs) act as negative regulators of MMPs and it has been shown that they can prevent the spread of cancer in animal models by preserving ECM integrity (3–4).

Matrix metalloproteinases, also called matrixins, constitute a family of zinc–dependent endopeptidases. Twenty-eight members of this family have been identified. Collectively, MMPs play important roles in ECM homeostasis, mediating such normal physiological processes as embryogenesis, organ morphogenesis, reproduction, angiogenesis, and tissue resorption and remodeling (5). The proteolytic activities of MMPs are tightly regulated by endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) (5). Any disruption of this fine balance can contribute

to the pathogenesis of serious diseases such as arthritis, periodontal disease, and cancer metastasis (6).

THE MMP FAMILY AND STRUCTURE

At present, the human MMP family consists of 23 structurally related members (Table 1). Historically, the MMPs were divided into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs, and other novel MMPs, on the basis of their specificity for ECM components. As the list of MMP substrates has grown and several MMPs can degrade a number of different ECM components, a sequential MMP numbering system has been adapted, and the MMPs are now grouped according to their structure. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (Figure 1) (7).

MMPs are produced and secreted by a number of cell types, including fibroblasts, smooth muscle cells, and endothelial cells. They share several highly conserved domains, including an N-terminal propeptide domain that contains a "cystein switch" sequence that enfolds the zinc atom of the catalytic site to maintain the latency of pro-MMPs, a catalytic domain with a zinc binding site and a conserved methionine, and a C-terminal hemopexin-like domain linked to the catalytic domain by a proline rich hinge region. The catalytic domain contains a zinc binding motif HEXXHXXGXXH, in which the three histidine residues represent the three zinc ligands and the glutamic residue the active site. The hemopexin domain contains a single Cys-Cys bond and plays a role in substrate recognition (for example, it is required for collagenases to cleave triple helical interstitial collagens), interaction with TIMPs, and binding of the enzyme to ECM or cell surface (4–5).

The substrates of MMPs are primarily insoluble proteins of ECM, including interstitial and basement membrane collagens, glycoproteins such as laminin, fibronectin, vitronectin, tenascin and elastin as well as proteoglycans. However, more recent data demonstrate that certain MMPs can degrade proteins other than ECM proteins. Many cell membrane bound precursors of growth factors (TGF-α, TGF-β), growth factor receptors (FGF receptor 1, HER2/neu, HER4) and cell adhesion molecules (CD 44, E-cadherin, αv integrin) have been reported to be MMP substrates. For example, MMP-11 can cleavage of insulin-like growth-factor-binding protein (IGF-BP) to release IGFs (8); MMP-12 can proteolytically process plasminogen to generate angiostatin, an inhibitor of angiogenesis (9); MMP-2 and MMP-9 can proteolytically activate TGF-β and promote tumor invasion and angiogenesis (10); and finally, cleavage of the αv integrin subunit precursor by MMP-14 enhances cancer cell migration (11). Although the significance of these observations is not entirely clear, they reflect the complex nature of MMPs in cancer progression.

REGULATION OF MMP ACTIVITY

The activities of MMPs are regulated at three major levels: transcriptional regulation, activation of latent MMP, and inhibition/deactivation by endogenous inhibitors such as α-macroglobulins and TIMPs.

