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ORIGINAL ARTICLE

Cancer-Specific Survival Stratification Derived from Tumor Expression of Tissue Inhibitor of Metalloproteinase-2 in Non-Metastatic Renal Cell Carcinoma

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Abstract Degradation of the extracellular matrix is a prerequisite for the processes of cancer cell invasion and metastasis. The purpose of our study was to assess the association of matrix metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-9) and their inhibitors (TIMP-1 and TIMP-2) with renal cell carcinoma (RCC) progression and cancer-specific survival (CSS), using immunohistochemical analysis of 60 formalin-fixed, paraffin-embedded sections of tumor tissue and normal tissue near the tumor from surgical T1-3bN0 M0 RCC specimens. Significant overexpression of MMP-2 in tumor and normal tissue was correlated with advanced stages, tumor size, sarcomatous differentiation and clinical symptoms. Overall survival was 31.7% (55.2% M0, 9.7% M1) and CSS 56.7% (100% M0, 16.1% M1) with a follow-up of 76 (5–230) months. Fuhrman grade [HR 2.87 (95% CI: 1.28–6.45); $p = 0.01$], tumor size [HR 1.13 (95% CI: 1.03–1.26); $p = 0.009$] and low TIMP-2 expression [HR 0.35 (95% CI: 0.16–0.78); $p = 0.01$] were independent predictive factors of CSS and stratified the patients into three groups with different rates of 10-year CSS; [100%, 73.9% and 20.5% for the good, intermediate and poor prognosis group respectively ($p = 0.000006$)] . This study offers strong evidence that TIMP-2 expression in tumor tissue may play a crucial role in progression and poor prognosis in human localized and locally advanced RCC.

Keywords Matrix metalloproteinases · Tissue inhibitors of metalloproteinases · Renal cell carcinoma, Metastasis, Survival

Introduction

Renal cell carcinoma (RCC) is the ninth most common cancer; accounting for 3% of all malignant tumors [8]. In cN0 M0 RCC, progression rates at 5 years following surgical resection are between 10 and 30%. Advanced disease stage, high tumor grade, large tumor size and the presence of tumor necrosis have been identified as the most powerful prognostic indicators of the development of metastases. Nevertheless, as these factors together still only account for 80–85% of the

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probability of developing metastatic disease [23], a better understanding of the underlying mechanisms may help improve individualized prognosis and risk-stratified clinical decision making. Specifically, there is a need to identify patients who may benefit from adjuvant treatment after surgical therapy and predict response to systemic therapies that are effective but toxic [18].

Metastasis of RCC cells depends on a range of factors including proteolysis, cell adhesion, angiogenesis, and migration, colonization and proliferation in distant organs [15]. Matrix metalloproteinases (MMPs) are responsible for degradation of the extracellular matrix (ECM), which is one of the earliest and most important steps in the process of cell migration and metastasis [11]. MMPs belong to a family of human zinc-dependent endopeptidases that are able to degrade components of the ECM. Their expression and activity is regulated at the genomic level by the H-ras oncogene mutation, which stimulates the MMP promoter via activator protein-1 (AP-1 complex). Specifically, this has been described for MMP-1, MMP-3 and MMP-9. On the other hand, most MMPs are produced as inactive zymogens (proMMPs) and are proteolytically activated extracellularly. In addition, the activity of MMPs, in the extracellular milieu is regulated by a group of endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). Four members of this family have been described to date. Although these inhibitors exhibit very similar inhibitory activities against most members of the MMP family, they differ in many respects, including their interactions with proMMPs, solubility, expression regulation, and tissue specificity [1]. As such, the balance between the levels of activated MMPs and free TIMPs regulates the ECM turnover, and the process of cell invasion [7, 12, 17].

The expression and involvement of several MMPs and TIMPs in human RCC have been investigated in several studies; however, these studies have provided somewhat conflicting results concerning clinical and pathological prognostic factors in RCC patients. The aims of the current study were therefore to evaluate the immunohistochemical expression of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 proteins in RCC, and to determine whether measuring the expression of these markers can improve our ability to predict the risk of metastasis and cancer-specific survival (CSS) in RCC.

Materials and Methods

Patient Samples

We conducted a retrospective study including 60 patients with sporadic RCC who underwent radical or partial nephrectomy between 1991 and 2000 in Clínic Hospital. The main features of the series are summarized in Table 1. The exclusion criteria were metastasis at the time of nephrectomy, pathological stage > pT3b, positive surgical margins, clinical follow-up <60 months in the case of M0 patients, and another malignancy within 5 years before or after the RCC diagnosis, except basal-cell carcinoma. All patients who developed M1 disease ($n = 30$) and a simple random sample of those with M0 disease (n = 30) were included; at the time of analysis, one patient from the M0 group had developed metastasis, and therefore, at this stage, RCC patients were divided into two groups for statistical analysis: one without metastases (cN0M0, $n = 29$) and one with distant metastases (cNxM1 = 31).

Table 1 Clinical/pathological features at the time of nephrectomy

Variables	N0 M0 ($n = 60$)
Age (range)	62 (35–87)
Sex	
Male (%)	42 (70)
Female (%)	18 (30)
Asymptomatic (%)	31 (51.7)
pT	
T1 (%)	19 (31.7)
T2 (%)	15 (25)
T3 (%)	26 (43.3)
Cell type	
Clear cell (%)	48 (80)
Papillary (%)	8 (13.3)
Chromophobe (%)	1 (1.7)
Unclassifiable (%)	3 (5)
Fuhrman grade	
I-II (%)	35 (58.3)
III-IV (%)	25 (41.7)
Size in cm (range)	8.3 (1–25)
Necrosis >20% (%)	19 (31.7)
Sarcomatous differentiation (%)	8 (13.3)

To identify metastases, all patients underwent a chest and abdominal computed tomography scan every 3 months until 3 years of follow-up, and then every year thereafter. Bone scintigraphy and computed tomography of the brain were performed as necessary.

