In situ gene expression and localization of metalloproteinases MMP1, MMP2, MMP3, MMP9, and their inhibitors TIMP1 and TIMP2 in human renal cell carcinoma

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Abstract. Matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) play a major role in the maintenance of extracellular matrix homeostasis. Alterations of MMP and TIMP expressions have been found in several malignant tumour entities. In this study the expression pattern of MMP1, MMP2, MMP3, MMP9, and their inhibitors TIMP1, and TIMP2 were investigated at mRNA and protein levels in human renal cell carcinoma (RCC). Formalin fixed paraffin embedded tumour samples of 10 patients and adjacent non-malignant controls were analysed by radioactive labelled riboprobe *in situ* hybridisation (*is*H) and immunohistochemistry. The slides were evaluated semiquantitatively. MMP1-antigen was strongly expressed in tumour epithelium with moderate stroma expression in one case. The gelatinases MMP2 and MMP9 showed moderate to strong signals in tumour epithelial cells at the mRNA and protein level, while the expression in tumour stroma was moderate. MMP3-mRNA and -antigen were expressed moderately to strong in tumour epithelium and focally in stroma cells. mRNA or TIMP1- and TIMP2-mRNA and -antigen were also predominantly expressed in tumour epithelium; only few samples showed positive expression in stroma cells. mRNA expression could be generally correlated to the protein expression in our study group, except for MMP1 (mRNA expression was only expressed in two cases). We found a pronounced expression for the gelatinases MMP2 and MMP9 and for MMP3 in RCC at the mRNA and protein level. The expression of TIMP1 and TIMP2 appears also to be relevant in RCC. Due to the small sample size further investigations need to be done to prove a statistical significant correlation between the MMP/TIMP expression and clinicopathological parameters.

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Introduction

A distinct and eventually life threatening aspect of malignant tumour cells is their ability to invade and metastasize at distant sites of the body. Degradation of the basal membrane and the extracellular matrix (ECM) is a prerequisite for tumour cell invasion. Different families of ECM degradation enzymes are known, consisting mainly of zinc-dependent matrix metalloproteinases (MMPs). The balance of secreted MMPs and their specific inhibitors (TIMPs) play an important role in maintaining connective tissue homeostasis in normal tissue (1). In neoplastic diseases an imbalance of MMPs and TIMPs, leading to an excess of degradative activity, is supposed to be linked to the invasive character of tumour cells (2-4).

MMPs are divided according to their target protein into several families (5): the collagenases (MMP1, MMP8, MMP13), the gelatinases (MMP2, MMP9), stromelysin (MMP3, MMP7, MMP10, MMP11, MMP12), the membrane-type MMPs (MT-MMP-1, -4, or MMP-14, -17), and the novel MMP20 (6,7). Expressions of MMPs are mainly transcriptionally regulated leading to the secretion of inactive (latent) enzymes. Their proteolytic activity is activated by a complex cascade, which is not yet completely understood. Activated MMP1, MMP3 and latent forms of MMP2 and MMP9 are regulated and inhibited by endogenous proteins known as tissue inhibitors of metalloproteinase TIMP1 and TIMP2 (8). Studies have shown that TIMP1 binds preferably to MMP9 and TIMP2 preferably to MMP2 (9,10). Over-expressions of MMPs are associated with various pathological events (11,12).

Gene expressions of MMPs and TIMPs have been extensively studied *in vivo* and *in vitro* in various tumours. It has been shown that expression of MMPs and TIMPs correlate with an increased metastatic potential of tumour cells (10,13,14). The clear cell subtype of human renal cell carcinoma (RCC) is the most common malignant renal tumour and comprises about 3% of all malignant tumour

diseases. It has been shown that gelatinase and stromelysin have an impact on tumourigenesis in a variety of malignant diseases with an elevated expression. So far data are rare concerning the expression of MMPs and TIMPs in RCC (15-18). Therefore, our goal is to clarify the general extent and the pattern of localization and cellular expression of MMPs and TIMPs in a group of patients with RCC. To our knowledge this is the first combined investigation on expression and localization of MMP1, MMP2, MMP3, MMP9, and TIMP1, TIMP2 in RCC studied by *in situ* hybridisation (*is*H) and immunohistochemistry.

