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ORIGINAL ARTICLE

Detection and molecular staging of bladder cancer using real-time RT-PCR for gelatinases (MMP-2, MMP-9) and TIMP-2 in peripheral blood

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Bladder neoplasia;
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Abstract
Introduction: Molecular staging of bladder cancer based on the detection of mRNA of urothelial specific genes in circulating cancer cells has been inconclusive. We analyze whether real-time RT-PCR evaluation of gelatinases (MMP-9, MMP-2) and TIMP-2 in peripheral blood allows diagnosing and characterizing patients with bladder neoplasm.
Materials and method: Total RNA is extracted from circulating blood cells in 42 individuals (11 healthy controls, 31 patients with bladder cancer in different stages) and real-time RT-PCR performed using specific primers for MMP-9, MMP-2, TIMP-2 and ribosomal 18S. The quantification values of mRNA are described as relative to 18S mRNA (ΔΔCt method) and the results are blindly compared with data obtained from histological diagnosis and clinical staging.
Results: Normalized levels of MMP-9 and MMP-2 mRNA are higher in patients with cancer than controls (1.82±0.6 times and 2.7±0.6 times, respectively; $P<0.05$). Patients with metastatic disease also have increased MMP-9, MMP-2 and TIMP-2 mRNA levels (9.6±0.20 times, 5.22±0.26 times and 1,97±0.22 times, respectively; $P<0.05$). MMP-9 and MMP-2 are also associated with advanced clinical stage and grade ($P<0.05$). A ratio between variables that increases the ability to segregate patients with Ta, T1, T2-4M0 and T2-4M1 tumors is proposed.
Conclusions: Both non-invasive bladder tumor recognition and molecular staging of the disease is possible using real-time RT-PCR-based detection of gelatinases and TIMP-2 in peripheral blood. The ability to distinguish metastatic disease is higher for MMP-9 but MMP-2 discriminates better levels of tumor invasion. Further investigation in this field could yield promising results regarding molecular evaluation of bladder neoplasia.

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PALABRAS CLAVE
Neoplasia vesical;
Estadificación molecular;
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MMP-2;
MMP-9;
TIMP-2

Detección y estadificación molecular del cáncer vesical mediante RT-PCR a tiempo real para gelatinasas (MMP-2, MMP-9) y TIMP-2 en sangre periférica

Resumen

Introducción: La estadificación molecular del cáncer vesical basada en la detección de ARNm de genes específicos de urotelio no ha sido concluyente. Analizamos si la evaluación de gelatinasas (MMP-9, MMP-2) y TIMP-2 en sangre periférica mediante RT-PCR a tiempo real permite diagnosticar y caracterizar pacientes con neoplasia vesical.

Material y método: Se ha extraído ARN total a partir de células sanguíneas circulantes en 42 individuos (11 controles sanos, 31 pacientes con cáncer vesical en diversos estadios) y se ha llevado a cabo RT-PCR a tiempo real empleando cebadores específicos para MMP-9, MMP-2, TIMP-2 y 18S ribosomal. Los valores de cuantificación del ARNm se describen como relativos a ARNm 18S (método ΔΔCt comparativo) y los resultados se comparan de forma ciega con los datos obtenidos mediante diagnóstico histológico y estadificación clínica.

Resultados: Los niveles normalizados de ARNm de MMP-9 y MMP-2 son más altos en pacientes con cáncer que en controles (1,82 ± 0,6 veces y 2,7 ± 0,6 veces, respectivamente; $p < 0,05$). Los pacientes con enfermedad metastática también tienen niveles mayores de ARNm de MMP-9, MMP-2 y TIMP-2 (9,6 ± 0,20 veces, 5,22 ± 0,26 veces y 1,97 ± 0,22 veces, respectivamente; $p < 0,05$). MMP-9 y MMP-2 también se asocian con estadio clínico y grado avanzado ($p < 0,05$). Se propone un índice entre variables que aumenta la habilidad para segregar pacientes con tumores Ta, T1, T2-4M0 y T2-4M1.

Conclusiones: La identificación de tumor vesical y la estadificación molecular de la enfermedad resulta posible mediante la detección de gelatinasas y TIMP-2 en sangre periférica empleando RT-PCR a tiempo real. La capacidad de distinguir enfermedad metastásica es mayor para MMP-9, pero MMP-2 discrimina mejor los niveles de invasión tumoral. La investigación futura en este campo podría aportar resultados prometedores en la evaluación molecular de la neoplasia vesical.

