Imbalance between serum matrix metalloproteinase-2 and its inhibitor as a predictor of recurrence of urothelial cancer

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Summary Serum levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-2 (TIMP-2) were evaluated as prognostic indicators of the recurrence of urothelial cancer. Sera were obtained from 127 healthy control subjects and 97 urothelial cancer patients who underwent complete resection and were measured for MMP-2 and TIMP-2 using a one-step enzyme immunoassay. The relationship between the serum MMP-2/TIMP-2 ratio and the recurrence of urothelial cancer was examined. The mean serum MMP-2/TIMP-2 ratio in the 31 advanced urothelial cancer patients with recurrence was significantly higher than that in the 22 patients without recurrence ($P = 0.0029$) and in the 44 superficial bladder cancer patients ($P < 0.0001$). The 1- and 3-year disease-free survival rates in the patients with high MMP-2/TIMP-2 ratios (50% and 12%) were significantly poorer than those of the patients with normal ratios (82% and 56%) ($P = 0.0152$). Univariate and multivariate analyses of recurrence demonstrated that the serum MMP-2/TIMP-2 ratio is a significant independent indicator of advanced urothelial cancer. Our results indicate that an imbalance between the serum levels of MMP-2 and TIMP-2 could be a new predictor of recurrence in advanced urothelial cancer patients.

Keywords: serum; matrix metalloproteinase-2; tissue inhibitor of metalloproteinases-2; urothelial cancer; recurrence

Matrix metalloproteinases (MMPs) has been shown to play a role in the degradation of the vascular basement membrane, whose major component is type IV collagen (Liotta et al, 1991; Nakajima et al, 1991). Matrix metalloproteinase-2 (MMP-2, gelatinase A, a 72-kDa type IV collagenase) is produced by both malignant cells and stromal cells such as fibroblasts, macrophages and vascular endothelial cells (Nakajima et al, 1991). Many investigators have demonstrated that human and rodent metastatic malignant cells produce larger amounts of MMP-2 than do non-metastatic cells, both in vitro and in vivo (Liotta et al, 1980; 1991). Tissue inhibitor of metalloproteinases-2 (TIMP-2), an unglycosylated protein of 21-kDa molecular weight, strongly inhibits the biological activity of MMP-2, and was shown to strongly inhibit cancer invasion, metastasis, growth and angiogenesis in some rodent and human tumours (Stetler-Stevenson et al, 1989; DeClerck et al, 1992). Therefore, it is likely that an imbalance in the MMP-2 and TIMP-2 ratio plays an important role in cancer invasion, metastasis and angiogenesis (Liotta et al, 1991; DeClerck et al, 1994). Some investigators have revealed a relationship between the recurrence and the expression of MMP-2 and TIMP-2 in bladder cancer tissues (Davies et al, 1993; Grignon et al, 1996). However, there are no previous reports concerning the relationship between the serum MMP-2/TIMP-2 ratio and the recurrence of human urothelial cancer. In this study, the serum levels of MMP-2 and TIMP-2

were determined in human urothelial cancer patients, and the relationship between the serum MMP-2/TIMP-2 ratio and invasion and metastasis in urothelial cancer was examined. We discuss the diagnostic value of an imbalance in the serum MMP-2/TIMP-2 ratio for the recurrence of advanced urothelial cancer after complete resection.

PATIENTS AND METHODS

Between January 1986 and October 1994, sera were obtained from 97 patients [44 with superficial bladder cancer ($\leq$ pT1) and 53 with advanced urothelial cancer with muscular invasion ($\geq$ pT2) or metastasis] before they underwent complete resection. Informed consent was obtained from all patients for measuring serum MMP-2 and TIMP-2. The study was carried out with ethical approval. The sera were stored at –80°C until use. The pathological stages were determined according to the TNM classification of urothelial cancer. The histology and differentiation of the tumours were determined according to the World Health Organization (WHO) classifications. Normal serum MMP-2 and TIMP-2 levels were determined from healthy control subjects (85 males and 42 females; 18–69 years old, median 59 years). The maximum diameter of each bladder tumour was determined endoscopically. The clinicopathological features of the patients are shown in Table 1.