Materials and methods

Patients and tissue samples. Ten patients (5 female, 5 male) were included in our study with diagnosed RCC. The mean age was 62.3 years (range from 35 to 75 years) All patients are summerized in Table I.

Fresh tissue samples were directly obtained in the operating room from 10 cancerous and 10 non-cancerous renal sections after radical tumour nephrectomy. An experienced pathologist performed the histopathological staging and grading according to the TNM classification (19). All tissue sections were evaluated microscopically. Adjacent non-malignant tissue sections with a security margin were taken from each patient and processed in the same way for control purposes. After collection, one half of tissue samples in each group was snap-frozen and stored in liquid nitrogen until processing and the other half was fixed in formalin and embedded in paraffin for histology evaluation and immunohistochemistry.

The investigators were blinded to information on *is*H and immunostaining until conclusion of this study.

Chemicals. The following materials were used and obtained from the indicated sources: T7 and T3 RNA polymerase, pBluscript KS (+) plasmid vector (Stratagene, Heidelberg, Germany), RNAse and DNAseI (Promega, Madison, WI), ^{35}S-UTP (DuPontNEN, Belgium), dithiothreitol (DTT), formamide, restriction endonuclease (Roche Diagnostics, Mannheim, Germany), autoradiographic emulsion (Eastman Kodak Co.), and MMP1 (Calbiochem, 41-1E5), MMP2 (Calbiochem, 42-5D11), MMP3 (Calbiochem, 55-2A4), MMP9 (Novocastra, 2C3), TIMP1 (Oncogene, 102D1), TIMP2 (Oncogene, T2-N1C3) monoclonal antibodies. All other chemicals used were of analytical grade.

Preparation of RNA probes. Antisense (complementary) riboprobes and sense (anti-complementary) were prepared from MMP1, MMP3 (stromelysin-1), MMP2, MMP9, TIMP1, and TIMP2 cDNAs, which cloned either pBluscript SK- (Stratagene) or SP^{64} vectors. MMP2, MMP9, TIMP1, and TIMP2 cDNAs were subcloned into the pBluscript SK$^-$ and transcribed with either T3 or T7 RNA polymerase after linearising with the appropriate restriction enzymes.

For the synthesis of antisense riboprobe for MMP1, the plasmid was linearised by SmaI and transcribed with SP6 RNA polymerase. For the synthesis of sense riboprobe for MMP1, HindIII + SmaI fragment was subcloned into the pBluscript KS (+) and transcribed with T3 RNA polymerase after linearising with XhoI. The human MMP3 receptor was derived from the BamH1-EcoR 11.7 kb fragment. For the synthesis of riboprobe in the antisense orientation for MMP3, the plasmid was linearised with HindIII and transcribed with T3 RNA polymerase. For the sense riboprobe, the plasmid was transcribed with T7 RNA polymerase.

Transcription was carried out essentially as recommended by the manufacturer's of the polymerase containing 1 μg of linearised DNA template. The riboprobe was hydrolysed in 0.1 mol/l DTT to an average size of 100 bases. The probes were stored at -20˚C until further processing.

In situ hybridisation (isH). Re-hybridisation and hybridisation steps were performed using the protocol of Hogan *et al* with small modification (20). Paraffin sections were dewaxed in xylene for 10 min followed by gradual hydration. Briefly, before hybridisation slides were treated with 0.2 M HCl, 2X SSC at 70˚C, digested with pronase (Sigma) for 10 min, fixed with 4% paraformaldehyde, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated in graded ethanol solutions, and air dried. ^{35}S-UTP labelled sense and antisense riboprobes (2-5x10^6 cpm) were applied to each slide in 25 μl hybridisation mixture (20). The slides were incubated at 55˚C overnight. The slides were then washed in 5X SSC containing 10 mM DTT at 55˚C for 1 h, followed by 2X SSC containing 50% formamide, and 50 mM DTT at 65˚C for 30 min. Unhybridised riboprobes were digested with 20 μg/ml RNAse A (Sigma) at 37˚C for 30 min, followed by a second incubation at 65˚C in 2X SSC containing 50% formamide solution. Slides were rinsed in 2X SSC and then 0.2X SSC for 15 min each, dehydrated in graded ethanol containing 0.3 M ammonium acetate and then air-dried. Finally, the slides were dipped in NTB-2 autoradiographic emulsion and stored in complete darkness at 4˚C for two weeks. The slides were developed with Kodak D-19 developer (Eastman Kodak Co.) and counterstained with hematoxylin and eosin.