Introduction

The therapeutic failure of distant metastasis in bladder cancer has hardly changed in decades, because occult invasive disease is often present at the time of primary treatment, and many high-grade invasive tumors develop metastases during follow-up early. Of course, the best predictors of cancer control and survival in bladder cancer are the extent of disease and its histological grade,[1] but many molecular markers are being investigated in depth in neoplastic tissue, urine or blood for supposed correlation with the increased likelihood of distant dissemination.

An important aspect of the metastatic cascade is the degradation of extracellular matrix (ECM) by specific proteolytic enzymes. The tumor cells have the ability to pool their proteolytic mechanisms with motility in a highly organized invasion to reach inappropriate places. A key role in this degradation process is attributed to matrix metalloproteinases (MMP), a family of zinc dependent endopeptidases that regulate the integrity and composition of the ECM. The activity of MMP-2 and MMP-9 (gelatinase) is regulated at several levels, including transcription, secretion, activation and inhibition by tissue inhibitors of metalloproteinases (TIMP). Overproduction of MMP by interacting with tumor vascular and lymphatic systems could lead to higher levels of MMP and TIMP, not only in the tissues but also in other biological fluids such as blood or urine. Many studies have highlighted the overexpression of MMP and TIMP in bladder cancer (table 1).[2-24] We investigated a new approach to evaluate the activity of MMP RT-PCR using real-time peripheral blood mononuclear cells, with special emphasis on gene amplification of MMP-9, MMP-2 and TIMP-2 in healthy controls and patients with bladder cancer.

Materials and methods

Population study

The study was conducted with 42 subjects (36 men, 6 women) with a mean age of 64 years (95% CI 59.7 to 68.3, range 29-90), which included controls for age and gender-matched healthy individuals (n=11) and patients with bladder cancer (n=31) diagnosed and treated at our center. All patients underwent transurethral resection of primary bladder cancer and 8 additionally underwent cystectomy. A preoperative study revealed metastatic proliferation in 5 cases (16%) and defined histopathological 2 grade 1, 6 grade 2 and 23 grade 3 tumors (WHO criteria). The tumor stages were Ta (n=7), T_1 (n=8), T_2 (n=7), T_3 (n=4) and T_4 (n=5), according to the criteria of the AJCC 2006. All patients gave their consent to donate biological material in accordance with the requirements of the Clinical Research Ethics Committee of the center.

Table 1 Outcome of the evaluation of MMP-2, MMP-9 and TIMP-2 to characterize bladder neoplasia

Author (ref.)	Number of patients	Sample source	Detection method	Conclusions proposed
Margulies et al (2)	55	Supernatant / Urine	ELISA, Western blot, Zymography	MMP-2 levels increased in cancer in control
Davies et al (3)	49	Homogenized/ Tissue	Zymography	MMP-9 and MMP-2 associated with stage and grade
Gohji et al (4)	233	Serum	ELISA	High MMP-2 in advanced disease
Grignon et al (5)	42	Frozen/ tissue	Immunohistochemistry	TIMP-2 in tumour cells or stroma worse survival
Moses et al (6)	10	Supernatant/ tissue	Zymography	MMP-9 and MMP-2 detected in cancer patients
Bianco et al (7)	65	Bladder lavage	Zymography	MMP-9 associated with stage and grade
Gohji et al (8)	224	Serum	ELISA	High MMP-2/ TIMP-2 ratio low survival
Kanayama et al (9)	41	Frozen/ tissue	RT-PCR	MMP-2 & TIMP-2 associated with stage
Hamasaki et al (10)		T24 Cell line	RT-PCR, EIA	MMP-2/ TIMP-2 imbalance suggests progression
Furukawa et al (11)		UCT1 and 2 lines	RT-PCR	MMP-2 higher in invasive UCT-1 line implants
Ozdemir et al (12)	33	Supernatant/ urine	ELISA	MMP-9 detectable in cancer but not in controls
Ozdemir et al (12)	60	Paraffin/ tissue	Immunohistochemistry	MMP-9 associated with lysis of the basement membrane
Kanda et al (13)	61	Frozen/ tissue	Zymography	MMP-2 associated with grade, stage and result
Monier et al (14)	101	Urine	ELISA, immunotransfer	Imbalance between MMP-2, -9 and TIMP-2
Guan et al (15)	52	Serum	ELISA	MMP-9 associated with grade and stage
Eissa et al (16)	110	Urine/ bladder lavage	ELISA	MMP-9 increases sensitivity of urine cytology
Gakiopoulou et al (17)	106	Paraffin / tissue	Immunohistochemistry	TIMP-2 associated with grade, stage and result
Durkan et al (18)	118	Urina and paraffin tissue	ELISA Immunohistochemistry	MMP-9 associated with stage and size
Vasala et al (19)	54	Paraffin/ tissue	Immunohistochemistry	MMP-2 associated with stage and worse survival
Sumi et al (20)	20	Paraffin/ tissue	Immunohistochemistry	MMP-2 associated with grade and stage
Chaffer et al (21)		TSU-Pr1 lines	RT-PCR	MMP-9 & TIMP-2 increase in metastatic lines
Vasala et al (22)	94	Serum	ELISA	High pro-MMP-2 and TIMP-2 better prognosis
Vasala et al (23)	84	Serum	ELISA	Low TIMP-2 and MMP-2:TIMP-2 worse prognosis
Fernández et al (24)	530	Urine	ELISA and zymography	MMP-9 used to control patients
Angulo et al (present)	42	Peripheral blood mononuclear cells	Real time RT-PCR	MMP-9, MMP-2 and TIMP-2 allows diagnosis and molecular staging

