Elevated Membrane-Type Matrix Metalloproteinases in Gliomas Revealed by Profiling Proteases and Inhibitors in Human Cancer Cells

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Abstract

Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) regulate proteolysis of the extracellular matrix and other extracellular proteins, including growth factors and their receptors. The aberrant expression of these genes is common in most cancers. We profiled the RNA levels of every human *MMP* and *TIMP* in a variety of cell types (fibroblast, endothelial, hematopoietic, carcinoma, melanoma, and glioma) using quantitative PCR, with the aim of identifying novel expression patterns. Almost all members of the membrane-type (*MT-*) *MMP* and *TIMP* families were elevated in glioma lines compared to carcinomas. In clinical glioma specimens, there were positive correlations between glioma grade and RNA levels of *MT-1*, *MT-2*, and *MT-6 MMP*, *TIMP-1* and *TIMP-2*, and for several growth factors and receptors. These findings suggest that advanced malignant gliomas have elevated levels of membrane-associated MMPs and TIMPs, which may potentially regulate vascularization and invasion. Concurrent elevation of signaling molecules suggests potential bidirectional relationships that enhance tumor aggressiveness.

Introduction

Degradation of the extracellular matrix (ECM) is essential for tumor invasion and vascular formation and is mediated by the concerted action of proteolytic enzymes and their inhibitors (1–3). The largest group of ECM-degrading enzymes is the matrix metalloproteinases (MMPs), comprising 23 human enzymes (see Table 1) that collectively degrade all proteinaceous components in the ECM (4, 5). These enzymes are synthesized as inactive zymogens and require proteolytic cleavage of a pro-peptide to become active. For most MMPs, activation occurs extracellularly and is mediated by proteinases, including other MMPs and serine proteinases (4). Unlike the majority of MMPs, which are secreted, membrane-type MMPs (MT-MMPs) are anchored to the cell membrane either via a single transmembrane domain, or a glycophosphatidylinositol anchoring domain, such that the catalytic site is extracellular (6, 7).

A major group of MMP inhibitor is the tissue inhibitors of metalloproteinases (TIMPs), which bind in a 1:1 stoichiometry to the active site of the MMPs (8). For the most part, each of the four TIMPs can nonselectively bind to all MMPs, with one exception being the inability of TIMP-1 to inhibit effectively MT1, 2, 3, and 5 MMPs (MMPs 14, 15, 16, and 24; Ref. 9). The balance or imbalance of MMPs with TIMP levels is therefore a potential predictor of ECM production and/or degradation. In seeming disagreement with its inhibitory activity, TIMP-2 is also an important mediator of MMP-2 activation. An MT1-MMP protein first associates with TIMP-2 extracellularly, with this complex then binding pro-MMP2. The MMP-2 is then brought into close proximity with a second MT1-MMP, resulting in cleavage of the MMP-2 pro domain (10). It now appears that all MT-MMPs may have the ability to activate pro-MMP-2 (11–15).

In addition to their direct role of ECM degradation and cell invasion, MMPs have other functions that may mediate tumor growth. Many can cleave growth factors, their receptors, or other growth factor-associated proteins. MMP-3 can cleave heparin-binding epidermal growth factor (HB-EGF), releasing it from the cell membrane (16). MMP-9 liberates vascular endothelial growth factor (VEGF) during angiogenesis (17), while metalloproteinases are involved in cleavage of members of the EGF receptor (EGF-R) family and the hepatocyte growth factor receptor, c-Met (18–20). Through these actions, MMPs can have profound effects on the pericellular environment that act to promote or inhibit tumor growth depending on tissue context.

Although different cancers may share many similar properties, including enhanced proliferation, increased angiogenesis, and local tumor cell invasion, the precise mechanisms that each cancer employs often differ. Although all malignant cancers demonstrate elevated levels of at least one MMP compared to normal tissue or less malignant tumors (1),

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Table 1. The Genes Analyzed by Quantitative PCR

Gene Name	Other Names		Primer/Probe Sequence[a]	Genebank Accession No.
MMP-1	Collagenase 1; Interstitial collagenase		[a]	NM002421
MMP-2	Gelatinase A; 72 kDa type-IV collagenase		[a]	NM004530
MMP-3	Stromelysin 1; Transin		[a]	NM002422
MMP-7	Matrilysin		[a]	NM002423
MMP-8	Collagenase 2; Neutrophil collagenase		[a]	NM002424
MMP-9	Gelatinase B; 92 kDa type-IV collagenase		[a]	NM004994
MMP-10	Stromelysin 2; Transin 2		[a]	NM002425
MMP-11	Stromelysin 3		[a]	NM005940
MMP-12	Metalloelastase		[a]	NM002426
MMP-13	Collagenase 3		[a]	NM002427
MMP-14	MT1-MMP		[a]	NM004995
MMP-15	MT2-MMP		[a]	NM002428
MMP-16	MT3-MMP		[a]	NM005914
MMP-17	MT4-MMP		[a]	NM016155
MMP-19	RASI 1		[a]	NM002429
MMP-20	Enamelysin		[a]	NM004771
MMP-21			[a]	AY121358
MMP-23			[a]	NM006983
MMP-24	MT5-MMP		[a]	NM006690
MMP-25	MT6-MMP		[a]	AJ239053
MMP-26	Matrilysin 2; Endometase		[a]	NM021801
MMP-27			[a]	NM022122
MMP-28	Epilysin		[a]	NM032950
TIMP-1		Forward primer:	GACGGCCTTCTGCAATTCC	NM003254
		Reverse primer:	GTATAAGGTGGTCTGGTTGACTTCTG	
		Probe:	ACCTCGTCATCAGGGCCAAGTTCGT	
TIMP-2		Forward primer:	GAGCCTGAACCACAGGTACCA	NM003255
		Reverse primer:	AGGAGATGTAGCACGGGATCA	
		Probe:	CTGCGAGTGCAAGATCACGCGC	
TIMP-3		Forward primer:	CCAGGACGCCTTCTGCAA	NM000362
		Reverse primer:	CCCCTCCTTTACCAGCTTCTTC	
		Probe:	CGACATCGTGATCCGGGCCA	
TIMP-4		Forward primer:	CACCCTCAGCAGCACATCTG	NM003256
		Reverse primer:	GGCCGGAACTACCTTCTCACT	
		Probe:	CACTCGGCACTTGTGATTCGGGC	
EGFR		Forward primer:	CGTTTGGGAGTTGATGACCTTT	NM005228
		Reverse primer:	GGCTGAGGGAGGCGTTCT	
		Probe:	AGCCATATGACGGAATCCCTGCCAG	
erb-b2	Her2/*neu*	Forward primer:	GGCTCTCACACTGATAGACCCAA	NM004448
		Reverse primer:	CGCGTCAGGCTCTGACAAT	
		Probe:	CTCTCGGGCCTGCCACCCCT	
erb-b3		Forward primer:	TTCTGATCACCGGCCTCAA	NM001982
		Reverse primer:	CCCGTACTGTCCGGAAGACA	
		Probe:	CCCTGGCACAAGATCCCTGCCC	
TGFα		Forward primer:	CTAGTTGGTTCTGGGCTTTGATCT	NM003236
		Reverse primer:	GGTTTTGGGCATTTGAGTCATT	
		Probe:	TTCCAACCTGCCCAGTCACAGAAGG	
hb-EGF		Forward primer:	TCTGGACCTTTTGAGAGTCACTTTATC	NM001945
		Reverse primer:	CGTGCTCCTCCTTGTTTGGT	
		Probe:	TCCAAGCCACAAGCACTGGCCA	
VEGF-A		Forward primer:	TGGAATTGGATTCGCCATTT	XM052673
		Reverse primer:	TATGTGGGTGGGTGTGTCTACAG	
		Probe:	CTGCTAAATCACCGAGCCCGGAAGA	
KDR	VEGF-R2; flk-1	Forward primer:	CATGTACGGTCTATGCCATTCCT	AF035121
		Reverse primer:	GCTCGTTGGCGCACTCTT	
		Probe:	CATCACATCCACTGGTATTGGCAGTTGG	
flt-1	VEGF-R1	Forward primer:	CCCTCGCCGGAAGTTGTA	AG063657
		Reverse primer:	TTAACGAGTAGCCACGAGTCAAAT	
		Probe:	TTACCTGCGACTGAGAAATCTGCTCGC	
EMMPRIN		Forward primer:	TGCTGGTCACCATCATCTTCAT	NM001728
		Reverse primer:	CCGGCGTCGTCATCATC	
		Probe:	ACGAGAAGCGCCGGAAGCCC	

