Plasma matrix metalloproteinase-7 as a metastatic marker and survival predictor in patients with renal cell carcinoma

Azizbek Ramankulov,[1,2] Michael Lein,[1,3] Manfred Johannsen,[1] Mark Schrader,[1] Kurt Miller[1] and Klaus Jung[1,3,4]

[1]Department of Urology, Charité University Hospital, Berlin, Germany; [2]Republic Center of Urology, Bishkek, Kyrgyz Republic; [3]Berlin Institute for Urologic Research, Berlin, Germany

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We evaluated the clinical usefulness of plasma matrix metalloproteinase-7 (MMP-7) as a diagnostic and prognostic biomarker in patients with renal cell carcinoma (RCC). MMP-7 was quantified in plasma of 50 healthy subjects and 97 RCC patients using a Fluorokine MultiAnalyte Profiling assay. RCC patients were stratified into the following groups: without metastases (N0M0; $n = 39$), with lymph nodes (N1M0; $n = 13$), and with distant metastases (M1; $n = 45$). Diagnostic performance of MMP-7 was analyzed by the receiver operating characteristics (ROC) curve. Kaplan–Meier analysis and the Cox regression model were used to estimate the impact of MMP-7 on the cancer-specific survival outcome of RCC patients. MMP-7 was significantly higher in both metastatic groups N1M0 and M1 (medians, 3.82 and 3.34 μg/L) compared to N0M0 group or controls (medians, 1.85 and 1.64 μg/L; all $P < 0.001$). In ROC analysis, the area under the ROC curve of MMP-7 was 0.80 in the detection of metastases in RCC ($P < 0.0001$). In the Kaplan–Meier analysis, patients with MMP-7 above the 95th percentile of controls showed less favorable survival rates compared to those with normal MMP-7 (log-rank test, 15.7; $P < 0.0001$). High MMP-7 was associated with cancer-related mortality estimated by univariate Cox regression (risk ratio, 4.34, 95% CI, 1.12–10.6; $P = 0.032$). The multivariate Cox regression model determined MMP-7 (risk ratio, 2.70, 95% CI, 1.39–5.24; $P = 0.003$) and metastases (risk ratio, 5.81, 95% CI, 2.77–12.2; $P < 0.0001$) as independent determinants of cancer-related survival outcomes. In conclusion, increased plasma MMP-7 could be related to metastatic disease and poor prognosis in patients with RCC. (*Cancer Sci* 2008; 99: 1188–1194)

RCC was estimated to have caused 51 190 new cases and 12 840 deaths in the USA in 2007.[1] Due to its asymptomatic clinical course, RCC is being detected incidentally in two-thirds of patients.[2] By the time of diagnosis about 25% of these patients will present with metastatic disease.[3] Frequent sites of metastasis include the lung, bone, liver, and brain.[4] Metastatic spread that often involves more than one organ system significantly impacts upon the survival of RCC patients.[3,5] Therefore, early diagnosis of RCC is a critical issue in the management of these patients. One of the strategies to improve this situation is the identification of biomarkers in plasma or serum samples whose level is sensitive to detect early tumor forms and to monitor for disease progression in RCC. Numerous tumor markers in RCC have been tested in the past, but there are no definitive biomarkers available for such purposes to date.[6] Search in free available mRNA data bases and immunohistochemistry data showed that, among different MMP, matrilysin (MMP-7) is overexpressed in RCC.[7–10]

MMP form a family of zinc-containing enzymes showing the common ability to degrade various components of the extracellular matrix. Both *in vitro* and *in vivo* investigations have shown that increased MMP are associated with the invasive and metastatic potential in several malignant tumors such as breast, colorectal, pancreatic, prostate, head/neck region carcinomas, and melanoma.[11–17] Unlike the most MMP, MMP-7 exhibits a wide spectrum of proteolytic activity against various elements of extracellular matrix.[18,19] It is of interest that MMP-7 is mainly localized in malignant cells and preferentially expressed at the invasive front of tumors.[10,20] Thus, MMP-7 may play a key role in tumor invasion. In support of this view, recent studies linked the overexpression of MMP-7 to the advanced tumor forms and unfavorable prognosis in various human tumors.[10,20–26]