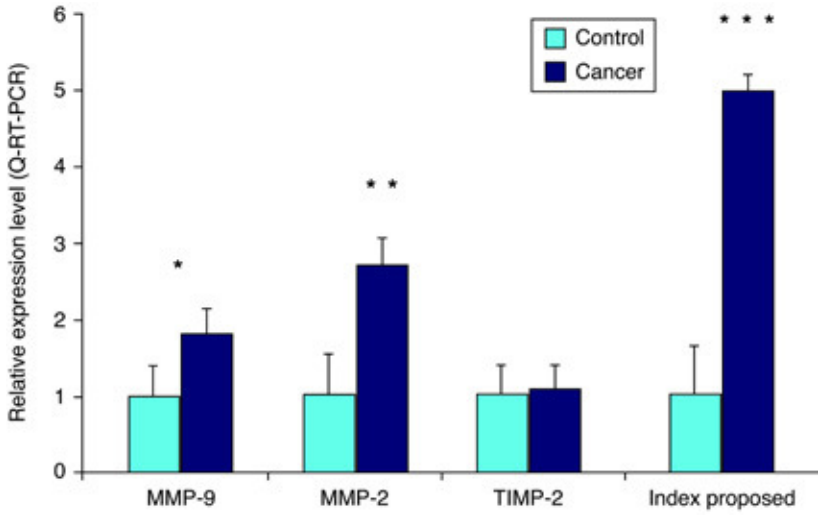

Figure 1 Comparison of MMP-2 expression, MMP-9 and TIMP-2 by quantitative RT-PCR in peripheral blood cells of patients with or without bladder cancer. The proposed index was defined as [(MMP-9 + MMP-2), TIMP-2 / (MMP-9 + MMP-2)]. Total RNA was used for synthesis of first strand cDNA. The results are expressed as relative expression levels (arbitrary units) versus control (without cancer). The mean ± SD for the 4 groups were compared using the Mann-Whitney U test. *p<0.05; **p<0.01; ***p<0.001.

Samples and RNA isolation

Samples were taken from peripheral blood of patients during surgery at the time of anesthetic induction and rapid morning extraction in the case of controls (9-12ml), collected in tubes containing EDTA, transported to the laboratory and processed within 2 hours. The mononuclear cell fraction was isolated as an intermediate layer by density gradient centrifugation in the presence of Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) for 20 minutes at 4°C. 2.100rpm Total RNA was prepared from sample cells using RNeasy (Qiagen, Valencia, CA, USA) according to the instructions suggested by the manufacturer. The production of RNA was determined spectrophotometrically at 260 and 280nm and the relationship of density (OD_{260}/OD_{280}) are measures to ensure the quality of RNA isolated using the spectrophotometer ND-100 (Nanodrop Technology). The average ratio was 1.8 to 2.1 for all samples. For IT, an aliquot with 1-2µg of total RNA from each sample was used for first strand cDNA in a total volume of 20-40µl using 0.75 ul (0.5 mg) of random primers (Promega, Madison, WI) and 0.5 ul (12.5 mmol / l), dNTPs mix (Ecogen, Spain), incubated at 70°C for 10 minutes and cooled immediately on ice to prevent renaturation. The following TI mixture was prepared in 10-20µl for each sample: 4-8µl IT MMLV buffer (5x) (Sigma), 0.5 ul (20 U) RNase inhibitor (RNasin, Promega) and 1µl (100 U) Moloney murine leukemia virus (MMLV) reverse transcriptase and incubated at 25°C for 5 minutes and 37°C for 50 minutes, the reaction was 95°C for 5 minutes to inactivate the TI.

