# Expression Pattern of Matrix Metalloproteinase 20 (MMP20) in Human Tumors

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**Abstract**. *Background/Aim: Matrix metalloproteinase 20 (MMP20) is a member of the family of matrix metalloproteinases. Under normal conditions the expression of MMP20 is restricted to ameloblasts and odontoblasts. In order to identify a possible expression of MMP20 under pathological conditions, we investigated three major human tumor entities, i.e. colon, breast and lung tumors, on the mRNA and protein level. Materials and Methods: Real-time RT-PCR and immunocytochemical analyses of established human tumor cell lines were employed for our study; immunohistochemical analysis was performed on both primary tumors and normal control tissues. Results: MMP20 was identified on both the mRNA and the protein level in breast MCF-7, colon HT-29, and lung A549 cell lines. MMP20 was also detected in primary tumor tissue by immunohistochemistry. Conclusion: MMP20 is a new potential candidate for tumor diagnosis or therapy.*

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that exhibit a variety of functions under normal as well as pathological conditions (1, 2). In the human genome, twenty-three distinct MMPs have been identified that can be classified into five groups: collagenases, gelatinases, stromelysins, matrilysins and membrane-type MMPs (3). MMPs are involved *inter alia* in tissue remodelling and organ development, in inflammatory processes as well as in cancer progression (1). Especially in cancer, MMPs participate in tumor growth, invasion and metastasis, as well as angiogenesis (4). These characteristics have rendered MMPs as attractive therapeutic targets. However, all of the more than 50 MMP inhibitors tested so far have failed in clinical trials (2). The reasons behind this result seem to be numerous but include problems due to the clinical trials themselves and the use of multitarget MMP inhibitors and, thus, the appearance of antitarget effects (2). MMP20 is one of two major proteinases present during enamel development (5). Beside cementum and dentin, enamel is the third mineralized tissue present in normal teeth (6). Enamel covers the crown of the tooth and its development can be subdivided into four consecutive stages that are defined by morphological and functional changes of enamel intrinsic ameloblasts that cover the developing enamel as a single cell layer: presecretory, secretory, transition and maturation (6). MMP20 is expressed by ameloblasts during the secretory through early maturation stage. As it was originally thought that MMP20 expression is restricted to enamel and, thus, MMP20 was named enamelysin. However, later on evidence was provided that during normal development MMP20 is also expressed by odontoblasts of the pulp organ (6). Under pathological conditions, MMP20 was identified in odontogenic (7) and oral tumors (8), esophageal cancer (9), and human tongue carcinoma cells (10). It has been suggested that the expression pattern of MMP20 in tumorigenic tissue is very restricted, therefore an extensive analysis aimed to investigate the molecule's appearance in a high number of different tumors did not reveal any positive result (11). In light of the lack of reliable tumor biomarkers the present study was undertaken to answer the question if the tumor-

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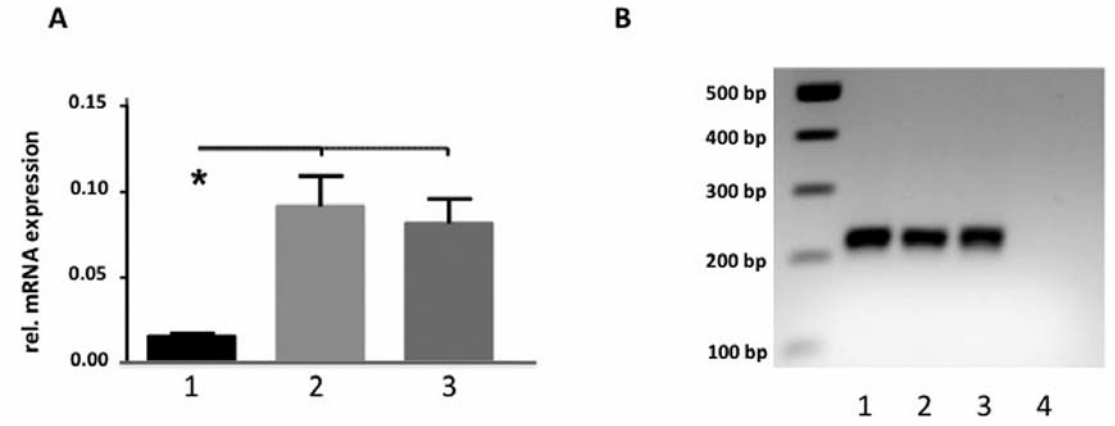