Note: Common gene names are included in the first column, with alternative names included in the second. Sequences for the primers and probes are in the third column and are shown in 5′ to 3′ orientation. Probes contain a FAM fluorescent reporter on the 5′ end and a TAMRA quencher on the 3′ end.
[a]For the MMPs, primer and probe sequences are the property of Applied Biosystems (Warrington, UK).

the elevated expression of some MMPs is restricted to certain cancers. For example, MMP-11 is elevated in breast, lung, and colon cancers, but not in non-Hodgkin's lymphoma (21). MMP-1 is elevated in breast (22) and brain cancers (23), but not in prostate cancer (24), while MMP-3 is elevated in breast cancer (22), but not in prostate (24) or brain (25). Thus, determining which metalloproteinases are involved in cancer will likely be dependent on the tissue of origin.

MMPs and TIMPs can be synthesized by tumor cells, non-transformed host epithelial or stromal fibroblast cells, or infiltrating cells such as macrophages and monocytes (1, 2), and expression is regulated by numerous autocrine, paracrine, and/or endocrine factors present in the tumor environment. Signal transduction via the EGF-R family (erb-B receptors) is altered in numerous cancers. Many breast cancer samples have an overproduction of EGF-R and erbB2 (HER-2/neu; 26), while many brain cancer patients have elevated EGF-R, erbB2, and the EGF mutant receptor vIII (27). VEGF, which is overexpressed in many tumor cells, binds to receptors found on endothelial cells to mediate angiogenesis (28) and is another mediator of MMP and TIMP production (29, 30). Other factors, such as extracellular matrix metalloproteinase inducer (EMMPRIN), stimulate MMP production in several cancers, including breast and brain cancer (31, 32).

We established a quantitative real-time PCR (qPCR) assay that allowed analysis of the RNA levels for all *MMPs* and *TIMPs* in human-derived cells of cancerous and non-cancerous origin (Table 2) with the aim of identifying relatively unique *MMP/TIMP* profiles in different cancers. We found that glioma-derived cells had elevated levels of *MT-MMPs* compared with other cancerous and non-cancerous cells and this elevated *MT-MMP* was present in malignant clinical samples. Gliomas are the most prevalent form of brain cancer, have a high degree of vascularity and extensive intracerebral invasion, and are unique from most other types of cancers in that they rarely metastasize (33). Co-expression pattern analysis showed several positive correlations between *MT-MMP* and *TIMP* levels with levels of growth factors and/or receptors.

Table 2. The Cell Lines Analyzed by qPCR

Classification	Number	Cell Name	Origin
Normal	1	HUVEC	Human umbilical vein endothelial cell
	2	MRC-5	Human fetal lung fibroblast
	3	IMA	Internal mammary artery
Hematopoietic	4	THP-1	Monocyte
	5	HL60	Promyelotic leukaemia
	6	Jurkat	T-cell leukemia
Carcinoma	7	T47D	Ductal breast carcinoma
	8	BT549	Breast carcinoma
	9	MB-MDA231	Breast carcinoma
	10	HeLa	Cervical adenocarcinoma
	11	G361	Melanoma
	12	PC3	Prostate carcinoma
	13	ECV304	T24 Bladder carcinoma
Glioma	14	U251	De novo glioblastoma
	15	U251N	De novo glioblastoma
	16	U251NH	De novo glioblastoma
	17	U87	De novo glioblastoma
	18	U118	De novo glioblastoma
	19	U178	De novo glioblastoma
	20	U188	De novo glioblastoma
	21	U373	De novo glioblastoma
	22	LN18	De novo glioblastoma
	23	LN71	De novo glioblastoma
	24	LN215	De novo glioblastoma
	25	LN229	De novo glioblastoma
	26	LN308	De novo glioblastoma
	27	LN340	De novo glioblastoma
	28	LN405	De novo glioblastoma
	29	LN428	De novo glioblastoma
	30	LN464	De novo glioblastoma
	31	LN992	De novo glioblastoma
	32	LN827	Glial recurrence of glioblastoma
	33	U343	De novo anaplastic astrocytoma
	34	LN319	De novo anaplastic astrocytoma

Note: Classification is our designation used for comparisons, while Number refers to the numbers used in the figures. Information on the cells can be obtained from American Type Culture Collection (ATCC) or from Ref. (35).

Results

Development and Validation of the qPCR Primers and Probes

Quantitative real-time PCR was used to profile the RNA levels for all human *MMPs*, all human *TIMPs*, and several growth factors and receptors in numerous cell lines and clinical samples, with the aim of identifying novel expression patterns for these genes. Numerous reports have shown disregulated *MMP* and *TIMP* RNA levels in various cancers (including Refs. 15, 21, 24, and 25), but these reports have used techniques such as Northern blot analysis and semi-quantitative PCR. qPCR is more sensitive than either of these techniques in that it can reliably detect less that 100 copies of RNA in a 5-ng pool of total RNA (equivalent to less than 1 copy per cell), and the capability to monitor gene amplification at each cycle means that qPCR is more quantitative than other PCR techniques, with amplification being linear over at least a 6-log dynamic range (data not shown). Additionally, we have shown that results obtained using qPCR are similar to, and more reproducible than, semi-quantitative PCR techniques (34). These advantages make qPCR the technique of choice for validation of data obtained from microarray profiling and for comprehensive analysis of particular gene families in large sample/tissue collections.

Primer/probe sets were designed for all 23 human MMP members, (MMPs 1–28), all 4 human TIMPs, *VEGF-A*, *KDR*, *flt*, *EGFR*, *erb-b2*, *erb-b3*, *TGF-α*, and *HB-EGF* (Table 1). Specificity was verified by comparing the primer and probe sequences to gene sequences found within publicly accessible databases using the NCBI BLAST website (blastn). We also performed a conventional PCR reaction using the qPCR primers without the probe (data not shown); the synthesized product was confirmed as the gene of interest by direct sequencing.

We were unable to detect the RNA for *MMP-20* in any of the cell lines studied. However, we have successfully used these primers and probes to detect *MMP-20* RNA in odontoblast cells and tooth pulp tissue (data not shown).