Quantitative RT-PCR at real-time

We carried out PCR analysis using the iCycler Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). We used gene expression assays (TaqMan Assay, Applied Biosystems): MMP2, Hs00234422_m1; MMP9, Hs00234579_m1; TIMP2, Hs00234278_m1, and 18S, Hs99999901_s1. One hundred ng of template cDNA was added to 10µl TaqMan Universal PCR Master Mix (Applied Biosystems) and TaqMan Assay 1µl specific genes indicated for a final volume of 20µl. PCR reactions were incubated for 10 minutes at 95°C, after which the target was amplified with 45 two-phases cycles, for 15 seconds at 95°C and one minute at 60ºC. Each sample was analyzed in triplicate to verify results. Transcription levels, normalized to those of 18S (used as internal standard) to account for the variability in the amount of cDNA in each sample and relative expression levels were calculated using the $\Delta\Delta C_T$ method. The relative expression level of the target gene is given by $2^{-\Delta\Delta CT,}$ where $\Delta\Delta C_T = \Delta C_{T\,target\,gene} - \Delta C_{T18s}$. The comparative C_T calculation is valid if the amplification efficiency of target amplification tends to match the reference. All TaqMan gene expression assays have amplification efficiencies very close to 1.

Statistical method

The comparison of weighted scales between groups was performed with the Mann-Whitney U test and exact probability Fisher test. Analyses were performed using SPSS 13.0 for Windows (SPSS, Chicago, IL) and statistical significance was considered at 0.05.

Results

Expression of MMP-9, MMP-2 and TIMP-2 mRNA in peripheral blood

The sample analyzed in this study shows the steady-state levels of MMP-9, MMP-2 and TIMP-2. In peripheral blood of patients with bladder cancer standardized mRNA levels of