Using light microscopy the degree of expressions (hybridisation signals) were determined in a semi-quantitative fashion ranging from 0, not detectable; +, (focal positive, <20% of tumour cells stained); ++, (minimal diffuse positive, 20-50% of tumour cells stained) to +++, (moderate positive, 50-80% of tumour cells stained); ++++, (strong positive, >80% of tumour cells stained). Two experienced pathologists evaluated all specimens independently.

Immunohistochemistry. The blocks of formalin-fixed paraffin-embedded tissue were cut (3 μm) and mounted on superfrost® slides. The slides were deparaffinized with xylene and gradually hydrated (100%, 70%, 50%, 30%, TBS). Heating times for antigen retrieval in 0.01 M citrate buffer were 5 min for MMP1 (dilution 1:40), MMP2 (dilution 1:20), MMP3 (dilution 1:100), and MMP9 (dilution 1:100) and 3 min for TIMP1 (dilution 1:50), and TIMP2 (dilution 1:50). As diluents a background-reducing dilution buffer (Dako) was used. No other blocking agents were employed. Adjacent non-malignant tissue sections were immunostained as controls. The primary antibody was incubated at room temperature for 1 h. Detection took place by the conventional labelled-streptavidin-biotin (LSAB) method with alkaline phosphatase as the reporting enzyme

Summary of *in situ* hybridisation and immunohistochemical semiquantitative analysis of MMP1-, MMP2-, MMP3-, MMP9-, TIMP1-, and TIMP2-mRNA and -protein expressions in paraffin sections from 10 patients with renal cell carcinoma.

No.	Age	Sex	pT	pN	M	G	MMP1 E/S		MMP2 E/S		MMP3 E/S		MMP9 E/S		TIMP1 E/S		TIMP2 E/S	
							mRNA	Protein[a]	mRNA[b]	Protein[a]	mRNA[b]	Protein[a]	mRNA[b]	Protein[a]	mRNA	Protein[a]	mRNA	Protein
1	73	M	2	0	X	2	+++/0	++++/0	++/0	++/0	++/0	+++/***	++/***	+++/***	0/0	++/0	0/0	+/0
2	69	F	1	0	X	2	0/0	++++/***	+/0	++/0	++/**	0/0	++/**		0/0	++/+	+++/*	++/0
3	60	F	1	0	X	2	0/0	++++/0	++/0	+/0	++/0	++/0	+++/0	+++/0	+++/0	0/0	+/0	0/0
4	58	M	1	0	X	2	0/0	++++/0	+++/0	+++/0	++/0	++/0	++/0	++/0	+++/0	++/0	+/0	++/0
5	75	M	3	0	X	3	0/0	++++/0	++++/0	+++/0	+++/**	+++/**	0/**	++++/**	+/0	++/0	++/0	+/0
6	73	F	2	0	0	2	0/0	+++/0	0/0	0/0	0/0	++/0	0/**	++/****	0/0	0/0	++/*	0/**
7	35	M	4	0	1	3	0/0	++++/0	0/**	++/0	++/0	+++/0	++/***	0/**	0/0	+/0	+/0	+/**
8	56	F	3	0	0	2	0/0	++++/0	++/0	++/**	++/0	+++/0	0/**	++/****	++/0	++/0	0/0	0/0
9	52	F	1	0	0	2	0/0	+++/0	+++/0	++/0	++/0	++++/0	+++/**	+/***	0/0	0/0	0/*	0/0
10	72	M	3	x	0	3	+++/**	++++/0	+/0	++/0	++/0	+++/0	+++/**	0/****	+/*	++/0	++/0	0/0

Comparisons are indicated (statistical significance at least $p<0.05$, analysis with Fisher's exact test): [a]Between controls and protein expression in epithelial cells of RCC; [b]Between controls and mRNA expression in epithelial cells of RCC. MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; E, malignant epithelial cell staining; S, malignant stroma (fibroblasts, endothelium, macrophages, lymphocytes). Expression data based on *in situ* hybridisation (MMPs/TIMPs-mRNA) and immunohistochemistry (MMPs-antigens, TIMPs-antigens) are given in a semi-quantitative fashion ranging from 0, no signal; +/*, focal positive; ++/**, minimal diffuse positive; +++/***, moderate positive; ++++/****, strong positive. Expressions of MMPs/TIMPs-mRNA or MMPs/TIMPs-antigens in adjacent non-malignant renal tissue (negative controls) were not detectable or very low due to backround staining.