Since these changes on the cellular levels may be reflected in body fluids, determinations of MMP in blood have been recommended as non-invasive tools in diagnostics and monitoring of diseases.[27] While increased circulating MMP-7-values in the blood were recently described in patients with colorectal and pancreatic cancer,[24,28] there has been only one report on RCC patients with limited clinical data that appeared after we had finished our study.[29] Taking into consideration the distinct implication of MMP-7 in tumor biology, we hypothesized that circulating MMP-7 could be of diagnostic and prognostic value for RCC. Therefore, the present study was aimed to answer to the following questions: (a) comparison of plasma MMP-7 in controls and different groups of RCC patients; (b) diagnostic performance of plasma MMP-7 in the detection of early or metastatic forms of RCC; (c) correlation of plasma MMP-7 to clinico-pathological parameters in RCC; and (d) association of plasma MMP-7 concentrations with the cancer-specific survival outcomes of RCC patients.

Materials and Methods

Study groups. This is a retrospective study that included 97 RCC patients who were investigated at the Department of Urology, Charité University Hospital, Berlin during 1998–2001 with the end of follow-up in 2007. Cancer stage and grade were assigned according to the Tumor Node Metastasis Classification of 1997 system.[30] Tumor grading was characterized as well differentiated (G1), moderately differentiated (G2), or poorly differentiated (G3). Metastases were diagnosed by bone scintigraphy, X-ray, computerized tomography, magnetic resonance imaging, and ultrasound diagnostics. Regional lymph node dissections with histological examinations were performed in certain cases for staging purposes. RCC patients were therefore subdivided into three groups: those without metastases (pN0M0, referred to as N0M0 group; $n = 39$), patients with surgically resected lymph node metastases (pN1, 2M0, referred to as N1M0 group; $n = 13$), and patients with distant metastases

[4]To whom correspondence should be addressed. E-mail: klaus.jung@charite.de
Abbreviations: AUC, area under the ROC curve; CI, confidence interval; MMP, matrix metalloproteinase; RCC, renal cell carcinoma; ROC, receiver operating characteristics; RR, relative risk.

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Table 1. Characteristics of the renal cell carcinoma groups

Variables	Patients with renal cell carcinoma (n = 97)		
	Stage N0M0	Stage N1, 2M0	Stage M1
No.	39	13	45
Female	14	2	16
Male	25	11	29
Age (years)†	60 (58–65)	62 (55–66)	59 (56–65)
Tumor stage			
T1	23	4	3
T2	5	2	12
T3	11	6	23
T4	–	1	3
Unavailable	–	–	4
Tumor grade			
G1	4	–	–
G2	29	7	20
G3	6	5	16
Unavailable	–	1	9
Histological subtypes			
Clear cell	30	8	31
Papillary	6	1	–
Chromophobe	2	1	–
Other	1	2	1
Unavailable	–	1	13

†Medians with interquartile ranges.
G, histopathological grading; N0M0, N1, 2M0, M1, lymph node and metastasis classification; T, tumor classification.

(M1 group, $n = 45$) as reported in Table 1. Patients without metastases were treated with curative intent with standard treatment consisting of radical nephrectomy. No adjuvant treatment was given to patients in groups N0M0 and N1M0. Patients with synchronous or metachronous distant metastases (M1 group) received systemic treatment consisting of interferon, interleukin-2, or both. The control group included 50 healthy individuals (22 females and 28 males). The subjects in the controls had no signs of infections, gastrointestinal, hepatic or renal disease, tumors, or immunologic disease. Participants in the latter group had values of basic laboratory parameters within the reference limit. The study was performed in accordance with the ethical standards of the Helsinki Declaration and was approved by the ethical board of the hospital.

Sample collection. Blood samples were collected into Monovette plastic tubes coated with lithium heparin (Sarstedt, Nümbrecht, Germany) and were centrifuged at 1600 *g* for 15 min at 4°C within 2 h of venipuncture. Separated plasma samples were archived at –80°C until analysis. Blood samples were collected before any treatment with some exceptions in the group of patients with distant metastases (M1 group). In 24 (53%) of 45 patients in M1 group blood samples were taken at the first visit of diagnosis of distant metastasis in untreated patients or one day before radical nephrectomy while in the remaining 21 (47%) patients, blood was collected between 1 and 76 months after surgery, but without further treatment at a control examination at the time of the diagnosis of metastases.