Paired formalin-fixed, paraffin-embedded samples of renal tumor and normal tissue near the tumor were obtained from surgical specimens. All of the hematoxylin-eosin (H&E)-stained slides from each case were reviewed and tumors were classified histologically according to the WHO classification [3]. Slides with extensive necrosis or hemorrhage were set-aside for further evaluation. Tumors were staged according to the 2009 TNM classification [4] and graded according to Fuhrman's system [5]. For statistical analysis, Fuhrman grades were divided into low (I and II) and high (III-IV) grades [16].

Follow-up data were obtained by reviewing patients' medical records.

This study was approved by the Medical Ethics Committee of the Clínic Foundation, and written informed consent was obtained from each patient prior to surgery and inclusion in the study.

Immunohistochemistry

To analyze the expression of MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 proteins, 5-μm sections were cut from a representative block of formalin-fixed, paraffin-embedded tissue. After deparaffinization, primary antibody incubation was performed using an automated system (TechMate 500® Plus and EnVision® DAKO; Carpinteria, CA). Further details of the antibodies and staining procedure are provided in Table 2. The rest of the staining procedure involved incubation with a biotinylated anti-mouse secondary DAB substrate and hematoxylin counterstaining. Positive- and negative-control slides were processed in parallel, negative-control slides being incubated with isotype-matched immunoglobulin with each batch of staining to confirm the specificity of the antibodies. All slides were processed simultaneously to minimize inter-batch variation.

P Pot; *EDTA* Ethylenediaminetetraacetic acid; *PEP* Pepsin; *C* Citrate; *MW* Microwave.

Staining Interpretation

Immunostaining was interpreted blindly by consensus between two observers. Both staining intensity and the approximate percentage of positive tumor cells were considered in the semi-quantitative assessment for all markers, as described previously [9]. Briefly, the distribution of positive staining in the tumors was described as focal (≤10%), regional (≤11–50%), or diffuse (≥50%). Moderate diffuse, intense regional and intense diffuse staining patterns were considered to indicate high expression of the corresponding protein.

Statistical Analysis

Statistical calculations were performed using the IBM SPSS Statistics for Windows, Version 20.0 (IBM, Armonk, NY). Associations between clinical and pathological variables in M0 and M1 groups were investigated using the χ [23] test or Student's *t*-test. The relationships between immunohistochemical expression in normal vs tumor tissue and inter-observer variability were assessed using the concordance index. Survival curves for all univariate analyses were plotted with the Kaplan-Meier method and compared with the log-rank test. Cancer-specific survival was defined as the interval between surgery and RCC-related death. Variables that reached statistical significance in the univariate analysis were subsequently entered into a multivariate analysis using a Cox proportional hazards model. The crude and adjusted effects on survival of immunohistochemical staining intensity and other risk factors were estimated by cox regression analysis. *P* values <0.05 were considered to be statistically significant.

Results

The median (range) follow-up was 76 (5–230) months and progression-free survival (PFS) was 65.2 (3–230) months. A total of 51.7% of patients developed metastasis (M1, $n = 31$), with a PFS after RCC surgery of 12 months (95% CI: 3.7–25.1) and 48.3% did not progress (M0, $n = 29$). OS was 31.7% (55.2% M0 and 9.7% M1) and CSS was 56.7% (100% M0; 16.1% M1).

Immunohistochemistry

The immunostaining pattern was cytoplasmic for all proteins, with mild, moderate or intense staining, although the distribution

Table 2 Antibodies and immunohistochemical procedure

Antibody	Type Ig	Clone	Antibody dilution	Primary antibody incubation	Antigen retrieval	Positive control
MMP-1	IgG1, kappa	3B6	1:5	60 min at 25 °C	P/EDTA	Colon carcinoma
MMP-2	IgG1, kappa	4D3	1:50	30 min at 40 °C	PEP	Colon carcinoma
MMP-3	IgG1, kappa	1B4	1:20	60 min at 25 °C	P/EDTA	Colon carcinoma
MMP-9	IgG1, kappa	2C3	1:40	Overnight at 4 °C	P/C	Macrophages
TIMP-1	IgG1, kappa	2A5	1:20	60 min at 25 °C	P/EDTA	Macrophages
TIMP-2	IgG2a	3A4	1:10	Overnight at 25 °C	MW	Colon carcinoma

was always diffuse, with more than half of cells being positive (Fig. 1). No significant inter-observer variability was noted (c-index 0.96; $p = 0.001$). MMP-2, −3 and −9 were significantly overexpressed in tumor tissue compared to normal kidney tissue ($p = 0.02$), and TIMP-2 expression was lower in tumor than normal tissue in M1 patients (41.9% vs. 83.9%; $p = 0.01$), but not in M0 patients (82.8% vs. 79.3%; $p > 0.05$). No differences were found in MMP-1 or TIMP-1 expression between tumor and normal kidney tissue.