Differential Expression of MMPs and TIMPs in Numerous Cell Types

RNA levels for every human *MMP* and *TIMP* were profiled in an array of cell types (Table 2). We used the cycle threshold (C_T) of each gene to classify its expression as either very high ($C_T \leq 25$), high ($C_T = 26–30$), moderate ($C_T = 31–35$), low ($C_T = 36–39$), or not detected ($C_T = 40$). Among the *MMPs* that were expressed in almost every cell type were *MMP-2*, *-11*, *-14*, *-15*, *-16*, *-17*, *-19*, and *-23*. *MMP-2* was detected in every cell type, with its highest levels in the normal cells and several cancer-derived cell types, while *MMP-11*, *-19*, and *-23* levels were moderate to high in most samples. Four *MT-MMPs*,

MMP-14 (*MT1-MMP*), -15 (*MT2*), -16 (*MT3*), and -17 (*MT4*) were present in almost every cell type, with *MMP-14* and *-15* showing the highest levels of expression; both *MMP-16* and *-17* were low to undetected in the hematopoietic cells (Fig. 1).

The *MMPs* that had a more restricted profile of expression were *MMP-1*, *-9*, *-10*, *-24*, and *-25*. *MMP-1* levels were highest in the normal cell types and several cancer-derived cell lines; *MMP-9* levels were highest in PC3

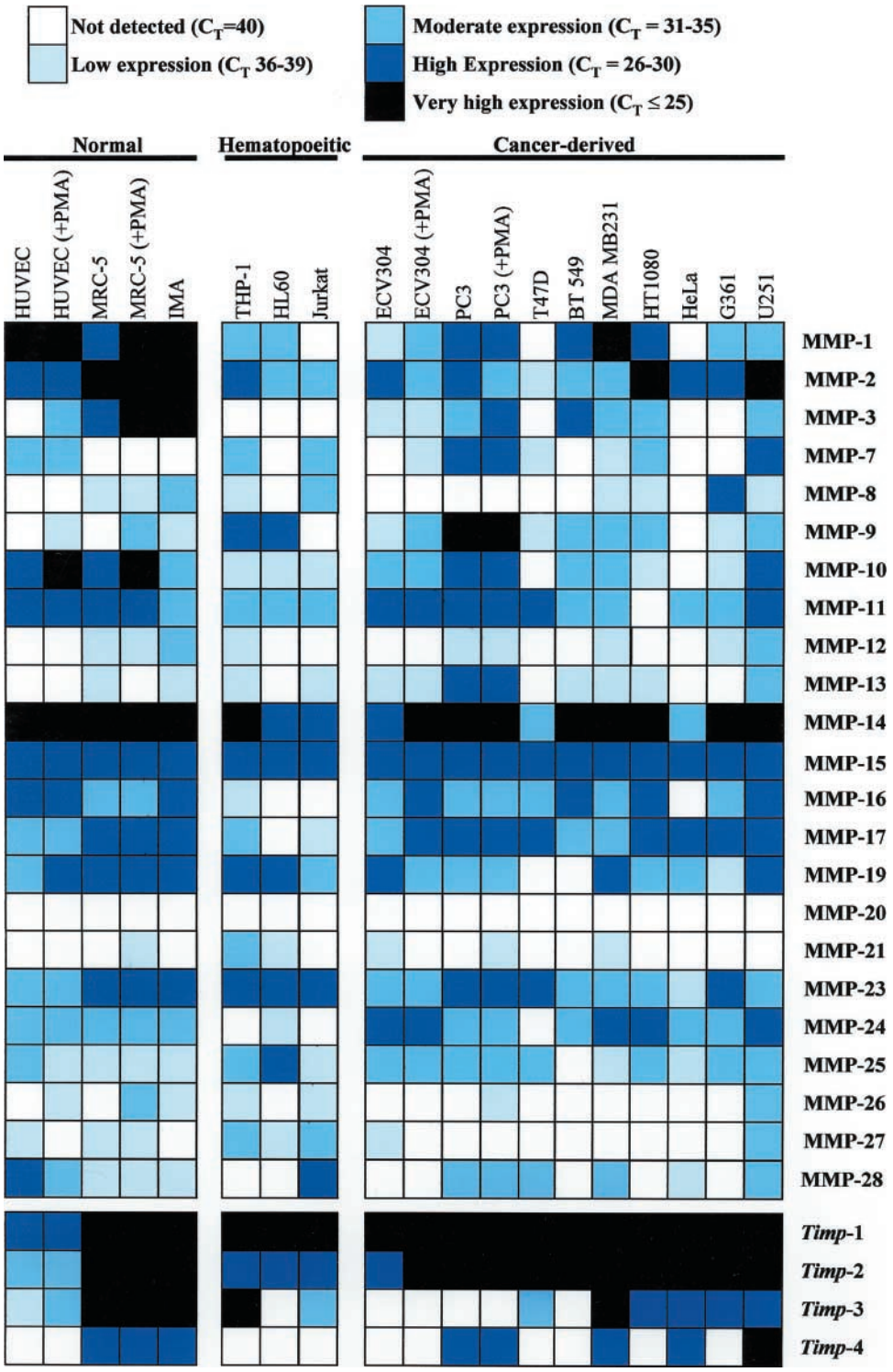


FIGURE 1. The relative mRNA levels for all human *MMPs* and *TIMPs* in several cell types, as listed in Table 2. Classification of expression level was determined from the cycle threshold (C_T) of each gene as either very high ($C_T \leq 25$), high ($C_T = 26-30$), moderate ($C_T = 31-35$), low ($C_T = 36-39$), or not detected ($C_T = 40$); see legend for color scheme. Some cell types were treated with PMA.

prostate cells; while *MMP-10* levels were highest in normal cells. For each of these three genes, their levels were either low or undetected in the poorly invasive T47D breast carcinoma, while in other cell types, phorbol 12-myristate 13-acetate (PMA) stimulation resulted in higher levels of expression. The levels of two additional *MT-MMPs*, *MMP-24* (*MT5*) and *MMP-25* (*MT6*), were moderate to high in most cell types.

The remainder of the MMPs had very restricted profiles of expression. Among the interesting observations were the absence of *MMP-3* from the hematopoietic cells and the T47D cells. In the few cells where *MMP-12*, *-21*, *26*, and *-27* were detected, levels were low to moderate. Levels of *MMP-26* were elevated on stimulation with PMA.

Among the *TIMPs*, *TIMP-1* and *-2* were detected at high to very high levels in almost every cell type, the only exception being moderate *TIMP-2* levels in the HUVEC cells. *TIMP-3* and *-4*, on the other hand, were not detected in many cell types. *TIMP-3* was not detected in several carcinoma-derived cells, including EVC304 (bladder), PC3 (prostate), and T47D (breast), while *TIMP-4* was not detected in any hematopoietic cell, in HUVECs, or in several cancer-derived cells. However, in the cells that did have *TIMP-4* RNA expression, levels were either high or very high.

Closer examination of the raw data revealed that the U251 cell line, derived from a glioblastoma, showed higher levels of the *MMPs* than most other cell lines. In particular, this line had the highest levels of *MMP-15*, *-17*, and *-24*, and the third highest levels of *MMP-16*, all of which are MT-MMPs. In addition, these cells had the highest levels of *MMP-26* and *-27* and *TIMP-4*, the second highest levels of *MMP-7*, and the third highest levels of *MMP-12*.