Quantification of plasma MMP-7. MMP-7 was quantified in plasma using Fluorokine MultiAnalyte Profiling assays (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. This assay measures, according to the information of the manufacturers, pro-, mature, and TIMP-1 complexed MMP-7 with less than 0.5% cross-reactivity to other MMP. Briefly, samples were diluted 10-fold with the respective diluents and incubated with microparticles coated with MMP-7-specific antibodies in wells of a microplate. During incubation, MMP-7 in samples or standards was captured by the immobilized antibodies. Then, the microplate underwent washing procedure to remove all unbound substances and biotinylated antibody specific to MMP-7 was added. The microplate underwent the second incubation and unbound biotinylated antibody was removed by washing. Streptavidin-phycoerythrin conjugate, which binds the biotinylated detection antibodies, was added. A final wash removed unbound conjugate and the microparticles were re-suspended in buffer. Phycoerythrin-derived signal, which is directly proportional to the amount of MMP-7, was read using the Luminex 100 Bioanalyzer (Luminex, Austin TX, USA), a dual laser sorting and measuring apparatus. One laser determines which analyte is detected and the other measures the phycoerythrin-derived signal. Application of microparticles coated with MMP-7-specific antibody allowed us to perform simultaneous quantification of different analytes of interest. The concentrations were then calculated using a cubic spline calibration curve. All measurements were run in duplicates in a blinded fashion. The assay performance was characterized by the coefficient of variation of 5.8% calculated from duplicate values.

Statistical analysis and sample size calculation. We used the Mann–Whitney *U*-test and Kruskal–Wallis test to compare MMP-7-values between the study groups using the software GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). The software MedCalc 9.3.9.0 (MedCalc, Mariakerke, Belgium) was employed to estimate the upper limit of the reference interval according to the non-parametric percentile approach,[31] and the diagnostic performance of MMP-7 using ROC analysis. Spearman correlation coefficients (r_s), Kaplan–Meier analyses, and univariate and multivariate Cox regression analyses were calculated using SPSS software (SPSS 15.0, Munich, Germany). All tests were two-sided and a $P < 0.05$ was considered statistically significant.

Sample size determinations and power calculations were performed using the software GraphPad Statmate version 2.0 for Windows (GraphPad Software) and MedCalc on the basis of a two-sided alpha error of 5% and a power of 80%. Calculations for comparing differences between groups were performed on the basis of serum MMP-7-values reported by Maurel *et al*.[24] Those authors found concentrations of 4.2 ± 2.2 μg/L in healthy controls. They used another ELISA method as we did, but MMP-7 elevations were found in colorectal patients within the level of 1 SD. Since MMP determinations are generally assay-dependent, we made sample size determinations based on changes of 1 SD. On the basis of this calculation, with controls matched to patients at a ratio of 1:1 or of 1:2, analyte changes of 1 SD could be detected by studying either 32 (16 controls and 16 patients) or 36 total subjects (24 controls and 12 patients). In addition, assuming that the clinical validity of a test or parameter is given with an area under the ROC curve of at least 0.75 in comparison to the null hypothesis value 0.5, a sample size of 41 in each group was calculated under the above-mentioned Type I and II error conditions. Sample size determinations for comparing survival curves as measures for the clinical endpoint 'cancer-specific mortality' showed that a total of 50 patients have to be included into a study to obtain a survival difference of about 0.25 between two survival curves when the survival rate of the controls was between 0.3 and 0.7. Instead of using 25 subjects in each group and without losing statistical power, the number of subjects in one group could be reduced to 16, but an increased number of patients in the second group would be necessary ($n = 64$). Although that already represents an unfavorable situation, we decided to increase the number of patients to 90–100 patients in order to realize a study with a power not less than 80%, because the differentiation between the groups (for example high and low MMP-7) could not be predicted.