Clinical and pathological characteristics

MMP-2 overexpression in tumor tissue was correlated with several clinical and histological features associated with a poor prognosis: clinical symptoms ($p = 0.045$), advanced T category ($p = 0.011$), tumor size ($p = 0.003$) and sarcomatous differentiation ($p = 0.005$). Low TIMP-2 expression was correlated with tumor necrosis >20% ($p = 0.034$). None of these factors was correlated with Fuhrman grade or histological type. (Table 3).

Tumor progression and survival analysis

In the whole series, the median OS was 74 months (95% CI: 53.5–94.5) [116 months in the M0 group (95% CI: 84.6–147.3) vs. 27 months in the M1 group (95% CI: 19.8–34.2); $p = 0.000002$]; and the median CSS was not reached [not reached in the M0 group vs. 27 months in the M1 group (95% CI: 19.8–34.2); $p < 0.0000001$].

The univariate analysis showed that 5- and 10-year PFS rates were correlated with advanced stage (67.6% and 56.7% for pT1–2 vs. 38.5% in both cases for pT3; $p = 0.047$), Fuhrman grade (65.7% and 58.6% for I-II vs. 40% and 36% for III-IV; $p = 0.029$), tumor size (76.7% and 69% for tumors <8 cm and 33.3% and 29.6% for tumors ≥8 cm, $p = 0.005$), elevated expression of MMP-2 in tumor tissue (67.4% and 59.1% for low MMP-2 expression vs. 23.5% and 23.5% for MMP-2 overexpression, $p = 0.01$) and low expression of TIMP-2 in tumor tissue (39.1% and 24.5% for low TIMP-2 expression and 64.9% in both cases for TIMP-2 overexpression, $p = 0.001$). These same factors were related to 5- and 10-year CSS rates in the univariate analysis: advanced stage (73.5%–69.2% for pT1–2 vs. 50%–37.3% for pT3, $p = 0.011$), high tumor grade (80%–66.2% for Fuhrman I-II vs 40% in both cases for Fuhrman III-IV, $p = 0.008$), tumor size (80% in both cases for tumors <8 cm vs. 46.7% and 28% for tumors ≥8 cm, $p = 0.0003$), MMP-2 overexpression (69.8% and 66.3% for low MMP-2 expression vs. 47.1% and 29.4% for MMP-2 overexpression, $p = 0.016$), and low expression of TIMP-2 (47.8% and 37.2% for low TIMP-2 expression, vs. 73% and 67% for TIMP-2 overexpression, $p = 0.023$).

The univariate analysis did not show significant correlations of 5- and 10-year PFS with MMP-1 (50% and 50% for low MMP-1 expression and 39.5% in both cases for MMP-1 overexpression, $p = 0.941$), MMP-3 (28.6% and 28.6% for low MMP-3 expression and 58.5% and 52.3% for MMP-3 overexpression, $p = 0.121$), MMP-9 (53.7% and 47.3% for low MMP-9 expression and 66.7% and 66.7% for MMP-9 overexpression, $p = 0.389$) or TIMP-1 (51.9% and 47.3% for low expression TIMP-1 and 62.5% and 62.5% for TIMP-1 overexpression, $p = 0.406$). Figs. 2 to 7 show time-to-event analysis (Kaplan-Meier curves and log-rank tests) of progression stratified by immunohistochemical results.

There were no significant differences in 5- and 10-year CSS as a function of the following immunohistochemical factors: MMP-1 (62.5% and 31.3% for low MMP-1 expression vs 63.5% and 56.9% for MMP-1 overexpression, $p = 0.772$), MMP-3 (28.6% and 28.6% for low MMP-3 expression vs 67.9% and 58.9% for MMP-3 overexpression, $p = 0.075$), MMP-9 (63% and 53.8% for low MMP-9 expression vs 66.7% and 66.7% for MMP-9 overexpression, $p = 0.548$), and TIMP-1 (61.5 and 54.5% for low TIMP-1 expression vs 75% and 60% for TIMP-1 overexpression, $p = 0.575$). In the multivariate analysis, the factors independently predictive of tumor progression were: Fuhrman grade [HR 2.14 (95% CI: 1.02–4.48); $p = 0.043$], tumor size [HR 1.1 (95% CI: 1.01–1.21); $p = 0.028$] and TIMP-2 expression [HR 0.29 (95% CI: 0.14–0.62); $p = 0.001$] (Table 4); and the factors independently predictive of CSS were Fuhrman grade [HR 2.87 (95% CI: 1.28–6.45); $p = 0.01$], tumor size [HR 1.13 (95% CI: 1.03–1.26); $p = 0.009$] and TIMP-2 expression [HR 0.35 (95% CI: 0.16–0.78); $p = 0.01$] (Table 5).

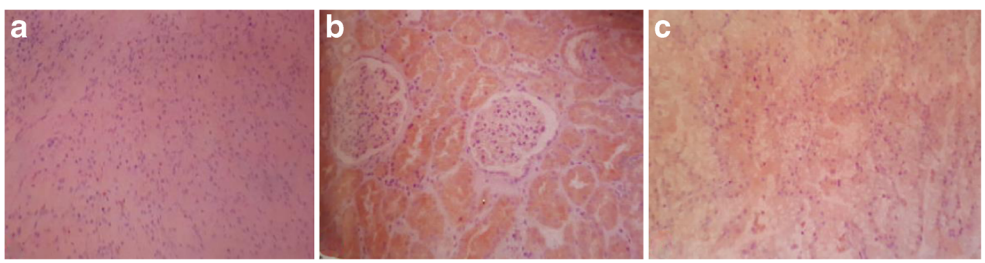

Fig. 1 **a** Conventional clear cell carcinoma with no immunoreactivity for TIMP-2, **b** Normal tissue near the tumor showing intense diffuse immunoreactivity for TIMP-2, C) Conventional clear cell carcinoma showing moderate diffuse cytoplasmic immunoreactivity for TIMP-2