Expression of MT-MMP and TIMP RNA in Glioma Cells

Given the predominance of several *MMPs* in the U251 glioma line, we quantified the RNA levels of the *MT-MMPs* and *TIMPs* in 20 additional glioma cells lines, and then compared the gene expression in these lines with that in the other cell types (Fig. 2). For all genes, the glioma cells had higher RNA levels compared to at least one other group. Compared to normal cells, the glioma cells had higher levels of *MT3-MMP* ($P < 0.01$), *MT6-MMP* ($P < 0.05$), *TIMP-2* ($P < 0.05$), and *TIMP-4* ($P < 0.05$). Compared to the carcinoma cell lines, the glioma cells had higher RNA levels of *MT1-* ($P < 0.05$), *MT2-* ($P < 0.05$), *MT3-* ($P < 0.01$), *MT5-* ($P < 0.05$), and *MT6-MMP* ($P < 0.01$), and *TIMP-1* ($P < 0.01$), *-2* ($P < 0.01$), *-3* ($P < 0.05$), and *-4* ($P < 0.05$).

Expression of MT-MMP and TIMP RNA in Surgical Specimens of Gliomas

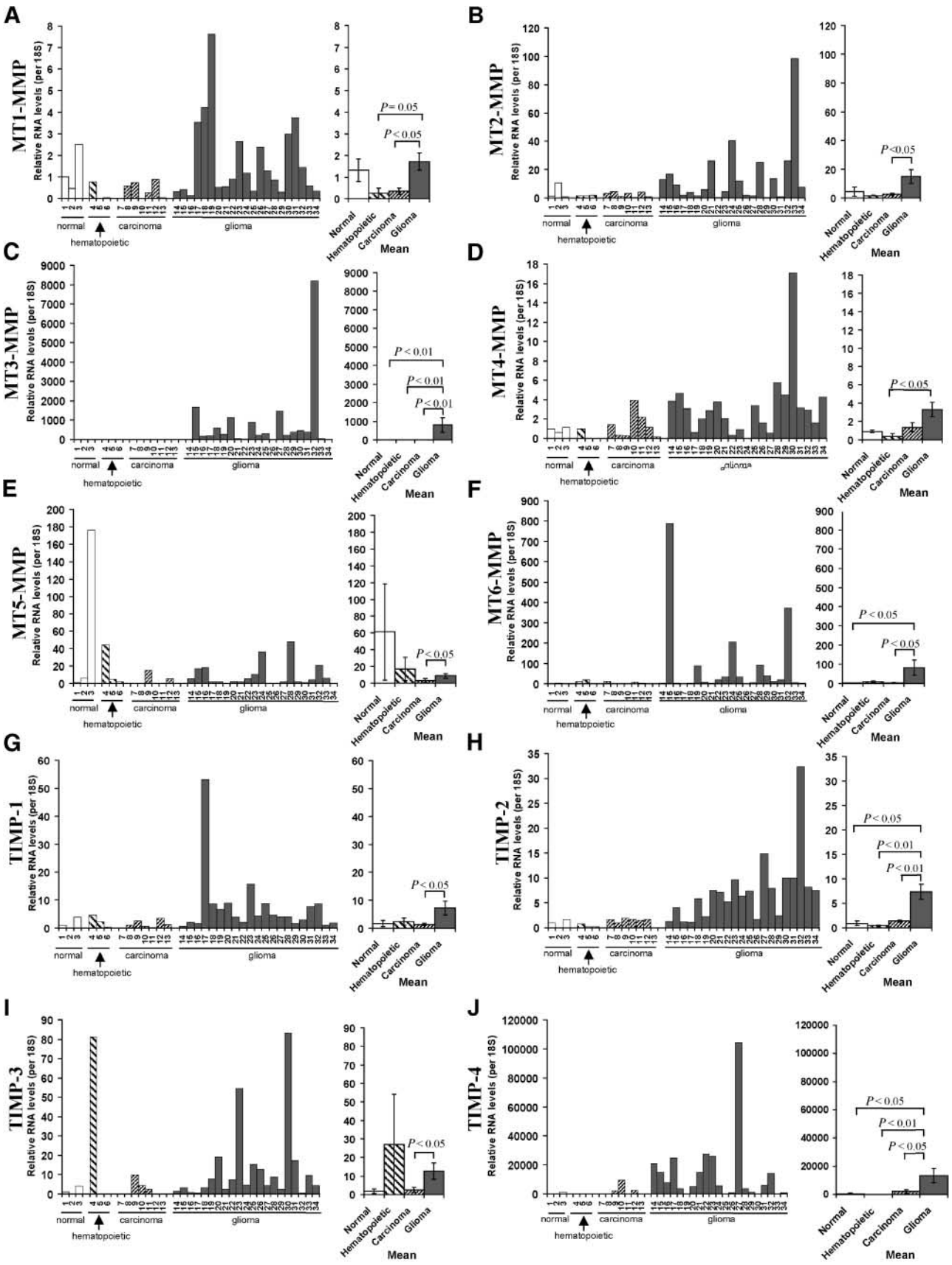
We examined the RNA levels for the *MT-MMPs* and *TIMPs*, in addition to *MMP-2* and *-9*, in surgical specimens of gliomas, to determine if elevated MT-MMP expression is a property of tumor progression or simply an artifact of tissue culture (Fig. 3). There were positive correlations between tumor grade and the RNA levels of *MT1-* ($P < 0.01$), *MT2-* ($P < 0.05$), and *MT6-MMP* ($P < 0.05$), *TIMP-1* ($P < 0.01$) and *-2* ($P < 0.05$), and *MMP-2* ($P < 0.01$) and *-9* ($P < 0.01$). For *MT3-MMP* (Fig. 3C), there was a significant difference ($P < 0.05$) between the low-grade gliomas (LG) and the mid-grade gliomas (MG), and although there were several patients in the GBM group with elevated *MT3-MMP* levels, as a whole, this group did not have elevated *MT3-MMP* expression. There was a significant difference ($P < 0.05$) between *MT4-MMP* levels in the normal samples compared to the MG samples; the levels of *MT4-MMP* decreased with advancing tumor grade (Fig. 3D). For *MT5-MMP*, there were no apparent changes in its levels in any of the groups, while *TIMP-3* and *-4* levels were higher in the LG and MG ($P < 0.05$) than in the normal brain samples.

We determined the absolute number of RNA transcripts for all *MT-MMPs* and *TIMPs* (Fig. 4) and found that in the normal brain, *MT3-* and *MT4-MMP* are the most abundant of the enzymes, while *TIMP-2* and *-4* are the most abundant inhibitors. During glioma progression, there is a 46.6-fold increase in the RNA levels of *TIMP-1*, a 21.6-fold increase in *MT1-MMP*, and a 12.8-fold increase in *TIMP-4*, while the RNA levels for *MT4-MMP* in the GBM patients are 17.7% of those in the normal brain. Totaling all the *MT-MMPs* and *TIMPs* revealed that in the normal brain, there are 1.25×10^5 copies of *MT-MMPs* per nanogram total RNA, compared to 0.72×10^5 copies of *TIMPs* per nanogram total RNA. However, in the GBM, there are 2.33×10^5 copies of *MT-MMPs* and 4.21×10^5 transcripts of *TIMPs*.