Results

Characteristics of the control group. The control group consisted of 50 subjects (22 female, 28 male) with the median age of 52 years (interquartile range, 41–62). There was no age difference between women and men (median age of 54 *vs* 50 years; $P = 0.969$). In addition, MMP-7 showed no difference between women and men in the control group (median values of 1.67 *vs* 1.52 μg/L; $P = 0.282$). Since age- and gender-dependent values of MMP-7 in this study were most probably excluded, we combined the control subjects to one group. The median value of MMP-7 of this combined group was 1.64 μg/L. Thus, the 95th percentile with 3.35 μg/L was used as the conventional upper limit of the reference interval calculated by the non-parametrical percentile method. As mentioned, blood samples in the M1 group were taken from 25 patients before any treatment and from 21 patients between 1 and 76 months after radical nephrectomy when metastases were diagnosed at control examinations. Median MMP-7 concentrations were not different between both groups (3.06 μg/L *vs* 4.44 μg/L; $P = 0.991$, Mann–Whitney *U*-test). Therefore, we combined these two collectives with distant metastases into one group.

Concentrations of MMP-7 in study groups. Figure 1 shows plasma concentrations of MMP-7 in controls and in patients with RCC subdivided into the groups without metastases (N0M0), with lymph nodes (N1M0), or distant metastases (M1). According to the statistical assessment, the results were as follows: (a) MMP-7-values in controls did not differ from that of N0M0 group of RCC patients ($P = 0.197$; Mann–Whitney *U*-test); (b) both metastatic groups N1M0 and M1 had significantly elevated MMP-7 compared to that of N0M0 group or controls (all $P < 0.001$, Kruskal–Wallis with Dunn's post test); and (c) there was no difference in MMP-7 concentrations between metastatic groups N1M0 and M1 ($P = 0.563$; Mann–Whitney *U*-test).

Diagnostic performance of MMP-7. Since the scatter plot revealed an elevation of plasma MMP-7 in both metastatic groups, we consequently considered its possible application as a metastatic marker. For that purpose we employed ROC analysis to estimate whether plasma MMP-7 can be used to differentiate between patients with metastases and those with local disease (Fig. 2). MMP-7 achieved an AUC of 0.80 (95% CI, 0.71–0.88) and this was significant compared to the reference AUC of 0.50 ($P < 0.0001$). The point with the highest diagnostic accuracy determined by ROC analysis was at the MMP-7 concentration of 2.47 μg/L with the sensitivity and specificity of 75.9% (95% CI, 62.8–86.1) and 71.8% (95% CI, 55.1–85.0), respectively. For the cut-off of 3.35 μg/L, established as the 95th percentile of the controls, a higher specificity of 82.1% (95% CI, 66.5–92.4), but lower sensitivity of 53.4% (95% CI, 39.9–66.7), resulted.

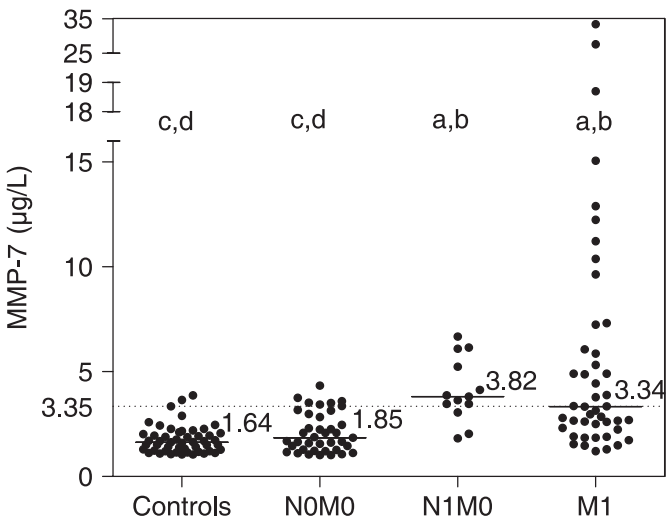

Fig. 1. Levels of matrix metalloproteinase (MMP)-7 in controls and different renal cell carcinoma (RCC) groups. Medians are indicated as short horizontal lines with respective figures; dotted horizontal line represents the cut-off point of 3.35 μg/L calculated as the 95th percentile of the control group. Significant differences between the study groups were estimated by the Kruskal–Wallis test with Dunn's post test indicated by the following symbols: (a) compared to controls; (b) compared to N0M0 group; (c) compared to N1M0 group; and (d) compared to M1 group ($P < 0.001$).