Table 3 Relationship of MMP-1,-2,-3,-9 and TIMP-1,-2 expression with clinical-pathological factors in RCC. For each protein, number of patients (n) and percentage (%) of overexpression are listed. NS (non-significant)

	MMP-1 n (%)	*p value*	MMP-2 n (%)	*p value*	MMP-3 n (%)	*p value*	MMP-9 n (%)	*p value*	TIMP-1 n (%)	*p value*	TIMP-2 n (%)	*p value*
Age, years												
< 65	23 (79.3)	*NS*	8 (27.6)	*NS*	25 (86.2)	*NS*	2 (6.9)	*NS*	4 (13.8)	*NS*	18 (62.1)	*NS*
≥ 65	29 (93.5)		9 (29)		28 (90.3)		4 (12.9)		4 (12.9)		19 (61.3)	
Sex												
Male	39 (92.9)	*NS*	12 (28.6)	*NS*	40 (95.5)	NS	4 (9.5)	NS	7 (16.7)	NS	27 (64.3)	*NS*
Female	13 (72.2)		5 (27.8)		16 (88.9)		2 (11.1)		1 (5.6)		10 (55.6)	
Clinical												
Asymptomatic	26 (83.9)	*NS*	5 (16.1)	*=0.045*	26 (83.9)	NS	3 (9.7)	NS	3 (9.7)	NS	20 (64.5)	*NS*
Symptomatic	26 (89.7)		12 (41.4)		27 (93.1)		3 (10.3)		5 (12.7)		17 (58.6)	
pT												
T1	15 (78.9)	*NS*	1 (5.3)	*=0.011*	15 (78.9)	NS	1 (5.3)	NS	2 (10.5)	NS	13 (68.4)	*NS*
T2	13 (86.7)		4 (26.7)		14 (93.3)		3 (20)		2 (13.3)		9 (60)	
T3	24 (92.3)		12 (46.2)		24 (92.3)		2 (7.7)		4 (15.4)		15 (57.7)	
Cell type												
Clear cell	41 (85.4)	*NS*	11 (22.9)	*NS*	41 (85.4)	*NS*	3 (6.3)	NS	7 (14.6)	NS	28 (58.3)	*NS*
Non-clear cell	11 (91.7)		6 (50)		12 (100)		3 (25)		1 (8.3)		9 (75)	
Fuhrman grade												
I-II	29 (82.9)	*NS*	10 (28.6)	*NS*	32 (91.4)	*NS*	4 (11.4)	NS	6 (17.1)	NS	23 (65.7)	*NS*
III-IV	23 (92)		7 (28)		21 (84)		2 (8)		2 (8)		14 (56)	
Size, cm												
< 8	26 (86.7)	*NS*	3 (10)	*=0.003*	26 (86.7)	NS	3 (10)	NS	2 (6.7)	NS	18 (60)	NS
≥ 8	26 (86.7)		14 (46.7)		27 (90)		3 (10)		6 (20)		19 (63.3)	
Necrosis, %												
< 20	34 (82.9)	*NS*	9 (22)	*NS*	36 (87.8)	NS	2 (4.9)	NS	6 (14.6)	NS	29 (70.7)	*=0.034*
≥ 20	18 (94.7)		8 (42.1)		17 (89.5)		4 (21.1)		2 (10.5)		8 (42.1)	
Sarcomatous differentiation												
No	44 (84.6)	*NS*	11 (21.2)	*=0.005*	45 (84.9)	NS	2 (4.9)	NS	7 (13.5)	NS	32 (61.5)	*NS*
Yes	8 (100)		6 (75)		8 (100)		4 (21.1)		1 (12.5)		5 (62.5)	

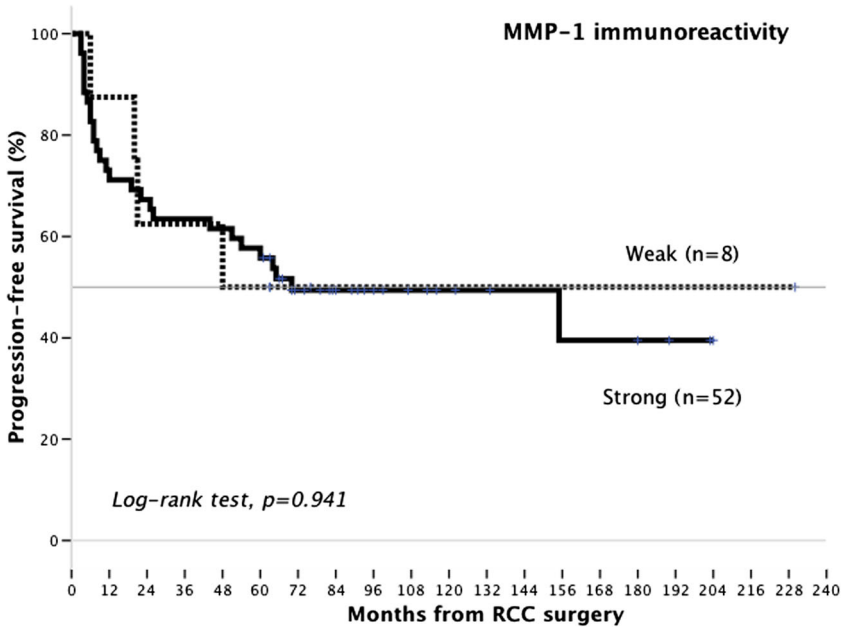

Fig. 2 Progression-free survival curves of the RCC patients based on MMP-1 staining. MMP-1, matrix metalloproteinase 1