Figure 1. *RT-PCR analysis of MMP20 expression in human tumor cell lines. (A) Validation of MMP20 gene expression as revealed by real time PCR. RNA from A549 (1), HT-29 (2), and MCF-7 (3) cells (n=6 for each cell line). The expression level of MMP20 in each cell line was normalized to GAPDH expression. Mean±SEM data are depicted. Statistical differences were analyzed by one-way-ANOVA and the posthoc Tukey's multiple comparison test and are marked with asterisks (*p<0.05). (B) Ethidium bromide stained agarose gel demonstrating the purity of MMP20-specific RT-PCR after realtime PCR (50 cycles). Lane 1: A549, lane 2: HT-29, lane 3: MCF-7 cells, lane 4: water control. Product size of the MMP20-specific transcript: 223 bp.*

specific expression pattern of MMP20 is not more widespread than previously suggested. Our data clearly demonstrate the expression of MMP20 in breast, colon and lung carcinoma. In addition, they point to the necessity of more highly sophisticated studies to elucidate the expression pattern of MMP20 expression during tumor development and progression in more detail, whereby one focus should be the possible correlation of MMP20 expression with distinct tumor subtypes.

## Materials and Methods

*Cell lines and culture conditions*. Human breast carcinoma MCF-7 cells, and non-small cell lung carcinoma (NSCLC) A549 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal calf serum (FCS) and colon carcinoma cells HT-29 were cultivated in DMEM/Ham's F12, 10% FCS.

*RNA isolation and RT-PCR analysis*. Total cellular RNA was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany). 1 μg of total cellular RNA was reverse transcribed with the Superscript III kit and random hexamer primers (Life Technologies, Waltham, USA). cDNAs were amplified (35 to 40 PCR cycles) with gene specific primers for MMP20 (sense: 5'-GGGAGATGGATAC AGCTACG-3', antisense: 5'-TTAGCAACCAATCCAGGAACTAGAT-3'; T=65°C; E=1.95; product size 223 bp) and glyceraldehydephosphate-dehydrogenase (GAPDH; sense 5'-TGGTATCGTGGAAGGACTCA-3', antisense 5'-CCAGTAGAGGCAGGGATGAT-3'; T=67°C; E=1.93; product size 131 bp), the PCR products electrophoresed on 1% agarose gels and visualized with ethidium bromide. For real-time PCR the 7300 Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany), SYBR® Green (Bio-Rad Laboratories, München, Germany), was used. Real-time PCR was performed by adding 50 ng cDNA to a master mix containing primers and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories) and PCR conditions were as follows: a 5 min preceding denaturation step at 95°C was succeeded by 50 cycles of 15 s at 95°C, 30 s at annealing temperatures specific for the primers, and 30 s at 72°C for elongation. Relative differential gene expression was calculated using the method described by Pfaffl (12) with GAPDH serving as house-keeping gene.

*Immunocyto- and histochemistry*. For immunocytochemistry, cells were fixed in 4% paraformaldehyde (15 min) and then permeabilized with PBS containing 0.1% Triton X-100 (15 min) at room temperature (RT). Unspecific binding sites were blocked with 10% rabbit serum in Tris-buffered saline (TBS) for 1 h at RT. Cells were then incubated with polyclonal rabbit IgG anti-MMP20 (#PAB4787 from Abnova Taipeh City, Taiwan); diluted 1:50 in TBS, 1% bovine serum albumin (BSA) in a humid chamber at 4°C overnight. After washing, cells were incubated for 1h at RT with Alexa Fluor 488-conjugated secondary goat anti-rabbit IgG (Dianova; diluted 1:250 in TBS, 1% BSA). For nuclear staining, cells were treated with 4',6-Diamidin-2-phenylindol (DAPI) for 5 min. Fluorescence microscopic analysis was performed with the axio imager system (Zeiss, Jena, Germany).

For immunohistochemistry, tissue sections (from US Biomax, Rockville, IL, USA) from tumor and normal control tissue were deparaffinized, rehydrated and rinsed with TBS. Endogenous peroxidase was blocked in a methanol/$H_2O_2$ solution and unspecific binding sites blocked with TBS, 1% BSA. Slices were then incubated with rabbit IgG anti-MMP20 (diluted 1:50) in a humid chamber at 4°C overnight. Antigen–antibody binding was visualized using the EnVision Detection System Peroxidase/DAB from Dako (Hamburg, Germany). Cell counterstaining was performed with Mayer's haematoxylin.