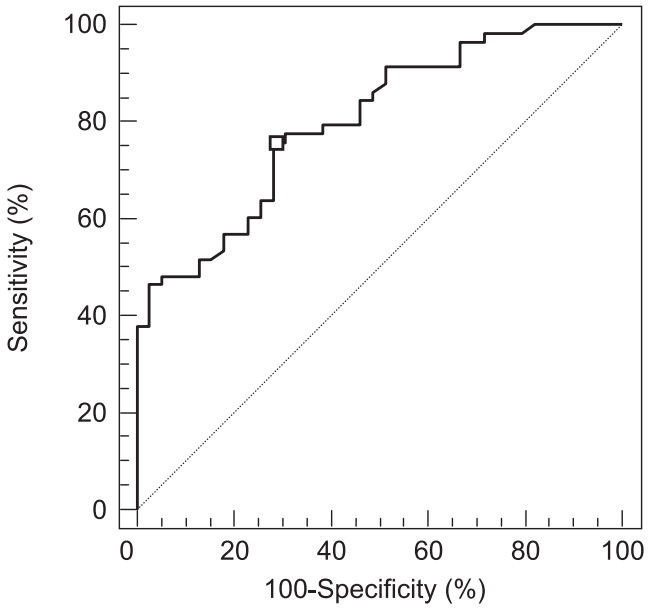

Fig. 2. Receiver operating characteristics (ROC) analysis of matrix metalloproteinase (MMP)-7 in the detection of metastatic renal cell carcinoma (RCC) patients. ROC analysis included RCC patients without metastases (N0M0, $n = 39$) as a negative group, and metastatic RCC patients (N1M0 and M1, $n = 58$) as a positive group.

MMP-7 and clinico-pathologic parameters. We examined plasma MMP-7 in all 97 RCC patients with respect to clinico-pathological variables as given in Table 1. MMP-7 correlated more closely with the stage ($r_s = 0.35$, $P < 0.001$) than with the grade ($r_s = 0.19$, $P < 0.05$). The medians of MMP-7 for clear cell, papillary, and chromophobe subtypes were 2.32, 3.44, and 2.84 μg/L, respectively, and this was not statistically different ($P = 0.507$, Kruskal–Wallis overall test). Further analysis was focused on the number and character of organs affected by metastases in M1 group of RCC (Table 2). MMP-7 showed no difference in patients with one, two or three, and more organs affected by metastases. MMP-7 levels were examined in patients grouped as osseous *versus* non-osseous metastases, liver *versus* other than liver metastases, or lung *versus* other than lung metastases, as reported in Table 2. Plasma MMP-7 showed no difference between these groups. Thus, plasma MMP-7 does not appear to correlate with clinico-pathologic parameters such as the histological subtype, metastatic burden, or site of metastases in RCC patients.

Prognostic performance of MMP-7. Each patient had complete follow-up data which was collected from the hospital records and provided by general practitioners. Therefore all 97 RCC patients were considered as eligible for the cancer-specific survival analysis. Median follow-up period for all RCC patients was 61.2 months (interquartile range, 16.4–87.7). Median follow-up in 44 (45%) patients who died of RCC was 13.3 months (3.4–34.8) whereas the same estimation for the remaining 53 (55%) patients who survived during the follow-up was 84.4 (65.1–98.8). We examined the prognostic significance

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Table 2. Plasma matrix metalloproteinase (MMP)-7 and clinico-pathologic parameters in renal cell carcinoma patients with distant metastases (M1 group)

		MMP-7 (μg/L)	*P*-value
No. of affected organs (*n*)			
	One (19)	3.36 (2.89–11.3)	
	Two (16)	2.78 (2.38–7.28)	
	> Three (10)	2.49 (1.88–5.60)	0.354†
Type of effected organs (*n*)			
(a)	Osseous (18)	3.00 (2.28–6.60)	
	Non-osseous (27)	3.35 (1.91–7.25)	0.728‡
(b)	Hepatic (12)	2.69 (1.86–3.89)	
	Non-hepatic (33)	3.57 (2.62–7.28)	0.248‡
(c)	Pulmonal (33)	2.89 (2.08–6.60)	
	Non-pulmonal (12)	3.35 (2.86–7.25)	0.531‡

Values are medians with interquartile range in parentheses.
†Calculated with the Kruskal–Wallis overall test. ‡Calculated with the Mann–Whitney *U*-test.