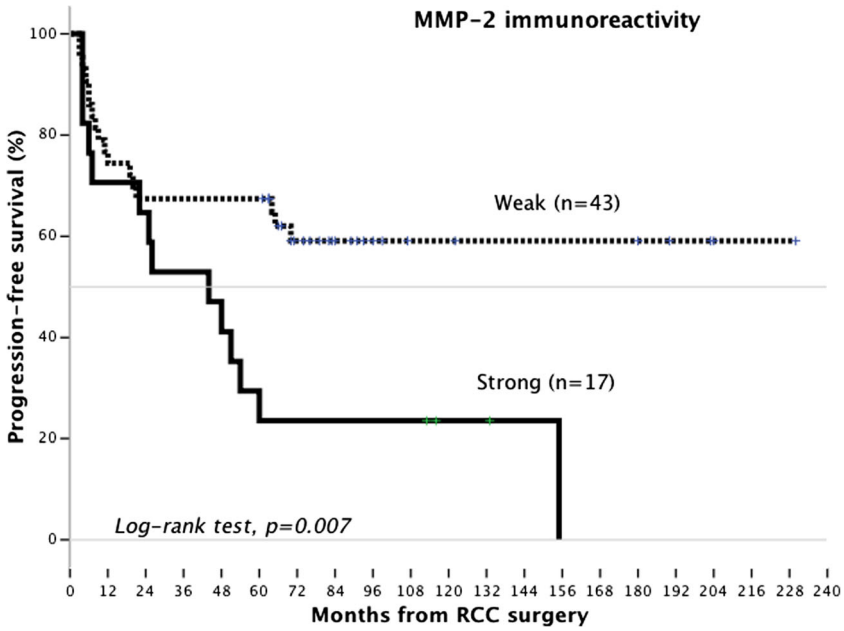

Fig. 3 Progression-free survival curves of the RCC patients based on MMP-2 staining. MMP-2, matrix metalloproteinase 2

These independent prognostic factors can be used to stratify the patients into three groups with different risks of progression and CSS: favorable prognosis group (n = 11): no risk factors; intermediate prognosis group (n = 23): one risk factor for a poor prognosis; and poor prognosis group (n = 26): two or three risk factors for a poor prognosis. The 5- and 10-year PFS rates were both 100% in the favorable prognosis group; 65.2% and 59.8% respectively in the intermediate prognosis group; and 29.9% and 19.2% respectively in the poor prognosis group; $p = 0.000003$ (Fig. 8).

Concerning CSS, 5- and 10-year rates were both 100%; 73.9% and 73.9%; and 38.5% and 20.5% in the groups with favorable, intermediate and poor prognosis respectively; $p = 0.000006$ (Fig. 9).

Discussion

Cancer development, through tumor growth, invasion, and metastasis, is a multistep process facilitated by the proteolytic degradation of components of the ECM and basement membrane. The role of MMPs in this process has been firmly established based on numerous previously published experimental and clinical studies [1, 7, 11, 12, 17]. In the present study, mean immunohistochemical expression of MMP-2, −3 and −9 was significantly higher in tumor tissue than in normal kidney tissue near the tumor, while TIMP-2 expression was lower in tumor than normal tissue in metastatic patients. In general, other researchers have reported higher protein concentrations or activity levels of MMP-2 [12, 21] and MMP-9 [6, 12, 13, 21] in tumor tissue than normal kidney tissue. In contrast, the findings are more heterogeneous concerning patterns of TIMP-2 expression in tumor and normal tissue. Specifically, Quiao [21] *et al.* observed high expression in the tumor, whereas Hageman [6] *et al.* and Lu [16] *et al.* observed stronger TIMP-2 expression in normal than in tumor tissue [16], and other authors [12, 13] have found no differences between tumor and normal tissue. As shown by our study, these discrepancies could be the result of low TIMP-2 expression in patients who develop metastases.

Table 4 Cox multivariate analysis of factors affecting tumor progression in RCC

Variable	HR (95% CI)	*p*-value
Stage	1.19 (0.45–3.12)	0.728
Grade	2.14 (1.02–4.48)	0.043
Size	1.11 (1.01–1.21)	0.028
MMP-2	1.81 (0.84–3.88)	0.130
TIMP-2	0.3 (0.14–0.62)	0.001

MMP-2 Matrix metalloproteinase 2; *TIMP-2* Tissue inhibitor of matrix metalloproteinases 2; *HR* Hazard ratio; *CI* Confidence interval

Table 5 Cox multivariate analysis of factors affecting cancer-specific survival in RCC

Variable	HR (95% CI)	p-value
Stage	2.66 (0.71–9.93)	0.145
Grade	2.87 (1.28–6.45)	0.010
Size	1.14 (1.03–1.26)	0.009
MMP-2	1.37 (0.59–3.14)	0.463
TIMP-2	0.35 (0.16–0.78)	0.010

MMP-2 Matrix metalloproteinase 2; *TIMP-2* Tissue inhibitor of matrix metalloproteinases 2; *HR* Hazard ratio; *CI* Confidence interval