The concentration of serum MMP-2 was measured by a one-step sandwich enzyme immunoassay (EIA) system using murine monoclonal antibodies raised against the purified proMMP-2 and an oligopeptide from residue 17 to 35 of the amino terminal region on the human MMP-2 sequence, as previously described (Fujimoto et al, 1993a). The sensitivity of this EIA system was

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Table 1 Clinicopathological features of urothelial cancer patients who underwent complete resection

Variables	Number of patients (n = 97)		
	Superficial Bca[a] (n = 44)	**Advanced Bca[b] (n = 29)**	**Upper urothelial[c] (n = 24)**
Sex			
Male/female	32/12	25/4	18/6
Age (years)			
Median (range)	64 (31–94)	66 (40–87)	66 (54–81)
Histology			
TCC[d]			
G1	19	1	1
G2	19	9	11
G3	6	15	12
SCC ADE[e]	0	4	0
PTstage			
Ta	12	0	0
T1	32	2[f]	3[f]
T2	0	12	11
T3a	0	3	0
T3b	0	12	8
T4	0	0	2
Lymphnode metastasis			
Negative	44	22	17
Positive	0	7	7
Lymphvascular involvement			
Negative	28	12	11
Positive	2	17	13
Unknown	14	0	0
Maximal tumour size in diameter (cm)			
≤ 1	8	0	ND[g]
1–3	10	7	
≥ 3	2	6	
Unknown	24	16	

[a]Superficial bladder cancer; [b]advanced (muscular invasive or metastatic) bladder cancer; [c]advanced upper urothelial cancer; [d]transitional cell carcinoma; [e]squamous cell carcinoma and adenocarcinoma; [f]all patients with PT1 disease had lymph node metastasis; [g]not detected.

2.4 pg per assay (0.24 ng ml⁻¹), and linearity was obtained between 10 and 5000 pg per assay (1.0–500 ng ml⁻¹). The concentration of serum TIMP-2 was similarly determined using a murine monoclonal antibody against human TIMP-2, as previously described (Fujimoto et al, 1993*b*). The sensitivity of this EIA was 16 pg per assay (1.6 ng ml⁻¹), and linearity was obtained between 63 and 500 pg per assay (6.3–50 ng ml⁻¹). As the monoclonal antibody used in the present study was raised against the N-terminal domain peptide of proMMP-2, it recognizes free proMMP-2 but does not detect inactivated MMP-2 bound to TIMP-2. There is no free active form of MMP-2 in the serum. When the sera were analysed by the combination of zymography and affinity chromatography with anti-TIMP-1 and anti-TIMP-2 monoclonal antibodies, the amounts of the active form of MMP-2 in sera were found to be extremely low and completely bound to TIMPs (data not shown). Thus, we measured only proMMP-2 level in the serum.

All superficial bladder cancers were resected endoscopically, and the patients were observed for recurrence over a median period of 30 months (10–67 months). Of the 29 advanced bladder cancer patients, 26 underwent radical cystectomy and pelvic lymph node dissection, and three underwent partial cystectomy and hemipelvic lymph node dissection; the median observation of these 29 patients was 28 months (4–98 months). All 24 upper urothelial cancer patients received nephroureterectomy, partial cystectomy and regional lymph node dissection; the median observation was 20 months (3–107 months).

The post-operative examinations for recurrence were carried out by pelvic and abdominal computerized tomography (CT), chest radiograph and routine blood tests at 3-month intervals for 3 years after the surgery, and bone scans were performed at 6-month intervals for the same period. These examinations were then carried out at 6-month intervals until 5 years after the surgery. After 5 years, the examinations were performed annually. When the patients had any neurological symptoms, such as headache or vomiting, they were examined by a brain CT scan. Only visceral, skeletal and lymph node metastases were regarded as recurrence for the purpose of this study.

The differences in the serum levels of MMP-2 and TIMP-2 among the superficial bladder cancer patients, the healthy controls and the advanced urothelial cancer patients were examined using the Mann–Whitney U-test. The significance of the elevations in the serum levels of MMP-2, TIMP-2 and the serum MMP-2/TIMP-2 ratio was categorized into groups and then a χ^2-test was carried out. The disease-free survival rate was calculated according to the method of Kaplan–Meier and compared using the

Table 2   Serum levels of MMP-2, TIMP-2 in healthy controls and urothelial cancer patients