Table 1. The matrix metalloproteinase (MMP) family

MMP subgroup	MMP	Domain class*	Common name (s)
Collagenase			
Collagenase-1	MMP-1	B	fibroblast collagenase, tissue collagenase, interstitial collagenase
Collagenase-2	MMP-8	B	neutrophil collagenase, granulocyte collagenase, PMN collagenase
Collagenase-3	MMP-13	B	todpole collagenase
Collagenase-4	MMP-18	B	found in Xenopus, no human homologue is known
Stromelysins			
Stromelysin-1	MMP-3	B	transin-1, proteoglycanase, procollagenase-activating protein
Stromelysin-2	MMP-10	B	transin-2
Stromelysin-3	MMP-11	D	
Matrilysins			
Matrilysin	MMP-7	A	matrin, PUMP1, small uterine metalloproteinase
Matrilysin-2	MMP-26	A	endometase
Gelatinases			
Gelatinase A	MMP-2	C	72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase
Gelatinase	MMP-9	C	92-kDa gelatinase, 92-kDa type IV collagenase
Membrane-type MMPs			
MT1-MMP	MMP-14	F	MT-MMP1
MT2-MMP	MMP-15	F	MT-MMP2
MT3-MMP	MMP-16	F	MT-MMP3
MT4-MMP	MMP-17	G	MT-MMP4
MT5-MMP	MMP-24	F	MT-MMP5
MT6-MMP	MMP-25	G	MT-MMP6, leukolysin
Other MMPs			
Metalloelastase	MMP-12	B	Macrophage elastase, macrophage metalloelastase
RASI-1	MMP-19	B	
Enamelysin	MMP-20	B	
XMMP	MMP-21	E	homologue of Xenopus XMMP
CMMP	MMP-22	B	found in chicken
Femalysin	MMP-23	H	cysteine array MMP
(no trivial name)	MMP-27	B	
Epilysin	MMP-28	D	
McoI-A	No designation	B	found in mouse
McoI-B	No designation	B	found in mouse
75-kDa gelatinase	No designation	C	found in chichen

*see Figure 1

MMP mRNA levels can be induced by a wide variety of chemical agents (e.g. phorbol esters), growth factors (e.g. epidermal growth factor, EGF), hormones (e.g. thyroid hormone, relaxin) cytokines (e.g. interleukin-1, IL-1 and tumor necrosis factor-α, TNF-α), and physical stress. They may also be down-regulated by suppressive factors such as transforming growth factor-β, retinoic acids and glucocorticoids (5,12). The promoter regions of several MMP genes (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13) contain some common regulatory DNA