*Statistical analysis*. Tissue sections were investigated by eye by four trained scientists independently. Tumor tissue was classified as positive when more than 20% of the tumor cells in at least two

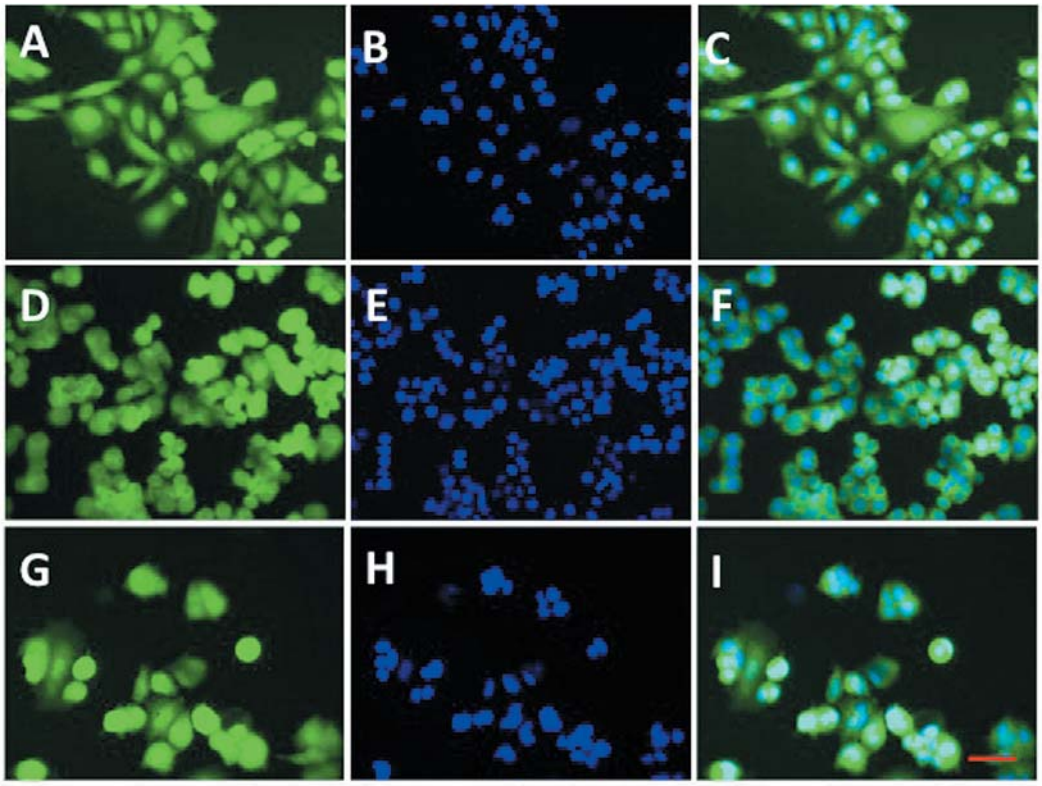


Figure 2. *Immunocytochemical analysis of MMP20 expression in human tumor cell lines. Permeabilized A549 (A-C), HT-29 (D-F), and MCF-7 breast carcinoma cells (G-I) were stained with MMP20 antibody and secondary Alexa Fluor (AF) 488 conjugated antibody (green; A, D, G). Cell nuclei were counterstained with DAPI (blue; B, E, H). C, F, I are the merged images of both AF 488 and DAPI staining. Scale bar in I: 50 μm.*

randomly selected areas (SIZE) were stained. Six tissue slides derived from three malignant and three normal tissues were analysed for each separate tumor type. For the statistical analysis of data obtained in real time PCR experiments the one-way-ANOVA and the posthoc Tukey's multiple comparison test were used.

## Results

*MMP20 expression in human tumor cell lines*. RT-PCR analyses were performed in breast MCF-7, colon HT-29 and A549 non-small cell lung carcinoma cells. Real-time RT-PCR experiments revealed expression in all three cell lines, while lowest expression level was observed in A549 cells (Figure 1A). A gel electrophoretic analysis of the RT-PCR products revealed a single band with the expected fragment length (Figure 1B). Sequencing of the PCR product (GATC Biotech, Cologne, Germany) confirmed its identity with the MMP20-specific transcript in all three cell lines. MMP20 expression could not be verified in five small cell lung carcinoma cell lines, in cervix carcinoma cell line HeLa and thyroid carcinoma FTC-133 cell line. In immunocytochemical studies a diffuse cytosolic staining for MMP20 could be observed in all three cell lines. Moreover, in A549 and MCF-7 cells (Figure 2A and G) a pronounced nuclear staining was obvious. The staining pattern of HT-29 cells (Figure 2B) also points to a plasma membrane-associated localization of MMP20.