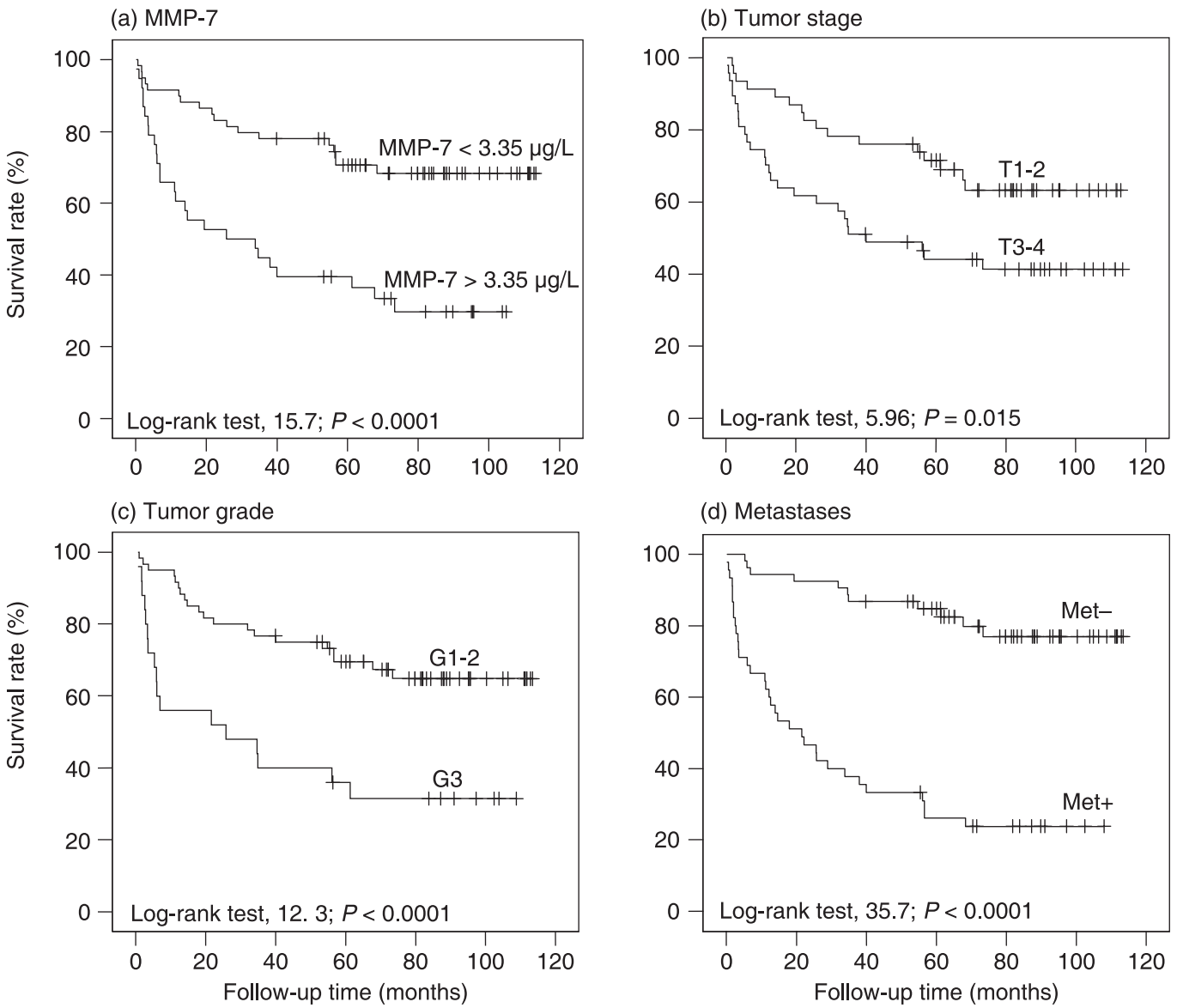

Fig. 3. Kaplan–Meier analyses of matrix metalloproteinase (MMP)-7 and clinico-pathological parameters. Kaplan–Meier analyses included all 97 renal cell carcinoma (RCC) patients stratified into the two groups as shown on the curves.