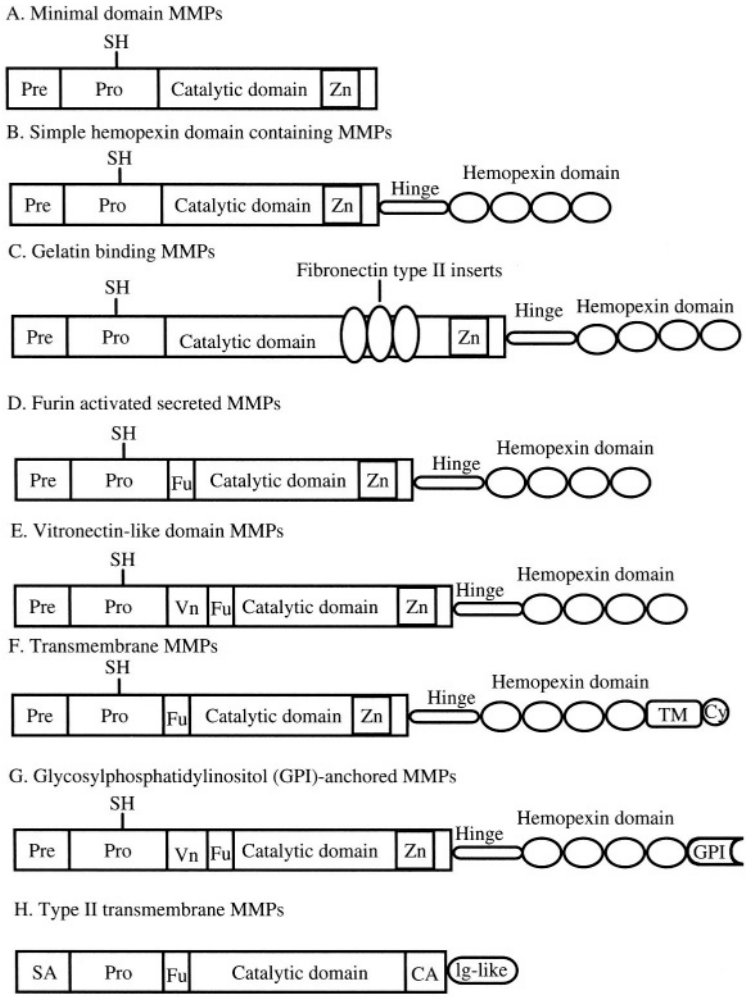

Figure 1. Matrix metalloproteinases groups.
Matrix metalloproteinases (MMPs) can be classified into eight distinct groups by their domain structure, five of which are secreted and three of which are membrane-type MMPs (MT-MMPs). Secreted MMPs: The minimal-domain MMPs contain an N-terminal signal sequence (Pre) that directs them to the endoplasmic reticulum, a propeptide (Pro) with a zinc-interacting thiol (SH) group (from cysteine) that maintains them as inactive zymogens and a catalytic domain with a zinc-binding site (Zn). In addition to the domains that are found in the minimal domain MMPs, the simple hemopexin domain MMPs have a hemopexin-like domain—that is connected to the catalytic domain by a hinge (H), which mediates interactions with tissue inhibitors of metalloproteinases, cell-surface molecules and proteolytic substrates. The first and the last of the four repeats in the hemopexin-like domain are linked by a disulphide bond. The gelatin-binding MMPs contain three inserts that resemble collagen-binding type II repeats of fibronectin (Fi) and is responsible for the specific binding to gelatins and collagens. The furin-activated secreted MMPs contain a recognition motif for intracellular furin-like serine proteinases (Fu) between their propeptide and catalytic domains that allows intracellular activation by these proteinases. This motif is also found in the vitronectin-like insert (Vn) MMPs and the membrane-type MMPs (MT-MMPs). MT-MMPs: MT-MMPs include transmembrane MMPs that have a C-terminal, single-span transmembrane domain (TM) and a very short cytoplasmic domain (Cy), and the glycosylphosphatidylinositol (GPI)-anchored MMPs. MMP-23 represents a third type of membrane-linked MMP. It has an N-terminal signal anchor (SA) that targets it to the cell membrane, and so is a type II transmembrane MMP. MMP-23 is also characterized by its unique cysteine array (CA) and immunoglobulin (Ig)-like domains instead of the hemopexin domain. Adapted from Ref. 7.

sequences. Two important elements for transcriptional regulation are an AP-1 binding site for AP-1 transcription factors which comprise of members of the FOS and JUN family of transcription factors, and a PEA-3 element that binds ETS transcription factors. The AP-1 site, located approximately 70 bp upstream from the transcriptional start site, has been considered to play an important role in the transcriptional activation of the MMP promoters, whereas interaction between AP-1 and PEA-3 site is necessary for basal transcription and trans-activation by cytokines and growth factors. The DNA binding and trans-activation of both AP-1 and ETS transcription factors are regulated by mitogen-activated protein kinases (MAPKs) (12). Interestingly, AP-1 site is not present in the promoter region of MMP-2, a critical metalloproteinase involved in cancer metastasis, and MMP-14, which is involved in the activation of MMP-2 (13). Another transcriptional control of MMP expression is the presence of naturally occurring sequence variation or single nucleotide polymorphisms (SNPs) in the promoters of MMP genes (14). These genetic polymorphisms have been shown to have allele-specific effects on the MMP promoter activities, e.g. an insertion of a guanine at position –1607 in the MMP-1 gene promoter creates the core sequence (5'-GGA-3') of a binding site for the ETS transcription factors. The 2G allele has a higher transcriptional activity in melanoma cells and is associated with more invasive tumors (15).

All MMPs are synthesized as prepro-enzymes. Most MMPs are secreted as inactive, latent pro-MMPs, with the exception of MT-MMPs, which are membrane bound and localize at the cell surface. Since MMP activation occurs after secretion into the extracellular milieu, an important control point for MMP activity is the proteolytic cleavage of pro-MMPs. It has been demonstrated that serine proteases such as trypsin, plasmin, or urokinase initiate activation of MMPs from the zymogen form (16). Some MMPs can also activate other members of the family. A good example is the activation of pro-MMP-2 at the cell surface by MMP-14 and TIMP-2 (17): TIMP-2 binds MMP-14 at its amino terminus and pro-MMP-2 at its carboxyl terminus, which allows an adjacent, non-inhibited MMP-14 to cleave the bound pro-MMP-2. MMP-14 does not fully activate MMP-2 and another already activated MMP-2 is required to remove a residual portion of the MMP-2 propeptide (18). Pro-MMP2 can also be activated by MMP-15 through TIMP-2 independent mechanism (19). Although most MMPs are activated outside the cells by serine proteases or other activated MMPs, MMP-11, MMP-28, and the MT-MMPs can also be activated by intracellular furin-like serine proteases before they reach the cell surface (20).