Variables	n	Mean ± s.d. (ng ml⁻¹)				Elevation (%)^a			
		MMP-2	P-value^b	TIMP-2	P-value^b	MMP-2	P-value^c	TIMP-2	P-value^c
Healthy controls	127	549 ± 90.5	0.820	60.3 ± 19.8	0.40	5 (4)	1.00	8 (6)	0.065
Superficial Bca^d	44	550 ± 142	–	57.3 ± 19.8	–	2 (4)	–	7 (16)	–
Advanced Uro Ca^e	53	702 ± 176	<0.0001	77.5 ± 31.0	0.0002	22 (41)	<0.0001	11 (21)	0.607
Without recurrence	22	647 ± 150	0.010] 0.060^f	89.9 ± 40.8	<0.0001] 0.011^f	7 (32)	0.0048] 0.268^f	9 (41)	0.035] 0.0044^f
With recurrence	31	739 ± 185	<0.0001	68.4 ± 16.7	0.013	15 (48)	<0.0001	2 (6)	0.292

^{a}Serum levels of MMP-2 ≥730 ng ml⁻¹; serum levels of TIMP-2 ≥ 94 ng ml⁻¹, ^{b}Mann–Whitney U-test. Healthy controls and advanced urothelial cancer were compared against superficial bladder cancer; $^c\chi^2$-test; ^{f}Mann–Whitney U-test; advanced urothelial cancer without recurrence vs advanced urothelial cancer with recurrence. ^{d}superficial bladder cancer; ^{e}advanced (muscular invasive or metastatic) urothelial cancer

log-rank test. Disease-free survival was defined as the time from the surgery to the detection of the first local recurrence or distant metastasis, or to the end of the study. Factors related to recurrence in the patients who underwent complete resection were analysed by Cox's proportional hazard regression model (Cox, 1972). P-values < 0.05 were regarded as significant.

RESULTS

Serum levels of MMP-2 and TIMP-2

The serum levels of MMP-2 and TIMP-2 obtained in this study are summarized in Table 2. The serum levels of these enzymes and inhibitors were not related to sex or age in the healthy control subjects (data not shown). The mean +2 s.d. levels of serum MMP-2 and TIMP-2 in the healthy controls were 730 ng ml⁻¹ and 94 ng ml⁻¹ respectively; any higher values were regarded as 'elevated'. There was no significant difference in these values between the healthy control subjects and the superficial bladder cancer patients. In contrast, the serum MMP-2 and TIMP-2 levels in the advanced urothelial cancer patients (702 ± 176 and 77.5 ± 31.0 ng ml⁻¹ respectively) were significantly higher than those in the superficial bladder cancer patients (P < 0.0001 and P = 0.0002 respectively). The elevation of serum MMP-2 in the advanced urothelial cancer patients was also significantly higher than that in the superficial bladder cancer patients (P < 0.0001). However, the elevation of serum TIMP-2 in the advanced urothelial cancer patients was not significantly different compared with that in the superficial bladder cancer patients. Moreover, there was no significant difference between the levels in the advanced bladder and upper urothelial cancer patients (data not shown). There was also no correlation between the serum levels of MMP-2 or TIMP-2 and tumour size (data not shown). Of the 53 advanced urothelial cancer patients, 31 had recurrence and the remaining 22 had no recurrence after the surgery. The serum levels of MMP-2 in the 31 patients with recurrence (739 ± 185 ng ml⁻¹) were significantly higher than those in the 44 superficial bladder cancer patients (550 ± 142 ng ml⁻¹) (P < 0.0001), but the value was not significantly different from those in the 22 advanced urothelial cancer patients without recurrence (647 ± 150 ng ml⁻¹) (P = 0.060). Moreover, the serum levels of TIMP-2 in the 22 patients without recurrence (89.9 ± 40.8 ng ml⁻¹) were significantly higher than those in the 44 superficial bladder cancer patients (57.3 ± 19.8 ng ml⁻¹) (P < 0.0001) and those in the 31 patients with recurrence (68.4 ± 16.7 ng ml⁻¹) (P = 0.011).

The serum MMP-2/TIMP-2 ratios

The mean+2 s.d. of the serum MMP-2/TIMP-2 ratio in the healthy control subjects was 11.0; any higher value was regarded as elevated. The serum MMP-2/TIMP-2 ratios were not related to tumour differentiation or pT stage (data not shown). A significant difference was found only between patients with positive lymph node metastasis (12.2 ± 3.16) and those who were negative (9.72 ± 4.02) (P = 0.04). The elevation of the serum MMP-2/TIMP-2 ratio in the advanced urothelial cancer patients with recurrence (15 out of 31, 48.4%) was significantly higher than that in the advanced urothelial cancer patients without recurrence (4 out of 22, 18.2%)(P = 0.0408) and the superficial bladder cancer group (3 out of 44, 6.8%)(P < 0.0001). It is noted that the mean ratio in the 31 advanced urothelial cancer patients with recurrence (11.2 ± 3.43, range 4.23–21.06) was significantly higher than that in the 22 without recurrence (8.48 ± 4.13, range 2.66–20.0) and than that in the 44 superficial bladder cancer patients (7.76 ± 1.55, range 3.52–13.3) (P = 0.0029 and P < 0.0001 respectively) (Figure 1).