Expression of Growth Factors and Receptors in Surgical Specimens of Glioma

We analyzed the expression of some members of the *EGF* and the *VEGF* families, to assess correlation with glioma grade (Fig. 5) and with *MMP* and *TIMP* levels (Table 3). RNA levels of *EGFR* did not increase with glioma progression, although there was one MG sample and three GBM samples that had elevated EGFR (Fig. 5A). *Erb-B2*, on the other hand, showed a significant correlation with glioma grade ($P < 0.05$), while *erb-B3* had a negative correlation with grade ($P < 0.01$). Two EGF receptor ligands, *TGF-α* (Fig. 5D) and *HB-EGF* (Fig. 5E), showed no significant correlation with grade. The levels of *EGFR* correlated with levels of *MT2-*, *MT3-*, and *MT6-MMP*, and *TIMP-2*, *-3*, and *-4*; levels of *erb-B2* correlated with *MT1-MMP*, *TIMP-1*, and *TIMP-3*; and there were negative correlations with *erb-B3* levels and *MT2-MMP*, *MT6-MMP*, *TIMP-2*, and *TIMP-4* (Table 3). Of two EGF receptor ligands examined, *TGFα* showed negative correlations with *MT6-MMP*, *TIMP-3*, and *TIMP-4*, but positive correlations with *TIMP-2*, whereas *HB-EGF* showed only a positive correlation with *MMP-9* (Table 3).

The RNA levels for *VEGF-A* ($P < 0.01$) and one of its receptors, *KDR* ($P < 0.01$), showed a positive correlation with tumor grade, while another receptor, *flt-1*, did not change with grade (Fig. 5). Both *VEGF-A* and *KDR* had positive correlations with *MT1-MMP*, *MMP-9*, and *TIMP-1*, while *KDR* alone showed a positive correlation with *TIMP-2* (Table 3). The levels of *KDR* also correlate with both *VEGF-A*



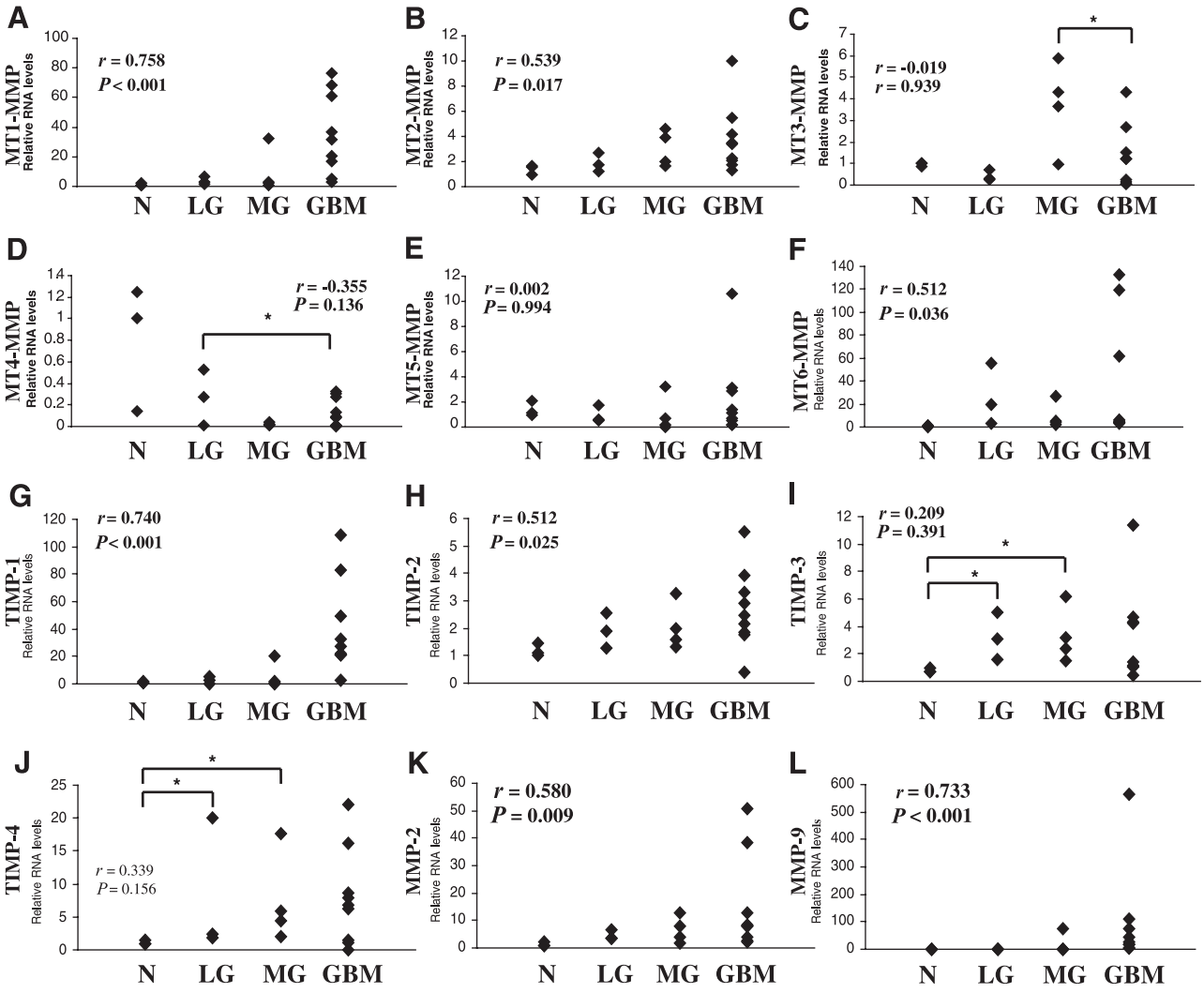


FIGURE 3. The relative mRNA expression of the *MT-MMPs* (**A**–**F**), *TIMPs* (**G**–**J**), *MMP-2* (**K**), and *MMP-9* (**L**) in clinical brain tumor samples. RNA levels are presented as ratios of target gene levels to 18S rRNA levels, with ratios normalized such that normal sample 1 equals a value of 1. Spearman's Rank Correlation was performed using all four tumor groups; correlation coefficient (*r*) and *P*-value are presented. Significant correlations ($P < 0.05$) are highlighted in *bold*. For those genes that did not show a significant correlation, Mann-Whitney non-parametric test was used to look for differences between groups. *, $P < 0.05$, **, $P < 0.01$. *N,* normal brain; *LG,* low-grade astrocytoma, *MG,* mid-grade anaplastic astrocytoma; *GBM,* glioblastoma multiforme.

and *flt-1*. *EMMPRIN* had a positive correlation with glioma grade, although the magnitude of this increase was small (Fig. 5F); *EMMPRIN* levels correlated with *MMP-9*, *MT2-MMP*, and *TIMP-1* and *-2* (Table 3).

Discussion

With approaches such as microarrays and qPCR, it is now possible to build up detailed knowledge of the expression of entire gene families in cancer cell lines and tumors. These techniques have the potential to identify patterns that relate to specific aspects of cell phenotype, and to reveal genes that associate with malignancy. The MMPs have long been linked with tumor invasion and metastasis, but their roles, and those of the TIMPs, are clearly complex because some of their actions promote malignancy, while others oppose it (1–5, 36). Also, MMPs have overlapping substrate preferences, so it is unlikely that an individual family member will be the sole determinant of cell behavior. It is more plausible that cell and tumor capabilities will reflect the integration of the expression of

FIGURE 2. The relative mRNA expression of the *MT-MMPs* (**A**–**F**) and *TIMPs* (**G**–**J**) in several human cell types. Numbers along the *X* axis refer to the designation in Table 2. RNA levels are presented as ratios of target gene levels to 18S rRNA levels, with ratios normalized such that sample 1 equals a value of 1. Cell types were classified as either normal (non-cancerous/non-cancerous origin), of hematopoietic origin, of carcinoma origin, or as a glioma-derived cell line. The mean expression level of target gene per 18S rRNA for each classification (±SE) is shown at the *right* of each graph. *P*-values are indicated.