A final and important control point of MMP activity is the inhibition of activated enzymes by endogenous inhibitors. The main inhibitor of MMPs in tissue fluids is α-macroglobulin, an abundant plasma protein (21). α-Macroglobulin binds to MMPs and the MMP/α-macroglobulin complex then binds to a scavenger receptor and is irreversibly cleared by endocytosis. In a similar way to α-macroglobulin, thrombospondin-2 forms a complex with MMP-2 and facilitates scavenger-receptor-mediated endocytosis and clearance (22). By contrast, thrombospondin-1 binds to pro-MMP-2 and -9 and directly inhibits their activation (23–24). Curiously, thrombospondin-1 has also been reported to increase MMP-2 and -9 activation (25).

Another group of endogenous MMP inhibitors are TIMP family of inhibitors. At present, four structurally related members have been characterized (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) with 40–50% sequence identity at the ammo acid levels (26). TIMPs are small, low molecular weight proteins (20–30 kDa). They differ in tissue-specific expression and ability to inhibit various MMPs. They reversibly inhibit active MMPs with relatively low selectivity by occupying the catalytic domain of activated enzymes (27–28). The TIMP/MMP complex is a tight binding, non-covalent complexes with a stoichiometric 1:1 molar ratio. Unlike TIMP-1, TIMP-2, and TIMP-4, which are secreted in soluble form, TIMP-3 has a unique association with ECM. Studies with Timp-2-deficient mice indicate that the dominant physiological function of TIMP-2 is activation of MMP-2 (29). Apart from inhibiting MMPs, TIMP-3 has been shown to promote apoptosis whereas TIMP-1 is active in blocking apoptosis and overexpression of TIMP-2 protect cancer cells from apoptosis (30–32).

MMPs AND TIMPs IN THYROID CANCER PROGRESSION AND METASTASIS

The expression and activity of MMPs are increased in many types of human cancer, and this correlates with advanced tumor stage, increased invasion and metastasis, and shortened survival. Many studies show a negative association between MMPs activity and prognosis (7). MMP-2 and MMP-9 are of particular importance in tumor cell invasion, because they degrade type IV collagen, the main structural component of the basement membrane. Tumor cells expressing high levels of these enzymes are highly metastatic. Cancer cells are not the only source of MMPs. Stromal cells are also participated in the production of MMPs (20). MMPs that are secreted by stomal cells can still be recruited to the cancer cell membrane, e.g. MMP-2 mRNA is expressed by stromal cells of human breast cancers, whereas MMP-2 protein is found on both stromal and cencer cell membranes (33). It has been shown that cancer cells can stimulate tumor stromal cells to produce MMPs in a paracrine fashion through secretion of cytokines, growth factors, and EMMPRIN (extracellular matrix metalloproteinase inducer). EMMPRIN is an intrinsic plasma membrane glycoprotein produced in high amounts by cancer cells, which stimulates local fibroblasts to synthesize MMPs (34). Tumor cell interactions with fibroblasts via EMMPRIN leads to fibroblast-induced local degradation of basement membrane and ECM components, thus facilitating tumor cell invasion. It has been shown that MMP-9 production in tumor infiltrating macrophages play a critical role in angiogenesis and progressive growth of human ovarian tumors in mice (35). Stromal cells and their products have been reported to even cause tumorigenic transformation of adjacent epithelial cells (36).

Earlier studies have shown that invasion by cultured human follicular thyroid carcinoma is correlated with increased production of beta 1 integrins and MMPs (37). Correlation between MMPs and ECM degradation is further demonstrated by the study of plasmin activation system in metastatic follicular thyroid carcinoma cell lines (38). As mentioned earlier, plasmin is a serine protease involved in the activation of MMPs. Plasmin is generated from plasminogen by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). UPA-mediated plasminogen activation is an important pathway in tumor invasion and can be inactivated by

plasminogen activator inhibitors (PAI-1 and –2) (39). Decreased activity of PAI-1 is associated with greater ECM degradation in follicular thyroid carcinoma cell lines (38).