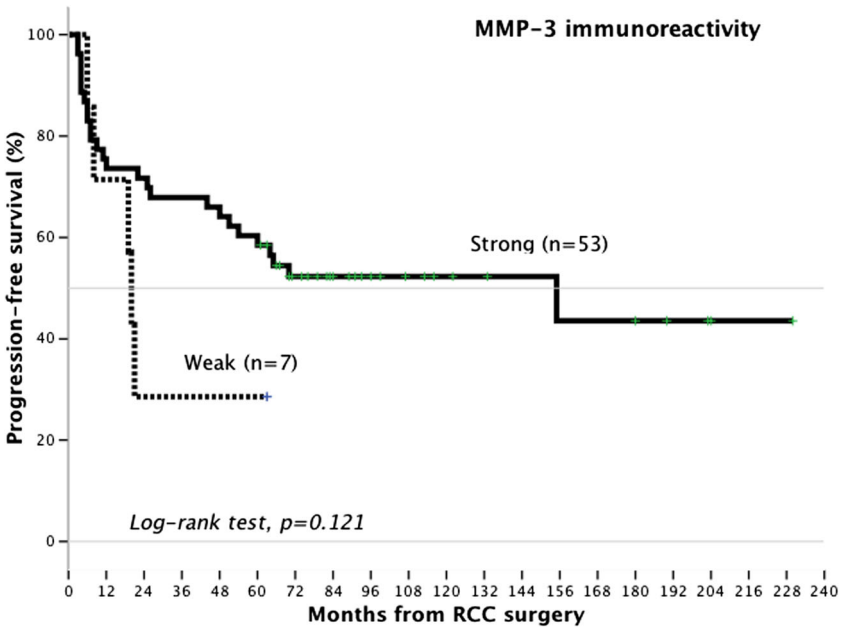

Fig. 4 Progression-free survival curves of the RCC patients based on MMP-3 staining. MMP-3, matrix metalloproteinase 3

It has previously been suggested that MMP-2 and -9 may have prognostic value, given their important role in degradation of type IV collagen of basement membrane, which is required for vascular invasion and hence the development of metastasis mediated by these proteins [21]. The relationships found in the present study between MMP-2 overexpression and some clinical and pathological prognostic factors, such as advanced tumor stage [21, 26], tumor size [22], sarcomatous differentiation [19], and systemic symptoms, are consistent with these data and with the findings of other authors. On the other hand, like most of these studies, we found no relationship between MMP-2 expression and Fuhrman grade [6, 12, 22, 24]. MMP-9 overexpression has also been found to be associated with advanced tumor stage [12, 21], high Fuhrman grade [9] or both [6, 14]. No such association was observed in our study or others [10, 22], and Pozzi [20] *et al*. in their study even note an association between MMP-9 overexpression and lower tumor vessel density.

Studies on MMP/TIMP expression and survival in RCC have been limited and results have been mixed, with MMP-2, −9, TIMP-1 and -2 expression being most frequently described in these previous retrospective studies. Among these, a study involving only 26 patients (23 samples of primary renal tumor tissue and 6 samples of

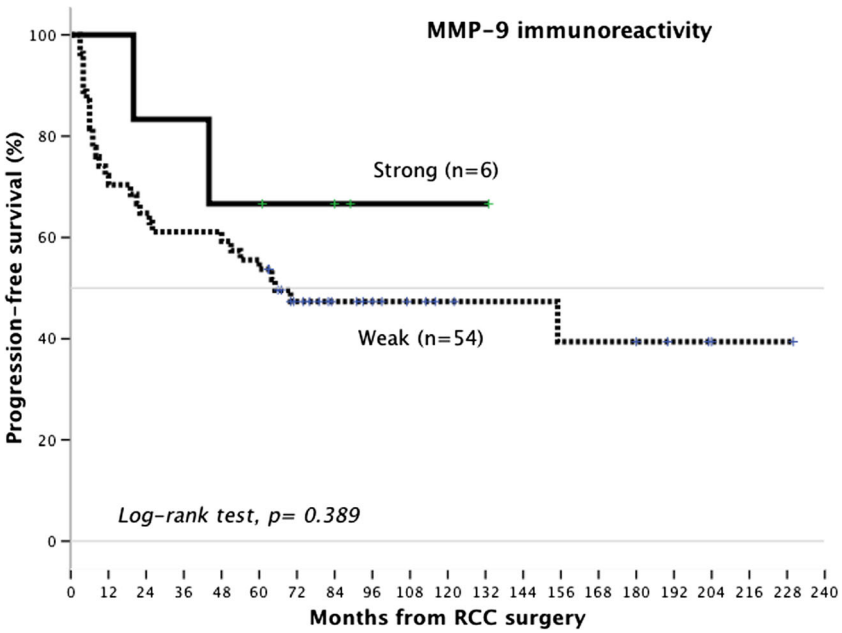

Fig. 5 Progression-free survival curves of the RCC patients based on MMP-9 staining. MMP-9, matrix metalloproteinase 9