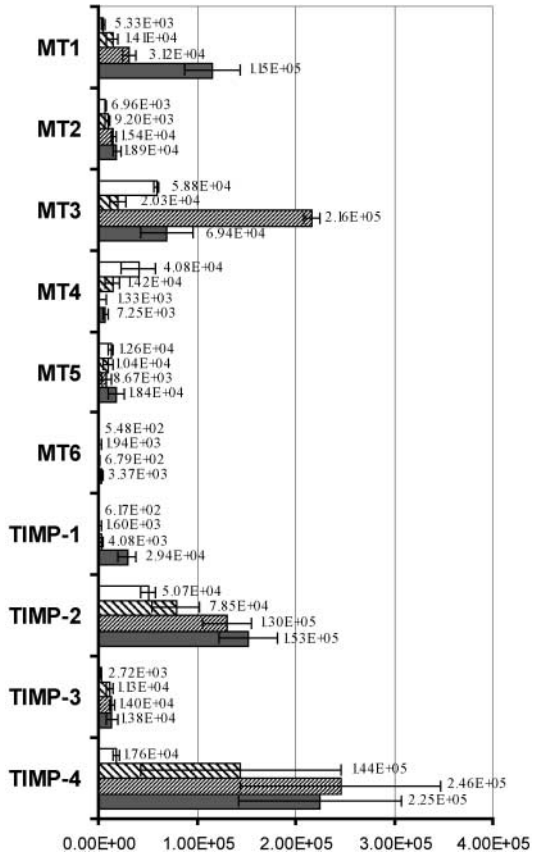


FIGURE 4. The absolute RNA transcript levels for the MT-MMPs and TIMPs in clinical brain tumor samples. Amounts are expressed as transcript numbers per nanogram total RNA. □, N; ▨, LG; ▨, MG; ■, GBM. Abbreviations as in Fig. 3.

protease and inhibitor genes from the repertoire that is available. This work represents the first comprehensive analysis of the human *MMP* and *TIMP* genes in a variety of cell types, which has led to the conclusion that deregulated expression of the *MT-MMP* subgroup of *MMPs* is a feature of glioma-derived cell lines and in clinical gliomas.

Several inferences of gene expression and cell behavior were made from the profile of *MMPs* and *TIMPs* in a panel of cell types. (*a*) The *MMPs* and *TIMPs* can be classified as those that are expressed in every cell type (*MMP-2*, *-14*, *-15*, and *-23*; *TIMP-1* and *-2*), those that are expressed in the majority of cell types (*MMP-1*, *-3*, *-7*, *-9*, *-10*, *-11*, *-16*, *-17*, *-19*, *-24*, and *-25*; *TIMP-3*), and those that are expressed in a small number of cells (*MMP-8*, *-12*, *-13*, *-20*, *-21*, *-26*, *-27*, and *-28*; *TIMP-4*). (*b*) For the most part, there were no substantial differences between the RNA levels in normal cells compared with those in cancer-derived cells. (*c*) In the cells of hematopoietic origin, RNA levels were either comparable or lower than in other cell types. (*d*) PMA treatment resulted in elevated RNA levels for several MMPs, including *MMP-1*, *-3*, *-9*, *-10*, and *-26*. (*e*) Several genes were not detected in the poorly invasive T47D breast carcinoma cell line, but were present in highly invasive cell lines (BT549 and MDA-MB231), including *MMP-1*, *-3*, *-10*, *-13*, and *-24*.

Lastly, and most interestingly, one of the most invasive glioma cell lines *in vivo*, U251 (37), showed higher levels of many MMPs than most other cell lines. When 20 additional glioma-derived cell lines were analyzed, levels of all *MT-MMPs*, except *MT4-MMP*, and all *TIMPs* were elevated in the glioma-derived cells compared to other cancer-derived cell types. These differences are unlikely a general property of the cancer phenotype because the glioma cell lines had elevated levels of several *MMPs* and *TIMPs* compared to other cancer-derived cell lines, and the RNA levels in the carcinoma cell lines were not significantly different from those in the normal cells. When surgical specimens of glioma were analyzed, the RNA levels of *MT1-*, *MT2*, and *MT-6 MMP* and *TIMP-1* and *-2* increased with glioma grade. Together, these findings suggest that elevated *MT-MMP* and *TIMP* levels are specific properties of glioma cells.

The involvement of the MT-MMPs in glioma progression has been understudied. One report, which used a non-radioisotopic, non-fluorogenic quantitative reverse transcription-PCR, also demonstrated high *MT1-* and *MT2-MMP* levels in GBMs, with no correlation for *MT3-MMP* (38), while we had previously been unable to detect a correlation between *MT1-MMP* mRNA levels and tumor grade using less sensitive conventional PCR (39). When the human *MT5-* (14) and *MT6-MMPs* (15) were identified, RNA levels for each were highly expressed in brain tumors compared to normal brain, based on Northern blot analysis. The present study suggests that *MT6-MMP*, but not *MT5-MMP*, is a potential contributor to glioma progression, although there was one GBM sample in the present study with significantly elevated *MT5-MMP* levels. The presence of *MT4-MMP* in gliomas had not been previously demonstrated, and the present study suggests that its levels may decrease during the early stages of glioma progression.

Differential regulation in glioma cells compared to other cancers is interesting in that gliomas, in contrast to other cancers, rarely metastasize, but have extensive local intracerebral invasion and are highly vascular (33). The MT-MMPs, cell-surface anchored MMPs, are well suited to mediate both of these traits. MT1-, 2-, and 3-MMPs promote fibroblast invasion (40) and are involved in tubulogenesis of endothelial cells in fibrin (41). Both MT1- and MT2-MMP have been localized to tumor cells in malignant gliomas (38, 42, 43), where they could mediate invasion, possibly through pro-MMP-2 activation, while the exact localization of the other MT-MMPs in the brain has yet to be determined.

On the basis of RNA transcript numbers, it would appear that the *MT-MMPs* are more abundant than the *TIMPs* in the normal brain, with *MT3-* and *MT4-MMP* being the most abundant enzymes and *TIMP-2* and *-4* the most abundant inhibitors; *TIMP-2* and *-4* are also the most abundant *TIMPs* in the normal mouse brain (44). However, in the GBM samples, there were greater numbers of *TIMP* RNA than *MT-MMP*, although this does not necessarily extrapolate to the creation of an environment in which there is net inhibition of protease activity, because a substantial contributor to the TIMP levels is TIMP-2, a co-factor for MT1-MMP activation of pro-MMP-2 (10). Because levels of *TIMP-2*,

MT1-MMP, and *MMP-2* were all elevated during glioma progression, this could explain the observations that there are elevated levels of active MMP-2 in GBM samples (23, 42) and in patients with extraneural metastasis (39). Yet another function for the TIMPs is that TIMP-1 and -2 have been shown to stimulate cell growth in a variety of cells, while these inhibitors, as well as TIMP-4, are anti-apoptotic and may promote tumorigenesis (36).