*MMP20 expression in human primary tumors*. To assess MMP20 expression in human tissues, we performed immunohistochemical staining of tissue sections derived from commercially available tumors (Figure 3A, C and E) as well as of normal control tissue (Figure 3B, D and F), whereby six different specimen were used for each organ. MMP20 protein expression was detected in all specimen examined that derived from tumor tissues, *i.e*. invasive ductal breast carcinoma (Figure 3A), colorectal adenocarcinoma (Figure 3B) and lung adenocarcinoma (Figure 3C). By contrast, in the respective non-malignant tissue no expression was observed in normal breast tissue (Figure 3B), and only a baseline expression in epithelial cells of the colorectal crypts (Figure 3D) and in the bronchus epithelium of the lung (Figure 3F). MMP20 expression was not detectable in other types of tumors, such as testicular seminomas or cervix squamous cell carcinomas (n=6 for each tumor type; not shown).

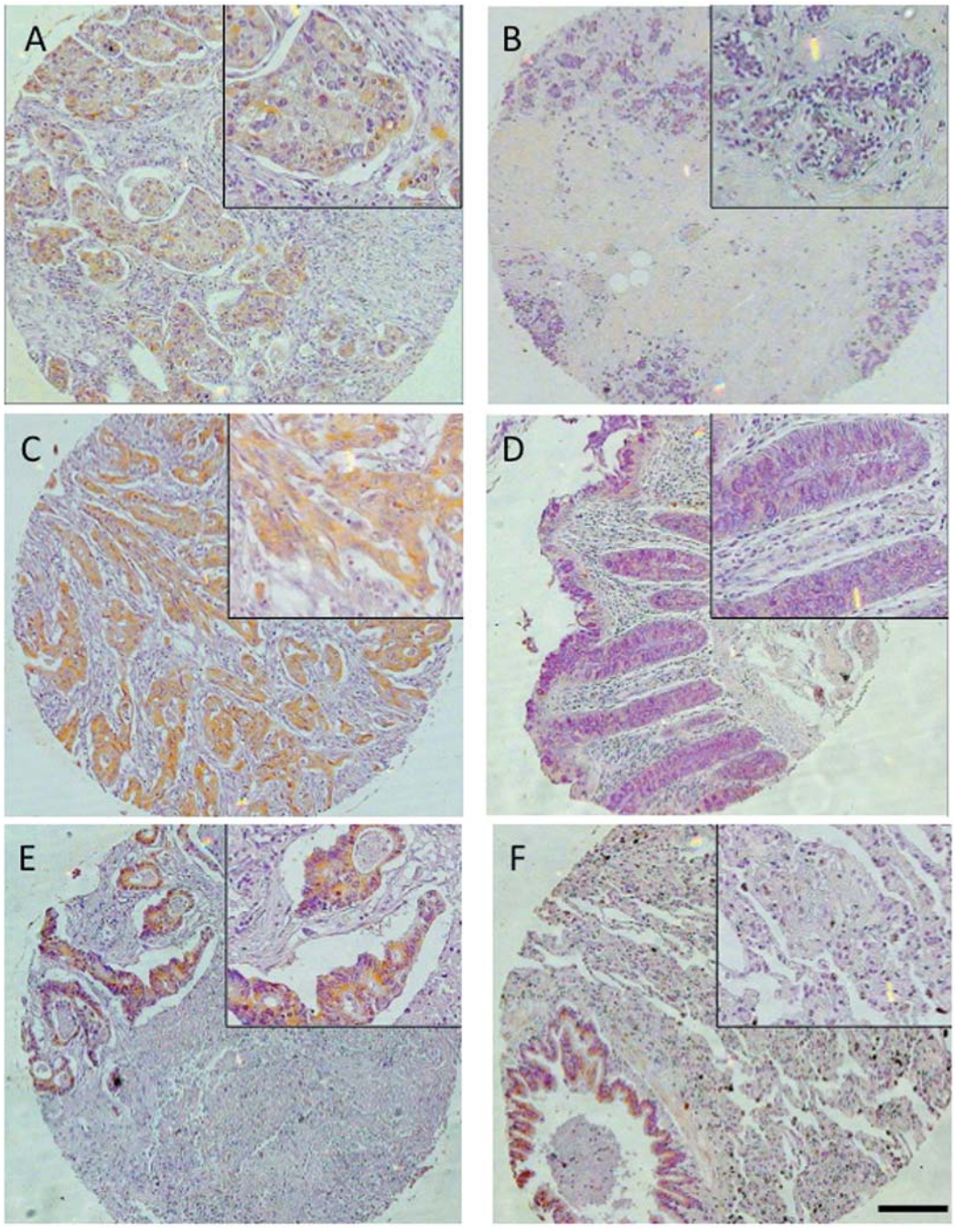


Figure 3. *Immunohistochemical analysis of MMP20 expression in primary human tumors. Tissue sections, derived from ductal breast carcinoma (A), mucinous colon adenocarcinoma (B) and lung adenocarcinoma (C) as well as from normal breast (B), colon (D) and lung tissue (F) were stained with MMP20 antibodies. Before antibody incubation, endogenous peroxidase activity was blocked. Finally, immunoreactivity was visualized with secondary horseradish peroxidase antibodies and diaminobenzidine as substrate leading to a brownish reaction product. Scale bar in F: 200 μm for overviews, 44 μm for inserts. Representative sections out of six sections for each tissue type are shown.*