(Dako) according to the manufacturer's instructions. Fast Red (Sigma-Chemicals) was used as the chromogen, afterwards the slides were briefly counterstained with hematoxylin and aqueous mounted (Aquatex®, Merck, Germany). Two experienced pathologists determined the degree of expression independently.

Statistical analysis. Statistical calculations were performed by Scientific Package for Social Science, version 11.5 for Windows (SPSS, Chicago, IL, USA). The χ^2 test was used to reveal statistical relationships between RCC specimens and the adjacent non-malignant renal tissues, the degree of gene expression (low/high), T-stage (T_{1+2}/T_{3+4}), histopathological grade (G_{1+2}/G_3), and local recurrence or metastasis (yes/no). The Fisher's exact test was applied for statistical calculations between mRNA/protein expressions and controls. Differences were considered statistically significant at p-values <0.05. The small study group did not allow further statistical analysis.

Results

Staging and histopathological grading and analysis. The majority of our patients were diagnosed with renal cell carcinoma (RCC) confined to the kidney (T_{1+2}) (Table I). In three patients the RCC invaded the renal capsule (T_3). In one patient the carcinoma was found beyond Gerota's fascia and already metastasized (T_4 M_1). Histopathological grade 2 was predominant (n=7) and the subtyping revealed a clear cell type RCC in all patients. Our pathologists confirmed independently 10 adjacent non-cancerous renal tissue specimens microscopically by H&E staining and immunohistochemistry.

In situ hybridisation (*is*H) and immunohistochemistry were performed to identify cells expressing MMP1-, MMP2-, MMP3-, MMP9-, TIMP1-, and TIMP2-mRNA and -protein in RCC and in non-malignant adjacent sections. To reduce the variability between the paired samples the cancerous and non-cancerous samples (controls) were always analysed together. Control sense RNA probes verified the specificity of the signal. Additionally, *is*H was also performed in corresponding frozen tissue samples. Nevertheless, mRNA was sufficiently preserved in paraffin sections to allow RNA hybridisation. The expression pattern in paraffin was similar to the expression in frozen sections, but rewarded with a clearer and more informative morphology.

There was no mRNA or protein expression detectable at the adjacent non-cancerous sections by *is*H and immunohistochemistry. No significant differences between mRNA or protein expression and clinico-pathological parameters, e.g., gender, tumour stage, grade, and lymph node involvement were obtained of all tested MMPs and TIMPs, most likely due to the small samples size.

mRNA and protein expression of gelatinases MMP2 and MMP9. mRNA expressions of the gelatinases MMP2 (72 kDa) and MMP9 (92 kDa) were detected in RCC samples with similar epithelial expression patterns. In general MMP2-mRNA showed a minimal diffuse, moderate to strong positive expression pattern of malignant epithelial cells. Only one probe presented minimal focal positive staining (*) for stroma cells. These findings were similar to the protein expressions analysed by immunohistochemistry. All results are presented in Table I. According to the Fisher's exact test MMP2-mRNA (p=0.018) and -protein (p=0.0007) expressions in epithelial

cells of RCC tissue samples were significantly overexpressed compared to the control group.