Disease-free survival in advanced urothelial cancer patients according to serum MMP-2, TIMP-2 levels and MMP-2/TIMP-2 ratios

There was no significant difference in disease-free survival between the patients with elevated serum levels of MMP-2 and those with normal levels of the enzyme (Figure 2A). The serum level of TIMP-2 itself was also not correlated with disease-free survival (Figure 2B). Among the patients with advanced urothelial cancer, 19 patients had high MMP-2/TIMP-2 ratios (≥ 11.0) and 34 showed normal ratios (< 11.0). The 1- and 3-year disease-free survival rates of the patients with high MMP-2/TIMP-2 ratios were 50% and 12%, respectively, significantly unfavourable compared with those with a low ratio (82% and 56% respectively) (P = 0.0152) (Figure 2C).

Univariate and multivariate analyses for recurrence in advanced urothelial cancer patients who underwent complete resection

The univariate analysis determined that significant predictors of recurrence were the serum MMP-2/TIMP-2 ratio (P = 0.0228), pT stage (P = 0.0056) and lymph node metastasis (P = 0.0448) (Table 3). The serum MMP-2/TIMP-2 ratio (P = 0.0249), pT stage
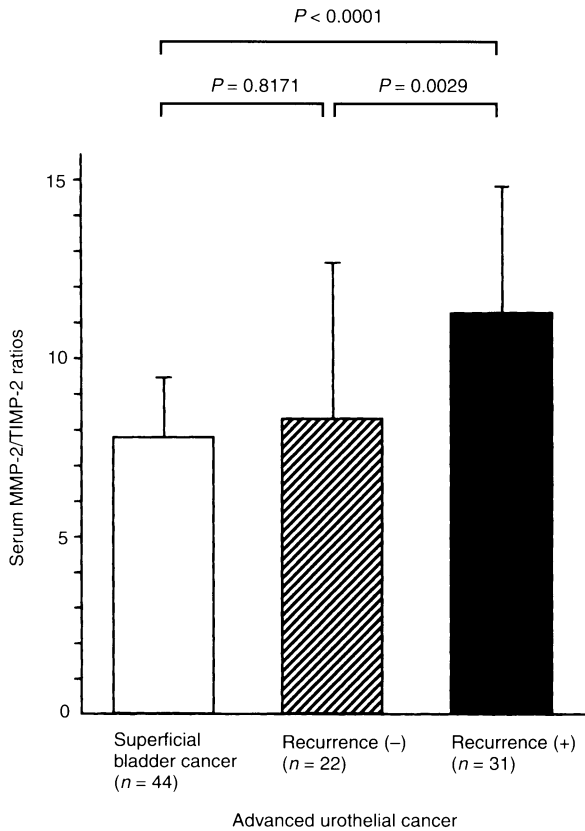
P < 0.0001

P = 0.8171   P = 0.0029

Figure 1   The serum MMP-2/TIMP-2 ratios in the 44 superficial bladder cancer and 53 advanced urothelial cancer patients. The mean serum MMP-2/TIMP-2 ratios in the advanced urothelial cancer patients with recurrence was significantly higher than that in the advanced urothelial cancer patients without recurrence and in the superficial bladder cancer patients ($P = 0.0029$ and ($P < 0.0001$, Mann–Whitney U-test). Data are means ± standard deviation

$(P = 0.0011)$, and lymph node metastasis $(P = 0.0260)$ were also defined as significant independent predictors of recurrence using multivariate analysis.

DISCUSSION

MMP-2 is one of the important proteases that degrades vascular basement membranes and extracellular matrices in the multiple metastatic process (Liotta et al, 1991; Nakajima et al, 1991; DeClerck et al, 1994). This enzyme also enhances the infiltration and migration of vascular endothelial cells, and thus induces neovascularization in malignant tumours (Liotta et al, 1991; Nakajima et al, 1991; DeClerck et al, 1994). The levels of MMP-2 and MMP-9 in high-grade or invasive bladder cancers were found to be significantly higher than those in low-grade and non-invasive bladder cancers, indicating that MMP-2 and MMP-9 may play a role in the invasion and metastasis of bladder cancer (Davies et al, 1993).