## Discussion

In the present study we provided evidence that MMP20 is expressed in major human tumor entities *in vitro* as well as *in vivo*. Because under non-pathological conditions MMP20 expression is most likely restricted to a narrow space and time window this molecule becomes an attractive candidate for tumor diagnosis and therapy. However, at least two questions

need to be addressed experimentally before potential clinical applications can be discussed in more detail: (i) What is the incidence and specificity of MMP20 expression in human cancer in general? Our data suggest that the irregular expression of MMP20 is not a cancer-subtype specific phenomenon and preliminary data of our group suggest an even more widespread distribution, as detailed in the present work. Moreover, only limited information is available concerning the expression pattern of MMP20 under normal or other non-cancerous pathological conditions, such as, for example, inflammatory processes. (ii) When does the expression of MMP20 in cancer occur? Is it an early event during carcinogenesis or does it take place late during cancer progression? Moreover, is the expression pattern of MMP20 related to specific subtypes of the disease? For breast cancer, for example, increasing knowledge has been accumulated during the last years that allowed discrimination between different major tumor subgroups (13) and it would be of considerable interest to compare the expression pattern of MMP20 in the context of this frame.

Concerning diagnosis, an ideal tumor marker should be exclusively expressed in the tumor tissue, but, unfortunately, the tumor markers currently available lack this kind of specificity (14). In addition, it is desired that tumor markers provide some information related to tumor type and/or tumor staging. This does not necessarily imply that lack of such specificity makes a marker useless for tumor diagnosis because it would still allow for a precise discrimination between normal and cancerous tissues. Thus, the expression of MMP20 could be an indicator for the presence of a tumor per se, but not for a specific type of tumor. It has to be noted, that in the context of breast cancer and breast cancer cell lines a study demonstrated an up-regulation, but not a neoexpression of some MMPs in breast cancer tissues (15). In contrast to us, these authors were not able to show an expression of MMP20 in primary breast cancer tissue and in MCF-7 cells. To be useful as a tool in routine diagnosis assays implicated to detect tumors already at early stages, the first litmus test will be the verification of MMP20-specific protein or transcripts in body fluids of cancer patients.

Concerning therapy, we are confronted with the disappointing fact, that, although more than fifty MMP inhibitors have been investigated in clinical trials, all of them failed (2). This failure has been attributed to the expression of MMPs by tumor as well as by surrounding stroma cells, varying functional roles of MMPs during cancer progression and benificial roles of MMPs, *i.e.* the generation of undesired anti-target effects (2, 16). According to our present knowledge MMPs are involved in a number of basic cellular processes that are only partially understood (2). As most of the inhibitors available are not MMP-specific, the appearance of undesired side-effects is very likely. *Vice versa*, the specific inhibition of a specific MMP may not be suffcient, based on redundancy regarding MMP function and expression pattern. Taking into account that MMP20, in contrast to other MMPs tested so far, exhibits a very restricted expression pattern under non-pathological conditions, its expression by tumor-surrounding stroma cells as well as anti-target effects are unlikely. Thus, if MMP20 provides any benefit for tumor cells, MMP20-specific drugs would be the first choice to attack such advantages. Unfortunately, most MMP inhibitors currently available are not specific for individual MMPs. Recently, however, for some MMP family members more specific tools have been developed: (phosphinic) peptide inhibitors for MMP9 (17) and MMP12 (18, 19) as well as a monoclonal antibody targeting the active form of MMP14 (20) and also a MMP20-specific inhibitor would be of considerable benefit. However, it has to be taken into account that a considerable amount of MMP20 could be expressed intracellularly (21). If MMP20 in tumor cells is localized in association with the plasma membrane, as suggested by the staining pattern in HT-29 cells (Figure 2), MMP20-recognizing agents, such as toxin-coupled antibodies could provide therapy-relevant tools. In sum, we have provided considerable evidence that MMP20 represents a promising candidate for tumor diagnosis or therapy. Thus, more sophisticated studies are legitimated aimed to uncover the molecule's usefulness in anticancer strategies.

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