of MMP-7 and clinico-pathologic parameters such as metastases, tumor stage, and grade. For that purpose we dichotomized all RCC patients in relation to the cut-off point of MMP-7 (normal *vs* high), metastases (absence *vs* presence), tumor stage (T1-2 *vs* T3-4), and grade (G1-2 *vs* G3). Kaplan–Meier analysis revealed that patients with high plasma MMP-7 had a markedly shorter survival time compared to those with normal MMP-7-values (Fig. 3a). The median survival of patients with high MMP-7 was

Table 3. Cox regression analysis of matrix metalloproteinase (MMP)-7 and clinico-pathologic parameters in renal cell carcinoma patients in relation to cancer-specific death

Variable	Stratification	Univariate		Multivariate Inclusion selection		Stepwise selection†	
		RR of death (95% CI)‡	*P*-values	RR of death (95% CI)‡	*P*-values	RR of death (95% CI)‡	*P*-values
MMP-7	3.35 μg/L	3.43 (1.12–10.6)	0.032	4.09 (1.94–8.64)	<0.0001	2.70 (1.34–5.24)	0.003
Tumor stage	T1-2/T3-4	2.13 (1.14–3.95)	0.017	1.00 (0.47–2.15)	0.995	–	–
Tumor grade	G1-2/G3	3.02 (1.58–5.78)	0.001	3.04 (1.47–6.29)	0.003	–	–
Metastases	No/yes	6.28 (3.16–12.5)	<0.0001	5.35 (2.36–12.1)	<0.0001	5.81 (2.77–12.2)	<0.0001

All 97 RCC patients dichotomized in relation to the indicated stratification criteria were included in the Cox regression analysis.
†All four variables were included when the stepwise selection procedure was used. Both forward and backward stepwise elimination procedures gave the same result.
‡RR = relative risk with 95% confidence interval in parentheses.
CI, confidence interval; G, histopathological grading; RR, relative risk; T, tumor classification.

25.7 months (Fig. 3a). In addition, median MMP-7-value was lower in patients who survived (2.25 μg/L, $n = 53$) compared to those who died (3.70 μg/L, $n = 44$) during the follow-up ($P < 0.0001$, Mann–Whitney test). Survival curves of RCC patients classified according to the presence and absence of metastases, and high and low tumor stage or grade, complied with well-known results (Fig. 3b–d). Prognostic significance of MMP-7 along with the selected clinico-pathologic parameters was also confirmed in the univariate model of Cox regression (Table 3). Thus, we included MMP-7 and the significant clinico-pathologic parameters into the multivariate model of Cox regression analysis in order to identify independent prognostic factors (Table 3). In the multivariate analysis, MMP-7 was shown a significant independent factor that also remained in the model using the forward elimination procedure of Cox regression analysis whereas tumor grade and stage were eliminated from the model as less significant.

Discussion

Our study showed that MMP-7 in plasma is significantly elevated in RCC patients with metastases compared to those with local disease or controls. This increased circulating MMP-7 also corresponds to its increased expression detected in advanced RCC.[9,10] Similar findings have been described for colorectal and pancreatic cancer.[24,28] This agreement between the circulating and tissue levels of MMP-7 is possibly due to its predominant expression in tumor cells compared to tissue stroma. MMP-7 is preferentially localized at the invasive front of tumors suggesting that it may facilitate destruction of surrounding extracellular matrix.[10,20,32] In fact, MMP-7 is capable of degrading the main elements of the basement membrane.[18,19,33] The latter, by separating the epithelium from the stroma, functions as a barrier for carcinomas *in situ*. Consequently, destruction of this vital component in extracellular matrix allows tumor cells to access lymph/blood vasculature and invade locally and distantly.[11,34] MMP-7 may enhance the destructive potential by activating other important MMP such as MMP-2 and MMP-9.[35,36] In addition, MMP-7 is known to regulate the function of other tumor-related cytokines such as insulin-like growth factors and osteopontin.[37,38] It is of interest that enzymatic cleavage of osteopontin caused by MMP-7 and MMP-2 generates active fragments.[38] Consequently, it potentiates adhesive and proliferative properties of osteopontin towards malignant cells.[39] In tissue specimens, increased MMP-7 was similarly shown to be associated with the poor survival of patients with esophageal, pancreatic, breast, and renal tumors.[10,21,22,26] A coincident expression of MMP-7 and CD34, an endothelial progenitor marker, was found in RCC tumors.[10] A possible role of MMP-7 in tumor-induced neovascularization can be postulated.