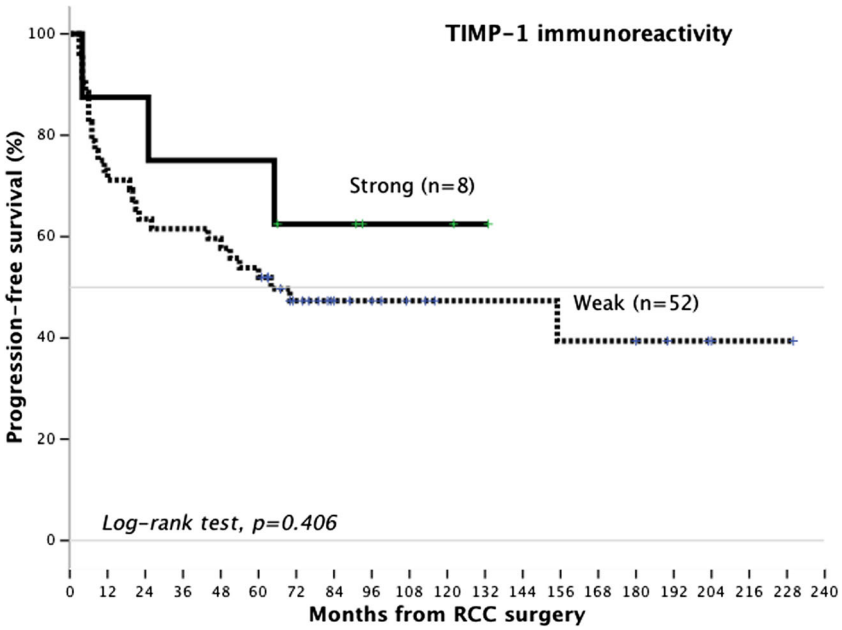

Fig. 6 Progression-free survival curves of the RCC patients based on TIMP-1 staining. TIMP-1, tissue inhibitor of matrix metalloproteinase 1

metastatic tissue) related levels of MMP-2 obtained by northern blot analysis and immunohistochemical expression of MMP-2 with overall survival, but did not analyze the impact of pathological factors [25]. A similar study in 46 patients (11 of them with synchronous metastases), with a mean follow-up of 48 months, concluded that the ratio of MMP-9 to E-cadherin, as measured by fluorescence in situ hybridization (FISH) was an independent predictor of metastasis [22]. Furthermore, the immunohistochemical overexpression of TIMP-1 and tumor stage (divided into high -stages I-II- and low - stages III-IV) was an independent predictor of shorter patient survival in another series of 153 patients (49 of them with M1 disease) with a follow up of 40 months [9]. One larger study involving 194 patients (47 of them with metastasis at the time of nephrectomy), with a mean follow-up of 35 months, evaluated the expression of MMP-2 and -9, as determined by reverse-transcription polymerase chain reaction (RT-PCR). These authors reported that high MMP-9 expression, along with advanced stage and the presence of tumor metastasis, were independent predictors of CSS [2]. Nevertheless, many other studies have found MMP-2, −9, TIMP-1 and -2 levels to have no significant prognostic value in RCC [12, 13, 21, 24, 26].

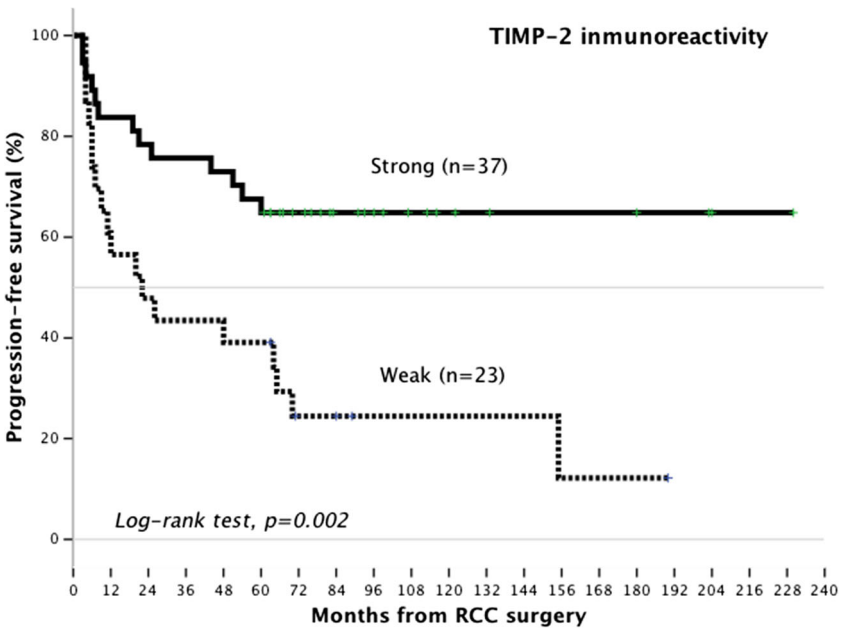

Fig. 7 Progression-free survival curves of the RCC patients based on TIMP-2 staining. TIMP-2, tissue inhibitor of matrix metalloproteinase 2