MMP9-mRNA was strongly expressed in malignant stroma cells leading to a 'starry sky' aspect, generally diffuse and less pronounced in malignant epithelial cells. Again, this observation correlated with the protein expression: MMP9-protein was strongly expressed in single stroma cells of macrophage origin. Additionally, MMP9 was detected to a variable extent in malignant epithelial cells with a mostly cytoplasmatic expression pattern. MMP9-mRNA ($p=0.0031$) and -protein ($p=0.0108$) expressions in epithelial cells of RCC tissue samples were significantly overexpressed compared to the control group (Table I).

mRNA and protein expression of interstitial collagenase MMP1 and stromelysin MMP3. MMP1-mRNA was moderately expressed in malignant epithelial cells in only two cases and minimally diffuse in stroma cells in one sample. In contrast to the mRNA expression MMP1-protein showed strong positive staining results of malignant epithelial cells in all samples. Interestingly, a different expression pattern was observed for different cellular compartments by immunohistochemistry: some cells presented a strong nuclear staining pattern with a highly variable cytoplasmatic expression for MMP1, whereas in others the cytoplasmatic or even membranous staining pattern was dominant. There was a significant difference between MMP1-protein expression in RCC samples in comparison to the adjacent non-malignant renal tissue ($p<0.001$). According to the Fisher's exact test no significance was obtained between MMP1-mRNA expression and the control (Table I).

MMP3-mRNA was detected in almost all samples of RCC with mainly expression in malignant epithelial cells and minimal diffuse positive staining in stroma cells. There was a significant difference between MMP3-mRNA expression in RCC samples in comparison to the adjacent non-malignant renal tissue ($p<0.001$). The expression of MMP3-protein was moderate to strong in malignant epithelial cells and diffuse positive to moderate positive in stroma cells of only two cases. Again, the protein expression for MMP3 appeared both nuclear and cytoplasmatic. In comparison to the adjacent non-malignant renal tissue MMP3-protein expressions in RCC samples were statistically significant ($p<0.001$).

mRNA and protein expression of tissue inhibitors TIMP1 and TIMP2. TIMP1-mRNA was detected with different degree of expression (minimal to strong) in half of the malignant epithelial cell probes and with almost no expression at stroma cells. TIMP1-protein was detected predominantly in malignant epithelial cells, mainly with moderate staining (Table I). It reached statistical significance in comparison to the control group ($p=0.0108$).

Because of few and weak expressions no statistical significance was reached between mRNA and protein expression of TIMP2 and the normal control group (Table I).

Discussion

In this study we evaluated the expression of MMP1, MMP2, MMP3, MMP9, TIMP1, and TIMP2 by *is*H and immunohistochemistry in human RCC. All analysed MMPs and TIMPs showed unique expression patterns. This reflects their specificity among the family members to differentially regulate their expression. Despite the clinical significance on the pathogenetic impact of MMPs in human RCC only a limited number of studies are available in the literature, which mainly analysed the expression in cell lines or in whole tissue extracts (15). In addition to the known regulators such as onco-proteins and TIMPs it has been shown that the expression of MMPs is regulated by cytokines and growth factors. Such regulatory mechanisms have been investigated previously in RCC cell lines. It has been suggested that TGF-ß1 and ß-FGF influences MMP2 and its invasive activity (16,17). These experimental approaches so far were not able to differentiate between various cell types and did not determine the differences between epithelial versus stroma cells in RCC.

Our study determined the expression of gelatinases MMP2 and MMP9 in malignant epithelium and stroma using morphological techniques such as ^{35}S-UTP labelled antisense riboprobes and metalloproteinase antibodies. Expression of MMP2-mRNA in epithelial carcinoma cells analysed by *is*H has been previously reported in several tumour entities including gastric, cervical, and lung cancer (21-23). Those observations are concordant to our findings. Additionally, we also studied the expression at the protein level by immunohistochemistry. The expression of the MMP2-antigen was well pronounced in epithelial cells of RCC with some diffuse expression of stroma cells. A pronounced stroma expression of MMP2, as reported in hepatocellular carcinoma and pancreatic cancer (24,25) could not be demonstrated in our study. The expression of MMP9-mRNA was diffusely distributed at the epithelium of RCC with a strong expression at scattered individual stroma cells. In our observation stroma cells (e.g., macrophages, inflammatory cells) expressed very pronounced MMP9 at the protein level. Elevated MMP9-protein expression in malignant epithelial and stroma cells have also been found in various tumours such as lung cancer, prostate cancer, pancreatic cancer, and bladder cancer (14,25-27). Taken together, we found a significantly pronounced expression of the gelatinases MMP2 and MMP9 in RCC tissue compared to the normal controls, which underscores the invasive capacity of human RCC. It remains unclear, if the pronounced MMP9 expression of stroma is due to inflammation or paracrine tumour functions.