The increased plasma MMP-7 concentration in advanced forms of RCC suggests that it can be applied as an analyte to detect metastatic lesions (Fig. 1). The MMP-7 assay used in this study measures pro-, mature, and TIMP1-complexed MMP-7. However, it was recently demonstrated that only proMMP-7 occurs in blood,[29] and that the MMP-7 concentrations in serum and heparin plasma are roughly comparable.[40] Thus, in contrast to other MMP, such as MMP-1, MMP-8, or MMP-9, not only plasma but also serum seems to be suitable as sample for accurate measurements of circulating MMP-7. The diagnostic accuracy of plasma MMP-7 corresponded to AUC of 0.80 and was confirmed as significant by the ROC analysis (Fig. 2). It is important that increased values were not only found in patients with distant metastases but also already in patients with lymph node metastases. Elevation of plasma MMP-7 could be helpful for selecting RCC patients prior to performing standard diagnostics to search for metastases. This selective attitude would possibly lead to more cost-effective and rational exploitation of diagnostic resources especially during follow-up. If validated as a metastatic marker in the extended studies plasma MMP-7 assay could be established as a non-invasive and routine method. To some extent our results correspond to the report of Maurel *et al*.[24] These authors also found that high circulating MMP-7 concentrations correlated with the advanced tumor forms but not with the metastatic burden in colorectal cancer.

In addition to this diagnostic potential of plasma MMP-7, our study also demonstrated a significance of MMP-7 with regard to predicting the survival outcome of RCC patients. According to the univariate analysis of Cox regression, elevated MMP-7 in plasma was associated with the 4.34-fold relative risk of cancer-related mortality (Table 3). Similar findings were documented in colorectal and pancreatic cancer.[24,28] Furthermore, our multivariate Cox regression models have identified plasma MMP-7 as independent prognostic factors in addition to well-known clinico-pathologic factors such as tumor stage and grade. In the multivariate Cox regression analyses with a stepwise elimination procedure, MMP-7 remained, along with the presence of metastases, in the established model as strong determinant of survival probability in RCC, whereas tumor stage and grade were eliminated from the model as less significant (Table 3). Thus, plasma MMP-7 is an independent factor related to survival outcomes.

We planned and performed our study and summarized the results while taking into account the 'Reporting Recommendations for Tumor Marker Prognostic Studies'[41] and additional suggestions given by Kyzas *et al*.[42–44] We widely followed these guidelines to provide sufficiently relevant information in order to enable readers to make their own judgement regarding the validity of the data and conclusions. However, some limitations of this study should be recognized and discussed. First, the small number of patients in the N1M0 study group is a major

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drawback. But despite the limited number of subjects in this group, the MMP-7 concentration was different in comparison with the N0M0 group and showed that our prior sample size determinations made correct assumptions. Thus, the risk of type II error as the problem with small studies does not exist in our study between patients without metastases and with metastases, regardless of lymph node and distant metastasis. In addition, the probability of a type I error could be excluded as far as possible because of the high significance level (Fig. 1, $P < 0.001$). However, retrospective power calculations suggested that about 200 metastatic RCC patients should to be included, taking into account the same proportion of N1M0 and M1 patients as in the present study to reach a significance of $P < 0.05$. Second, the present study is limited by its retrospective nature with blood sampling having occurred at different times. However, all measurements were performed in a blinded manner and additional analysis determined no difference in plasma MMP-7 concentration in relation to different timing of blood collections in patients with distant metastases. This allowed us to include all patients with distant metastases into one group for statistical assessment.

In conclusion, despite the above-mentioned limitations of this study, our results show that plasma MMP-7 should be considered as a sensitive analyte in the detection of metastases and as predictor of survival outcome in RCC patients. When we were revising this manuscript, a similar article on MMP-7 in RCC patients was published online.[29] The authors found increased serum MMP-7 concentrations already in patients with localized RCC using an own sandwich fluoroimmunoassay, but they did not carry out follow-up studies. Thus, further investigations taking into account potential analytical differences of the assays are urgently required to estimate the usefulness of plasma MMP-7 both in the diagnosis of RCC and in monitoring disease progression, as well as in assessing the response to new treatment options in RCC patients.

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