Overexpression of MMP-2, and MMP-9 has been found in thyroid carcinomas and is correlated with large tumor size, high intrathyroid invasion, presence of lymph node metastasis, and advanced disease stage (40). A more comprehensive study of MMPs profile involving seven secreted MMPs (MMP-1, -2, -3, -7, -8, -9, and -13) and three membrane-bound MMPs (MMP-14, -15, and-16) demonstrates that the major MMPs produced in papillary thyroid carcinomas are MMP-2 and MMP-14 (41). The pro-MMP-2 activation and the expression of MMP-14, known to activate pro-MMP-2 at the cell surface, are considerably higher in carcinomas with lymph node metastasis than those without metastasis. MMP-15 expression is confined to 26% of cases. MMP-2, MMP-14, and MMP-15 are immunostained in both carcinoma and stromal cells (41). In a separate study, increased MMP-2 expression is found in follicular and anaplastic thyroid carcinomas, but not in follicular adenomas (42). Interestingly, MMP-2 mRNA expression is restricted to fibroblasts in the stroma adjacent or close to invading tumor cells (42). MMP-1 expression is significantly greater among follicular and papillary thyroid carcinomas compared to benign lesions. However, there is no relationship between MMP-1 expression and invasion, metastasis, or disease recurrence (43). Both carcinoma and stromal cells have been shown to express MMP-1 (43–44). A recent cDNA and tissue microarray study shows that MMP-11 is up-regulated in 67% of papillary thyroid carcinoma tissues (45).

Both TIMP-1 and TIMP-2 expression are increased in thyroid carcinomas, and are correlated with large tumor size and advanced disease stage (40,46), which seem to be contradictory to the role of TIMPs as inhibitors of tumor cell invasion and metastasis. Further study shows stronger TIMP-1 immunostaining in the stromal cells surrounding the tumor, suggesting that the high levels of TIMP-1 transcripts in advanced stage of thyroid carcinoma are likely represent a stroma response to tumor cell invasion. Overexpression of TIMP-1 by gene transfer has resulted in a significant suppression of invasive potential of NPA cells, a poorly differentiated thyroid carcinoma cell line (46). Reduced TIMP-1 expression has been shown in recurrent papillary thyroid carcinoma when compared to non-recurrent carcinomas (43). Apparently, tumor invasion is not dependent on the absolute levels of TIMPs or MMPs. It is the balance between TIMPs and MMPs that determines the potential of thyroid tumor invasion and metastasis. Indeed, the molar ratio of total amounts of MMPs:TIMPs is significantly higher in the thyroid carcinoma samples than in the adenoma and normal samples (41).

Many MMP genes are transcribed at low or undetectable levels in normal thyrocytes. Analysis of MMPs and TIMPs expression *in vitro* demonstrates that MMP-1, -2, -9, -14, and TIMP-1, -2, -3 mRNA are present in normal thyrocytes, malignant thyroid cells and thyroid-derived fibroblasts. The basal levels of MMP-1, -9, and -14 are much lower in thyrocytes than in malignant thyroid cells and thyroid-derived fibroblasts, whereas high basal levels of MMP-2, TIMP-1, -2, and -3 are found in all three cell types without striking difference (47–48). IL-1 can upregulate MMP-1and MMP-9 mRNA in all the cell types through activating nuclear factor of κB (NF-κB), and has no significant effect on TIMPs, MMP-2, and MMP-14. TNF-α, also acting via

NF-κB passway, can stimulate MMP-9 mRNA expression in malignant thyroid cells and thyroid-derived fibroblasts. EGF, acting via protein tyrosine kinase, can only stimulate MMP-1 expression in malignant cells (49). Phorbol—myristate acetate (PMA, an active phorbol ester) can induce MMP-1, MMP-9 and TIMP-1 mRNA in all the cell types, MMP-14 in malignant thyroid cells and thyroid-derived fibroblasts (47–49). Since PMA, acting via protein kinase C (PKC), can induce *c-jun* and *c-fos* gene expression in human thyroid cells, and their gene products are AP1 transcriptional factors (50), it is likely that PKC is involved in the induction of MMP transcription. Although thyroid-stimulating hormone (TSH) has no significant effect on the basal MMP-1, or TIMP-1 mRNA levels, it can cause a dose-dependent inhibition in PMA or EGF-induced MMP-1 mRNA in malignant cells, and PMA-induced MMP-1 and TIMP-1 mRNA in benign thyroid cells. The repressive action of TSH on MMP-1 mRNA can be mimicked by the forkolin and 8-bromo-cAMP, and can be abrogated by a protein kinase A (PKA) inhibitor, H-89, suggesting that it is PKA-mediated (49). MMP-11, -13, and -18 genes are thyroid hormone responsive genes. Although they have not been shown to be involved in thyroid cancer, they have distinct functions during frog embrogenesis (51).