TIMPs are known for their anti-neoplastic activity (Albini et al, 1991; Montgomery et al, 1994). The transfection of sense TIMP-1 complementary DNA into metastatic cells decreased their invasive, tumorigenic and metastatic capacities (DeClerck et al, 1992; Khokha et al, 1994). Moreover, TIMPs are reported to inhibit angiogenesis, probably by affecting endothelial cell invasion, migration and proliferation (Moses et al, 1990; Takigawa et al, 1990; Liotta et al, 1991; DeClerck et al, 1994). The high level of
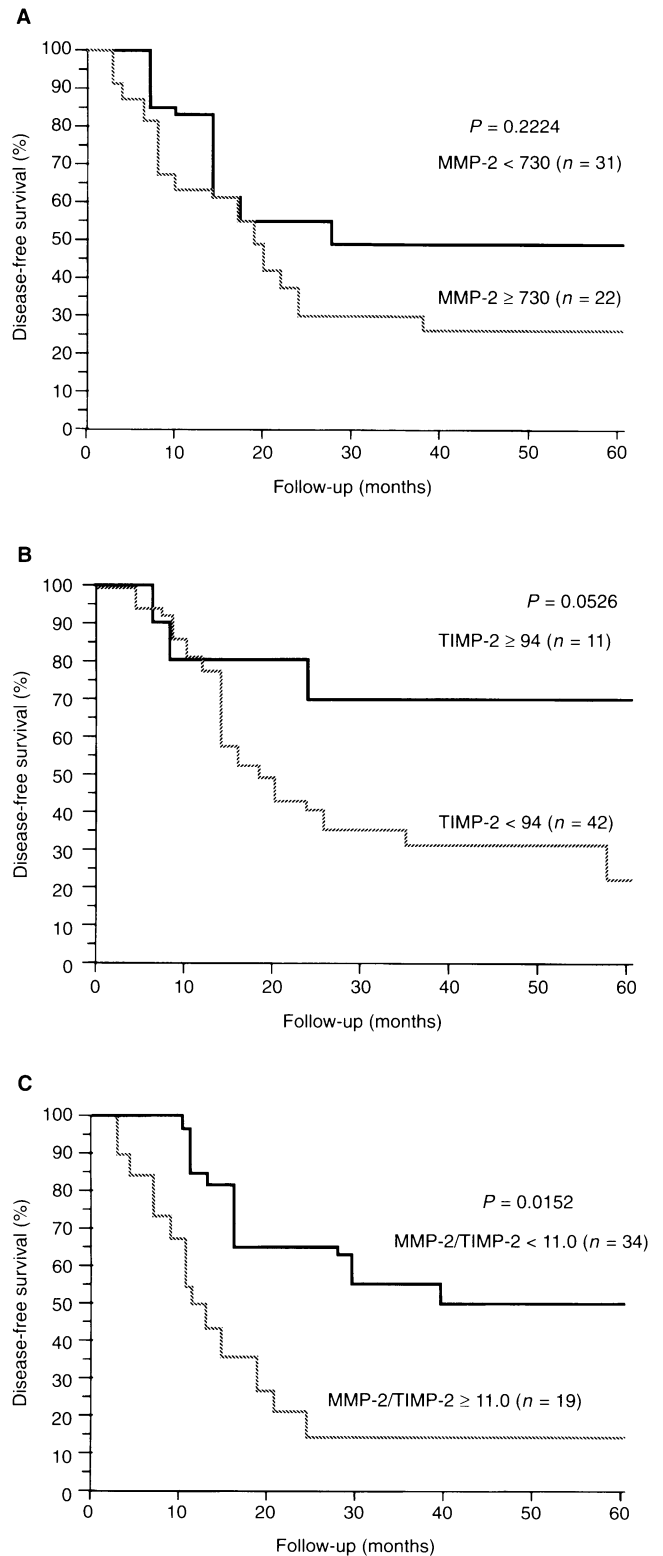
Figure 2   Disease-free survival of the 53 advanced urothelial cancer patients after complete resection according to the serum levels of MMP-2 (**A**), TIMP-2 (**B**) and MMP-2/TIMP-2 ratios (**C**). The 1- and 3-year disease-free survival rates of the patients with high MMP-2/TIMP-2 ratios (≥ 11.0) were significantly unfavourable compared with the patients with lower ratios (< 11.0)($P = 0.0152$; log-rank test)