With regard to their inhibitory activity, there are likely many additional *MMPs* elevated during glioma progression that would tip the balance of protease:inhibitor in favor of net proteolysis. Numerous studies have identified the presence of several other *MMPs* in gliomas, both *in vivo* and *in vitro* (23, 38, 39, 42, 43, 45, 46), and in particular suggest that there is a positive correlation between *MMP-2* and *-9* levels with increased malignancy, in agreement with the current findings. Some reports have also indicated a negative relationship between glioma aggressiveness and *TIMP-1* and *-2* levels (45, 46). However, these data are in contrast with the current findings, with our own previous work (47), and with those of Lampert *et al.* (42), that show elevated *TIMP-1* in GMB. Taken together, the data suggest that within the tumor environment, there are elevated levels of MMP-2, MMP-9, MT-MMPs, and TIMP-2, that together likely result in net proteolysis. These factors would promote intracerebral invasion and permit angiogenesis. In addition, the elevated TIMP-1, despite not being a mediator of pro-MMP-2 activation, and not being an inhibitor of several of the MT-MMPs (36), may act to regulate proteolysis by other MMPs, and may act as a promoter of cell growth and an inhibitor of apoptosis.

The RNA levels of several growth factors and their receptors were also profiled to determine if their expression correlates with levels of *MMPs* and *TIMPs*. Enhanced signaling through overexpressed or mutated *EGF-Rs* likely occurs in brain cancer progression (27). In this study, RNA levels of *EGFR* did not significantly increase with glioma progression, although there were a few patients with elevated *EGFR* RNA similar to another study that showed only a proportion of patients with GBM to have elevated *EGFR* (48). *Erb-B2*, on the other hand, showed a significant correlation with glioma grade, while *erb-B3* had a negative correlation, consistent with the findings of others (49). As production of MMPs and TIMPs has been associated with EGF stimulation (8, 29), we observed positive correlations between levels of *EGFR* and *erb-B2* with numerous *MMPs* and *TIMPs*, and negative correlations with *erb-B3* levels. Many of the genes that showed a negative relationship with *erb-B3* had a positive correlation with *EGFR*, suggesting that these two receptors could have contrary activity. Also, many

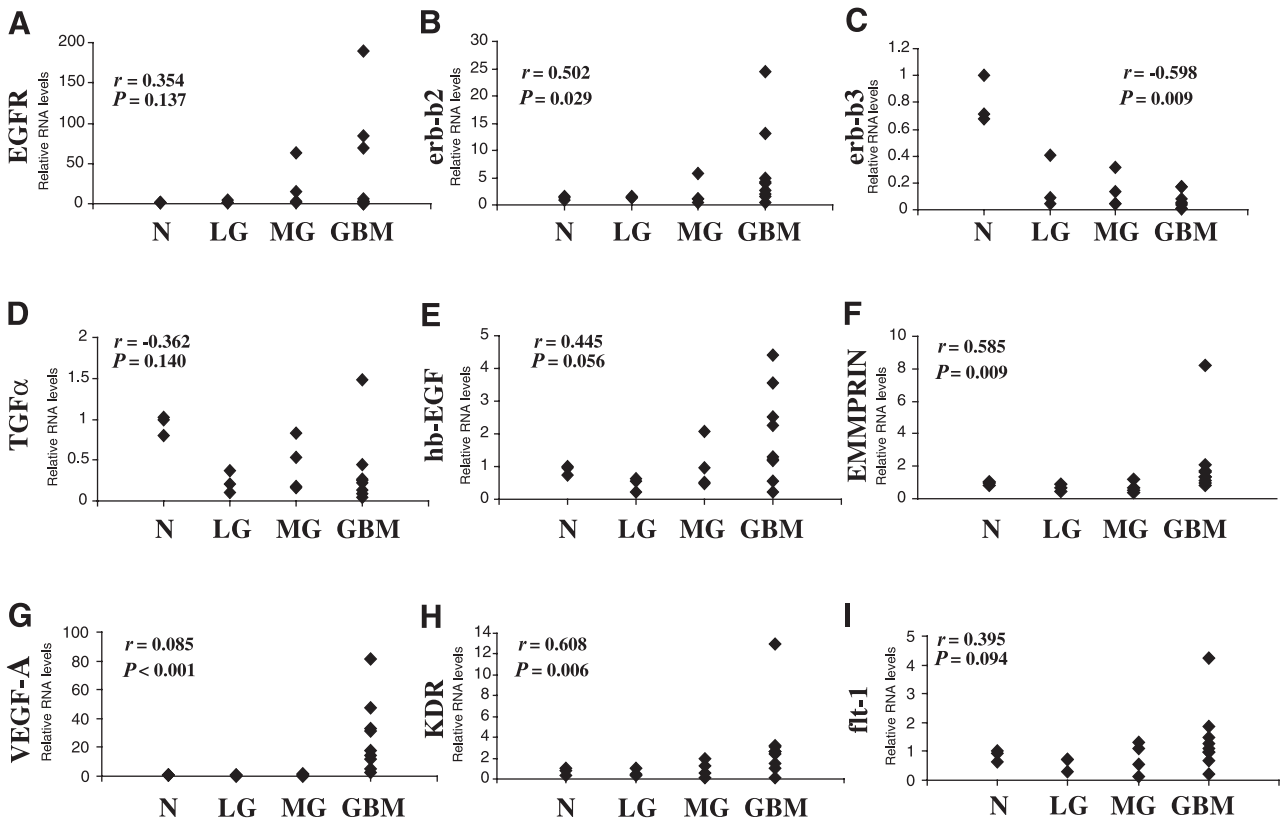


FIGURE 5. The relative mRNA expression of *EGF-R* (**A**), *erb-B2* (**B**), *erb-B3* (**C**), *TGF-α* (**D**), *HB-EGF* (**E**), *EMMPRIN* (**F**), *VEGF-A* (**G**), *KDR* (**H**), and *flt-1* (**I**) in clinical brain tumor samples. See legend in Fig. 3.

Table 3. Correlation Table Comparing Levels of *MMPs*, *TIMPs*, and the Growth Factors/Receptors With Each Other, in the Clinical Samples

	MT1-MMP	*MT2-MMP*	*MT3-MMP*	*MT4-MMP*	*MT5-MMP*	*MT6-MMP*	*TIMP-1*	*TIMP-2*	*TIMP-3*	*TIMP-4*	*MMP-2*	*MMP-9*	*EGFR*	*erb-b2*	*erb-b3*	*TGFα*	*HB-EGF*	*VEGF-A*	*KDR*	*flt-1*
MT2																				
MT3																				
MT4																				
MT5																				
MT6	*																			
TIMP1	**	*				*														
TIMP2	**	*				*	*													
TIMP3	*					**		**												
TIMP4			*			*		**	**											
MMP2	*					*	*	*	*											
MMP9						*	**		**		*									
EGFR		*	**			*		**		**										
erb-b2	**						**	*					*							
erb-b3		** (−)				* (−)		* (−)		** (−)			* (−)	* (−)						
TGFα						** (−)		*	* (−)	* (−)					**					
HB-EGF												*								
VEGFA	**						**					**		*			**			
KDR	*						**	*				**						*		
flt-1												*							**	
EMMPRIN		*					**	*				**		*			**	**		

Note: All correlations are positive, unless indicated by (−). Specific correlation coefficients and *P*-values are available on request.
*A *P*-value < 0.05.
**A *P*-value < 0.01.

of the genes that correlated with *EGFR* were different from those that correlated with *erb-B2*, suggesting that these two receptors may alter glioma progression through different mechanisms.