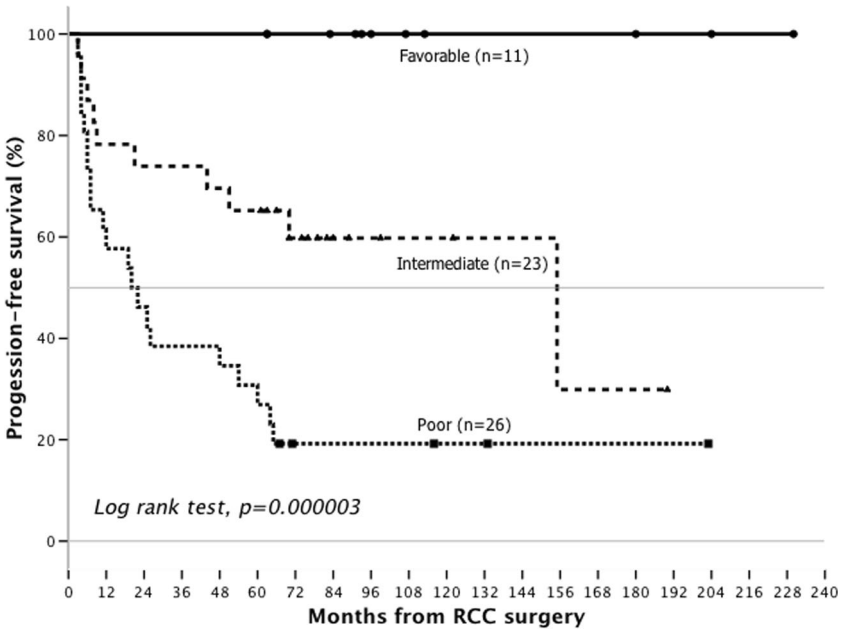

Fig. 8 Survival curves for progression-free survival based on prognosis group. Prognosis groups are based on three prognostic risk factors: tumor size ≥8 cm, Fuhrman grade 3 or 4, and low TIMP-2 expression in renal tumor tissue. Favorable prognosis group: no risk factors. Intermediate prognosis group: 1 risk factor. Poor prognosis group: 2 or 3 risk factors

Differences in the findings published to date may be explained by the heterogeneity of histological types of tumor in samples, especially as some authors have documented a papillary subtype [6, 9]; characteristics of the techniques used to determine MMP/TIMP expression, including RT-PCR, FISH and immunohistochemistry [1]; a follow-up of <60 months; and the inclusion of patients with metastases at the time of nephrectomy. To our knowledge, the only study investigating the relationship of immunohistochemical expression of MMP-2, −9, membrane type-MMP-1, and TIMP-1 and -2 with the CSS that has not included patients with metastasis at the time of nephrectomy involved 120 patients with T1-3N0M0, with unknown follow–up status, and according to the results, the only independent prognostic factor was tumor stage, the authors relating this to tumor expression of TIMP-2 in the univariate analysis [10].

Various limitations of this study should be recognized and discussed. First, the small number of patients is a major drawback. Nevertheless, there were clear differences in MMP/TIMP expression between tumor and normal tissue sample groups and this indicates that our sample size calculations were based on reasonable assumptions. Thus, the risk of a type II error, which is a common problem in small studies, was minimized in our study by increasing the size of the effect to be detected by considering positive cases only, irrespective of severe or moderately diffuse immunohistochemical

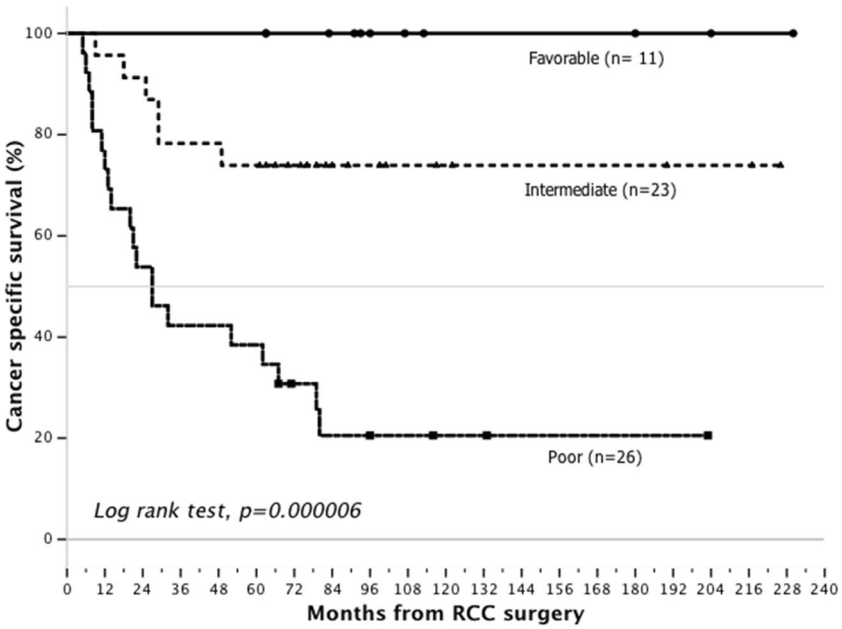

Fig. 9 Survival curves for cancer-specific survival based on prognosis group. Prognosis groups are based on three prognostic risk factors: tumor size ≥8 cm, Fuhrman grade 3 or 4, and low TIMP-2 expression in renal tumor tissue. Favorable prognosis group: no risk factors. Intermediate prognosis group: 1 risk factor. Poor prognosis group: 2 or 3 risk factors

expression. In addition, the number of patients and the normal distribution of the factors studied allowed the application of parametric tests. Similarly, the probability of a type I error should be low given the high significance level (Figs. 2 and 3, $p < 0.00001$). Secondly, the present study is limited by its retrospective nature, although all measurements were performed in a blinded manner (Figs. 4, 5, 6, 7, 8 and 9).

Conclusions

MMPs, and particularly their inhibitors, are key enzymes in tumor progression. Our results demonstrate a significantly poorer prognosis with higher Fuhrman grade, larger tumor size, and lower TIMP-2 expression in localized and locally advanced RCC after surgery. Increased MMP-2 activity may be important in the early stages of tumor growth, as suggested by immunohistochemical overexpression of this protein in RCC with respect to normal kidney tissue and by its correlation with advanced tumor stage, tumor size and sarcomatous differentiation. Prospective studies with large series of patients, analyzing all MMPs/TIMPS described to date with possible involvement in the progression of RCC, are now required to confirm these findings and identify patients who may benefit from adjuvant therapy.

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