Table 3   Prognostic factors of recurrence (univariate and multivariate analyses)

| | Disease-free survival | | | | | |
| | Univariate | | | Multivariate | | |
	Hazard ratio	(95% CI)	P-value	Hazard ratio	(95% CI)	P-value
MMP-2/TIMP-2 (<11.0 vs ≥ 11.0)	2.31	(1.12–4.74)	0.0228	2.39	(1.12–5.10)	0.0249
Age (<61 years vs ≥ 61 years)	1.14	(0.54–2.43)	0.734	2.18	(0.91–5.22)	0.0815
Sex (Male vs female)	0.91	(0.37–2.22)	0.835	0.998	(0.37–2.68)	0.999
Grade (G1 vs G2 vs G3)	0.93	(0.46–1.85)	0.826	0.73	(0.32–1.65)	0.446
Stage (T1 vs T2 vs T3 vs T4)	1.95	(1.22–3.14)	0.0056	2.94	(1.54–5.61)	0.0011
Lymphovascular involvement (negative vs positive)	1.58	(0.75–3.31)	0.231	0.74	(0.28–2.01)	0.5622
Lymph node metastasis (negative vs positive)	2.15	(0.99–4.69)	0.0448	2.78	(1.13–6.84)	0.0260

CI, confidence intervals.

TIMP-2 expression as detected by immunohistochemistry was associated with poor outcome in invasive bladder cancer patients (Grignon et al, 1996). However, several investigators have shown that the balance between MMPs and their inhibitors (TIMPs) modulated endothelial cell morphogenesis in vitro, and that TIMPs inhibited the early events in tube formation by endothelial cells on Matrigel (Schnaper et al, 1993). Therefore, MMPs and TIMPs in circulating body fluids may also contribute to the regulation of tumour metastasis, invasion and angiogesesis (Liotta et al, 1991; DeClerck et al, 1994).

The purpose of the present study was to determine whether the serum MMP-2/TIMP-2 ratio could be a predictor of invasion, metastasis and recurrence in advanced urothelial cancer patients who have undergone complete resection. Our results demonstrated that the serum levels of MMP-2 and TIMP-2 in the advanced urothelial cancer patients were significantly higher than those in the superficial bladder cancer patients. Moreover, the serum MMP-2/TIMP-2 ratio in the advanced urothelial cancer patients with recurrence was significantly higher than those in the superficial bladder cancer and advanced urothelial cancer patients without recurrence. In fact, the median disease-free survival of the patients with higher values of MMP-2/TIMP-2 (≥ 11.0) was significantly shorter than that of the patients with normal values (< 11.0). However, the disease-free survival was not correlated with the low level of serum MMP-2. In addition, although the results shown in Figure 2B suggest a possible correlation between the serum TIMP-2 level and the disease-free survival, high levels of serum TIMP-2 alone were not significantly correlated with the disease-free survival. Nevertheless, the imbalance of these enzymes and inhibitors is most likely an important factor in urothelial cancer invasion and metastasis, and, thereby, recurrence. There have been several studies on the inhibition of tumour invasion and metastasis by TIMP-1 (Schultz et al, 1988; Alvarez et al, 1990) and TIMP-2 (Albini et al, 1991; DeClerck et al, 1991). Koop et al (1994) found that the decreased metastatic ability of TIMP-overexpressing B16F10 melanoma cells was due to the effects of TIMP on tumour growth after tumour cell extravasation in the metastatic target organ. TIMP-2 has been shown to inhibit the basic fibroblast growth factor stimulation of endothelial cell growth (Murphy et al, 1993). In addition, synthetic MMP inhibitors have been demonstrated to suppress primary tumour growth (Naito et al, 1994; Wang et al, 1994). Therefore, MMP inhibitors could be inhibitory against not only tumour invasion but also tumour growth, through the inactivation of cell-associated MMPs.

In the present study, we noted that when the serum MMP-2/TIMP-2 ratio had been within the normal range (< 11.0), the secondary tumour at any metastatic site did not grow well. Therefore, even if micrometastatic lesions were formed before the operation, high levels of serum TIMP-2 would prevent metastatic tumour cells from developing further visible colonies.

Using multivariate analysis of recurrence we found that the serum MMP-2/TIMP-2 ratio is a new independent predictor comparable with traditional prognostic factors such as pT stage and lymph node metastatic status (Skinner et al, 1991).

In conclusion, our results indicate that the imbalance of serum MMP-2 and TIMP-2 (the MMP-2/TIMP-2 ratio) could be a new predictor of recurrence and may help us to determine whether or not patients with advanced urothelial cancer need intensive therapy, such as adjuvant chemotherapy, after complete resection.

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