The current study also demonstrated elevated levels during glioma progression of *VEGF-A* and one of its receptors, *KDR (VEGFR2)*, which has been shown previously by other groups (50, 51), who also observed that *KDR* and *flt-1* (VEGFR1) were present on vascular endothelial cells, while the ligand was a product of the tumor cells. The hypothesis is that VEGF from the tumor cells acts on the endothelial cells to stimulate angiogenesis, leading to the enhanced vascularity found in GBM. On the basis of our findings, potential genes that VEGF could regulate during angiogenesis are *MT1-MMP*, *MMP-9*, and *TIMP-1*. EMMPRIN is a tumor-derived stimulator of *MMPs* and *TIMPs* (31, 32) that may specifically stimulate the production of *MMP-1* (52), *MMP-2*, and *MT2-MMP* (53). This study correlated its levels with *MMP-9*, *MT2-MMP*, *TIMP-1*, and *TIMP-2*, although there was no substantial increase in *EMMPRIN* RNA with glioma grade.

These co-expression profiles are useful in forming hypotheses about the relationships between regulatory molecules, such as growth factors and their receptors, with downstream effector functions, including the *MMPs* and *TIMPs*. The samples analyzed in the current study were bulk tumors, which were comprised of a mix of tumor, endothelia, blood, astrocytes, and neurons, making sites of regulation and activity difficult to assess. Tissue localization studies will be important to determine sites of production, while *in vitro* studies using many of the cell types found in the tumor environment will elucidate regulatory mechanisms. However, while some genes may be regulated *in vitro* by single molecules, such as EMMPRIN and EGFR ligands, other genes may depend on multifaceted cell-cell interactions, making these studies more complex. Although the present work implicates MT-MMPs and TIMPs to have a functional role in the tumor environment, much work remains to be done to determine the exact mechanisms that exist *in vivo*.

Materials and Methods

Cell Lines

Thirty-four different human cell types (numbers 1–34 from Table 2) were studied to develop a profile of *MMP* and *TIMP* RNA levels. These cell types consisted of 3 normal (fibroblast, smooth muscle, and endothelial) cell types, 3 hematopoietic cell types, 7 cancerous cell lines (fibrosarcoma, and carcinoma), and 21 glioma-derived cell lines. Additional information on these cell lines can be obtained through ATCC or Ref. (35). Cells were cultured for several days at 37°C in serum-containing conditions, in the recommended media from ATCC. For some cell types, PMA (10^{-7} M, Sigma Aldrich, Poole, United Kingdom) was added to the cultures to assess the ability of cells to produce MMPs. At near confluence, media were removed, and cells were rinsed in PBS and harvested in RNAzol (Biogenesis, Poole, United Kingdom), with lysates frozen at −20°C until the RNA was isolated.

Clinical Samples

Human glioma samples were obtained from the University of Calgary and the Canadian brain tumor bank in London, Ontario, Canada. All patients gave signed, informed consent for their tissue to be used. Tissue was collected in the operating room immediately after removal and snap frozen in liquid nitrogen. The following grades of tissues were studied: three normal brain tissues (N; two obtained during surgery for epilepsy and one

obtained during autopsy; the specimens from epilepsy surgery were normal tissue that had to be resected to allow the neurosurgeon's access to the epileptogenic focus), three low-grade gliomas (LG), four mid-grade gliomas (MG; also called anaplastic gliomas), and nine glioblastoma multiforme (GBM). Tumors were classified and graded by neoropathologists at the two institutions supplying tissue. Samples, having been previously frozen in liquid nitrogen, were homogenized in RNAzol, and frozen at −20°C until the RNA was isolated.

RNA Isolation and Reverse Transcription

Total RNA was isolated from tissue lysates according to the instructions provided with the RNAzol. RNA was resuspended in diethyl pyrocarbonate-treated (Sigma Aldrich) water, and concentrations were determined by spectrophotometry using a GeneQuant *pro* RNA/DNA calculator (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). One microgram of total RNA was reverse transcribed using 2 μg random hexamers (Amersham) and Superscript II reverse transcriptase (Life Technologies, Paisley, UK) according to the supplier's instructions. cDNA was stored at −20°C until used in the PCR.

Quantitative Real-Time PCR

For PCR reactions, specific primers and fluorogenic probes for all human *MMPs*, all four *TIMP* genes, *TGF-α*, *HB-EGF*, *EGF-R*, *erb-B2*, *erb-B3*, *VEGF-A*, *KDR*, and *flt-1* were designed using Primer Express 1.0 software (PE Applied Biosystems) and synthesized by PE Applied Biosystems; sequences for primers and probes are given in Table 2. To control against amplification of genomic DNA, primers were designed to be close to intron/exon boundaries. The 18S rRNA gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample; 18S rRNA primers and probe were purchased from PE Applied Biosystems.

PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems), using the manufacturer's protocol. Each reaction was performed in 25 μl and contained the equivalent of 5 ng of reverse transcribed RNA (1 ng RNA for the 18S analyses), 50% TaqMan 2X PCR Master Mix (PE Applied Biosystems), 100 nM each of the forward and reverse primer, and 200 nM of probe. Conditions for the PCR reaction were 2 min at 50°C, 10 min at 95°C and then 40 cycles, each consisting of 15 s at 95°C, and 1 min at 60°C.

To determine the relative RNA levels within the samples, standard curves for the PCR reaction were prepared by using the cDNA from one sample and making 2-fold serial dilutions covering the range equivalent to 20–0.625 ng of RNA (for 18S analyses, the range was from 4 to 0.125 ng). To determine the absolute levels of *MT-MMPs* and *TIMPs*, standard curves were prepared by first cloning the full-length cDNA of each gene into a pBluescript KS(-) vector (Stratagene, Amsterdam, The Netherlands). Plasmids were then linearized with an appropriate endonuclease, and sense RNA for each gene was *in vitro* transcribed using the appropriate polymerase (T7 or T3, Roche Molecular Biochemicals, East Sussex, United Kingdom). The plasmid DNA was then digested with DNase I, RNase-free (Roche), the RNA precipitated and resuspended in RNase-free water, and the amount synthesized was determined by GeneQuant spectrophotometry. Knowing the sequence and length of the synthesized RNA, the molecular weight of each RNA was calculated, and the number of molecules synthesized was determined. One microgram of *in vitro* transcribed RNA was then reversed transcribed (as described above), and 10-fold serial dilutions of cDNA were prepared covering concentrations ranging from the equivalent of 10^{10} copies of RNA to the equivalent of 10^{1} copies of RNA. These dilutions were subject to real-time PCR as described above.

During each PCR cycle, the fluorogenic probe was digested by endonuclease activity of the polymerase, generating a fluorescent signal; the amount of fluorescence was proportional to the amount of cDNA amplified. The ABI Prism 7700 measured the cycle-cycle changes in fluorescence in each sample and generated a kinetic profile of DNA amplification over the 40-cycle PCR reaction. The cycle number (termed cycle threshold, or C_T) at which amplification entered the exponential phase was determined and this number was used as an indicator of the amount of target RNA in each tissue, that is, a lower C_T indicated a higher quantity of starting RNA. Relative and/or absolute standard curves for C_T *versus* input RNA were prepared, and relative and/or absolute levels of starting RNA in each sample were determined.

Statistical Analysis

The RNA levels for each gene obtained from the standard curves were corrected using the 18S rRNA levels, and all statistical tests were done on these ratios. For the cell line comparisons, a two-sided Mann-Whitney *U* non-parametric test was done to define differences between groups. For the clinical samples, two-tailed Spearman's Rank Correlation was performed first to define any relationship between RNA levels and tumor grade (from normal to GBM). For those genes that showed no significant correlation with grade, Mann-Whitney *U* non-parametric test was used to look for differences between groups. To determine if there were any correlations between gene levels in the clinical samples, regardless of grade, Spearman's Rank Correlation was performed between all genes. For all tests, a P-value < 0.05 was considered significant.

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