Several studies have shown that high serum levels of MMP-2, MMP-9, and TIMP-1 are associated with tumor invasion and poor survival in several types of cancer (52–54). Thus, they may be used as prognostic markers in cancer patients. Higher levels of MMP-2 and TIMP-2 are detected by ELISA in peripheral blood of thyroid cancer patients when compared to normal control, and increased blood levels of MMP-3 and MMP-9 appear to be associated with medullary thyroid cancer (55). It remains to be determined whether serum levels of MMPs and TIMPs can be used as diagnostic or prognostic markers for thyroid carcinoma.

MMP INHIBITION IN ANTICANCER THERAPY

Given that MMPs play important role in tumor invasion and metastasis, inhibition of MMPs activity has been the focus of much anticancer research and clinical trials. Pharmaceutical industries have invested considerable effort over the past decade to develop safe and effective MMP inhibitors for use in cancer patient. Three classes of synthetic MMP inhibitors have been developed (Table 2): the collagen peptidomimetics which mimic the collagen amino-acid sequence near the collagenase cleavage site; the collagen non-peptidomimetics which are synthesized based upon the conformation of MMP active site; and the tetracycline derivatives which inhibit the activity of MMPs without antibiotic activity (13, 56–57). Numerous preclinical studies using these MMP inhibitors in cancer models have demonstrated their effectiveness to delay primary tumor growth and inhibit experimental metastasis. Initiation of treatment when tumor burden is minimal has a more profound effect on tumor growth inhibition than at the time of large tumor bulk. Despite of positive preclinical results in the use of MMP inhibitors, most clinical trials have not yielded significant beneficial effects in patients with advanced cancer (57). In the case of BAY12-9566, alarming reports show significantly poorer survival for groups treated with the drug than for placebo-treated group.

Table 2. The matrix metalloproteinase inhibitors for cancer therapy

Inhibitor	Structure	Specificity
Marimastat (BB-2516)	Peptido mimetic	Broad spectrum (2nd generation of BB-94)
Batimastat (BB-94)	Peptido mimetic	Broad spectrum (e.g. MMP-1, 2, 3, 7, 9, 12)
Tanomastat (Bay 12-9566)	Non-peptido mimetic	Broad spectrum (e.g. MMP-2, 9, 11, 13, 14)
Prinomastat (AG3340)	Non-peptido mimetic	Broad spectrum
BMS-275291	Non-peptido mimetic	Broad spectrum
MMI 270 (CGS27023A)	Non-peptido mimetic	Broad spetrum
Metastat (COL-3)	Tetracycline derivative	Gelatinases (MMP-2, 9)

In view of the disappointing results of synthetic MMP inhibitors in clinical trials, we and other investigators have recently explored the potential applications of TIMP gene overexpression for cancer gene therapy (58–60). Antitumor effects have been shown following systemic or local delivery of TIMP-1, TIMP-2, and TIMP-3 genes in animal models (60–63). However, stimulation of mammary tumorigenesis has been reported following systemic TIMP-4 gene delivery. TIMP-4 has been shown to up-regulate Bcl-2 and Bcl-X(L) protein and inhibit apoptosis in human breast cancer cells (64). Given the multifunctional nature of TIMP proteins, further preclinical studies will be needed before initiation of clinical gene therapy trial in patients with cancer.

CONCLUSIONS

As compared with tumors from other organs such as lung, colon, and breast, a limited number of studies have been carried out so far on the involvement of MMPs and TIMPs in thyroid tumorigenesis. Based upon the available data, it is clear that MMPs, especially MMP-2 and MMP-9, and TIMP-1 are involved in thyroid tumor invasion and metastasis. Although TIMP-1 can reduce the invasive potential of thyroid cancer cells *in vitro,* therapeutic intervention *in vivo* has not been attempted yet in animal models to inhibit thyroid tumor growth, invasion, and metastasis, using either synthetic MMP inhibitors or TIMPs gene therapy. Clearly, more studies are needed to fully appreciate the important roles of MMPs and TIMPs in thyroid cancer.

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