The interstitial collagenase MMP1 degrades components of the extracellular matrix (ECM), e.g., the fibrillary collagens type I, II, III, VI, IX, and proteoglycans. MMP1 expression has been found in oesophageal, gastric, colorectal, head, and neck cancers, partly with prognostic significance (13,28,29). We found MMP1 expressions in RCC to a variable extent in malignant epithelial cells with strong immunohistochemical staining. Interestingly, only two cases showed a marked mRNA expression. We cannot explain this discrepancy between mRNA versus protein expression, which has been also found by other groups (30). For malignant epithelial cells we observed different protein expression patterns: predominantly nuclear or cytoplasmatic and membranous staining. The significance of these patterns remains unclear. It is reported that MMP1 was found exclusively in tumour cells in head and neck cancer (13), whereas in colorectal carcinoma MMP1

IP3 - see below) was described in small cancer deeply invasive or in marginal portion of the tumours (29).

The enzyme stromelysin-1 (MMP3) has broad substrate specificity. It degrades proteoglycan, laminin, fibronectin, gelatins, and collagens of types III, IV, and V (1). Oesophageal, gastric, and pancreatic carcinoma (25,28,29) are known to express MMP3. In this study we found a high level of MMP3-mRNA expression in RCC, which also correlated well with our findings at the protein level. This was mainly localized in epithelial cells of RCC samples, but two samples showed also a moderate signal at stroma cells. Comparable to MMP1 we found two patterns of expression for MMP3, either cytoplasmatic-membranous or predominantly nuclear. MMP3 has been identified in the tumour cell compartment, while the corresponding mRNA expression has been found in stroma cells (31). These findings imply that in addition to the gelatinases MMP1, MMP3 is also involved in RCC progression.

Because of the inhibitory and regulatory function of TIMPs a general down-regulation of TIMPs in malignant tumours might be expected. Apparently, the opposite has been observed for a variety of malignancies, e.g., in lung (32) and breast cancer (33). Kallakury *et al* observed a coexpression of MMP2, MMP9 and TIMP1, TIMP2 in RCC tissue by immunohistochemistry, which correlated with high tumour grade (34). Kugler *et al* stated that the balance of MMP2- and MMP9- to TIMP1- and TIMP2-mRNA expression is a prognostic factor of tumour aggressiveness in RCC (15). We also found a variable degree of elevated TIMP1 and TIMP2 expression in RCC. TIMP2 was expressed predominantly in tumour cells and some diffuse expression was seen in stroma cells. Especially, small blood vessels were outlined by an elevated expression of TIMP1 in this study. This association of TIMP1 in small blood vessels suggests its role in controlling tumour angiogenesis. One could speculate that the expression of TIMP1 around blood vessels leads to inhibition of basement membrane dissolution and promotes capillary remodelling, which is necessary for angiogenesis. In contrast, we did not observe MMP2 or MMP9 expressions near blood vessels. Our overall results emphasize that TIMP1 expression is mainly localized in epithelial cells of RCC samples. High levels of TIMP1-mRNA were found in all areas where tissue remodelling was evident, particularly in cells at the tumour-stroma interface. Our findings endorse the hypothesis that not only stroma but also epithelial cells could be a major source of TIMPs in RCC tissues.

We could demonstrate a statistically significant difference between the mRNA and protein expression for MMP2, MMP3, and MMP9 in RCC samples and the normal controls. For future studies larger sample sizes are necessary. Due to our small sample size no correlation of MMPs and TIMPs expression with clinical and histopathological grading was possible as reported by others (15). To enhance the feasibility of larger sample sizes we suggest to focus on the evaluation of MMP2, MMP3, and MMP9 (excluding collagenase MMP1).

In conclusion, our findings underline the value of MMP1, MMP2, MMP3, MMP9, TIMP1, and TIMP2 for RCC tumour detection. To our knowledge this is the first report, which describes a significant antigen expression of MMP3 in RCC. Further studies are needed to clarify if the expression patterns are of prognostic or clinical relevance and to provide a better understanding on the pathogenesis of human RCC.

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