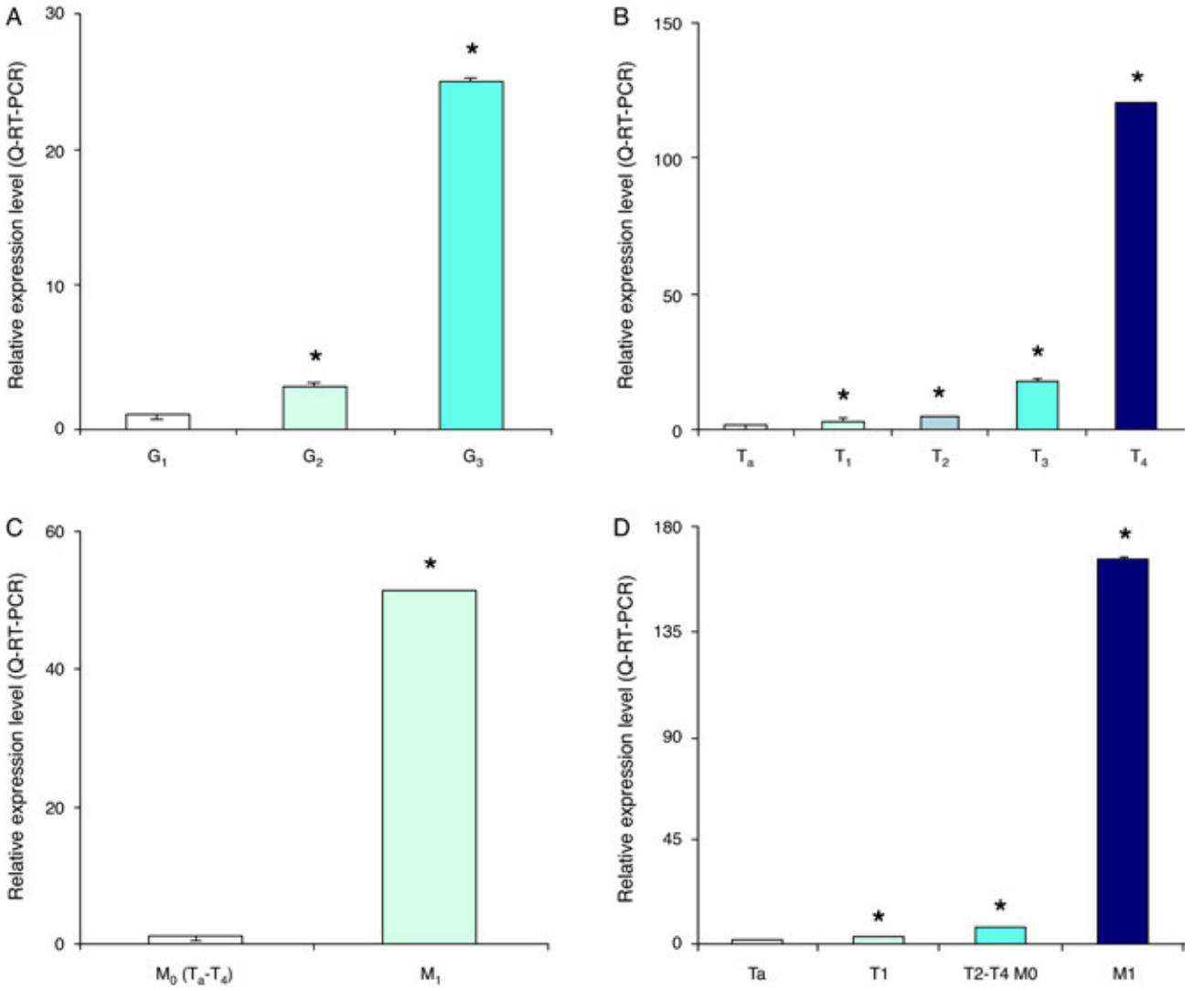

Figure 2 A. Expression of MMP-9 mRNA in peripheral blood cells of patients with bladder cancer of different histological grade. B. Clinical classification T. C. Metastatic state. D. Tumour stage. The results are expressed as relative expression levels (arbitrary units) versus reference sample A (*$p<0.05$).

MMP-9 and MMP-2 are respectively 1.82±0.6 and 2.7±0.6 times higher in cancer patients than controls ($p<0.05$), but normalized levels of TIMP-2 showed no differences between controls and patients with cancer (fig. 1).

In relation to the population of patients with transitional cell carcinoma of the bladder confirmed histologically, both mRNA levels of MMP-9 and MMP-2 were correlated with the assigned histological grade. MMP-9 is significantly higher in grade 3 tumors (1.97±0.34 times) compared with grade 1, yet there is no difference between the mRNA level of grade 2 (0.89±0.37 times) and grade 1 (fig. 2). MMP-2 level is higher in both grade 3 tumors (8.31±0.38 times) and grade 2 (8.80±0.33 times) compared to grade 1 (fig. 3). Standardized levels of TIMP-2 did not show the relative differences in the various classifications of degrees, although it showed a trend towards decreased expression in G3 tumors compared with G1 and / or G2 ($p=0.09$) (fig. 4).

In relation to clinical stage, both the degree of invasion into the bladder wall (T) as well as the presence of visceral metastases (M) have been analyzed and correlated with mRNA levels of MMP-9, MMP-2 and TIMP-2. Regarding the T classification, the level of MMP-9 is significantly higher in patients with T_4 tumors (13.6±0.36 times) and T_3 (2.3±0.3 times), but not in T_2 (1.15±0.38 times) or T_1 (1.43±0.27 times) when compared with T_a non-invasive lesions (fig. 2). The expression of MMP-2 correlates best with the depth of invasion, since the level of MMP-2 was significantly higher in T_4 (8.44±0.38 times), T_3 (7.7±0.32 times), T_2 (4.45±0.4) and T_1 (2.21±0.34) compared to T_a (fig. 3). Standardized levels of TIMP-2 did not show relative differences between classifications (fig. 4). As for the M classification, a statistically significant increase was observed in the expression of MMP-9 (9.6±0.2 times), MMP-2 (5.22±0.27 times) and TIMP-2 (1.97±0.22 times) when comparing

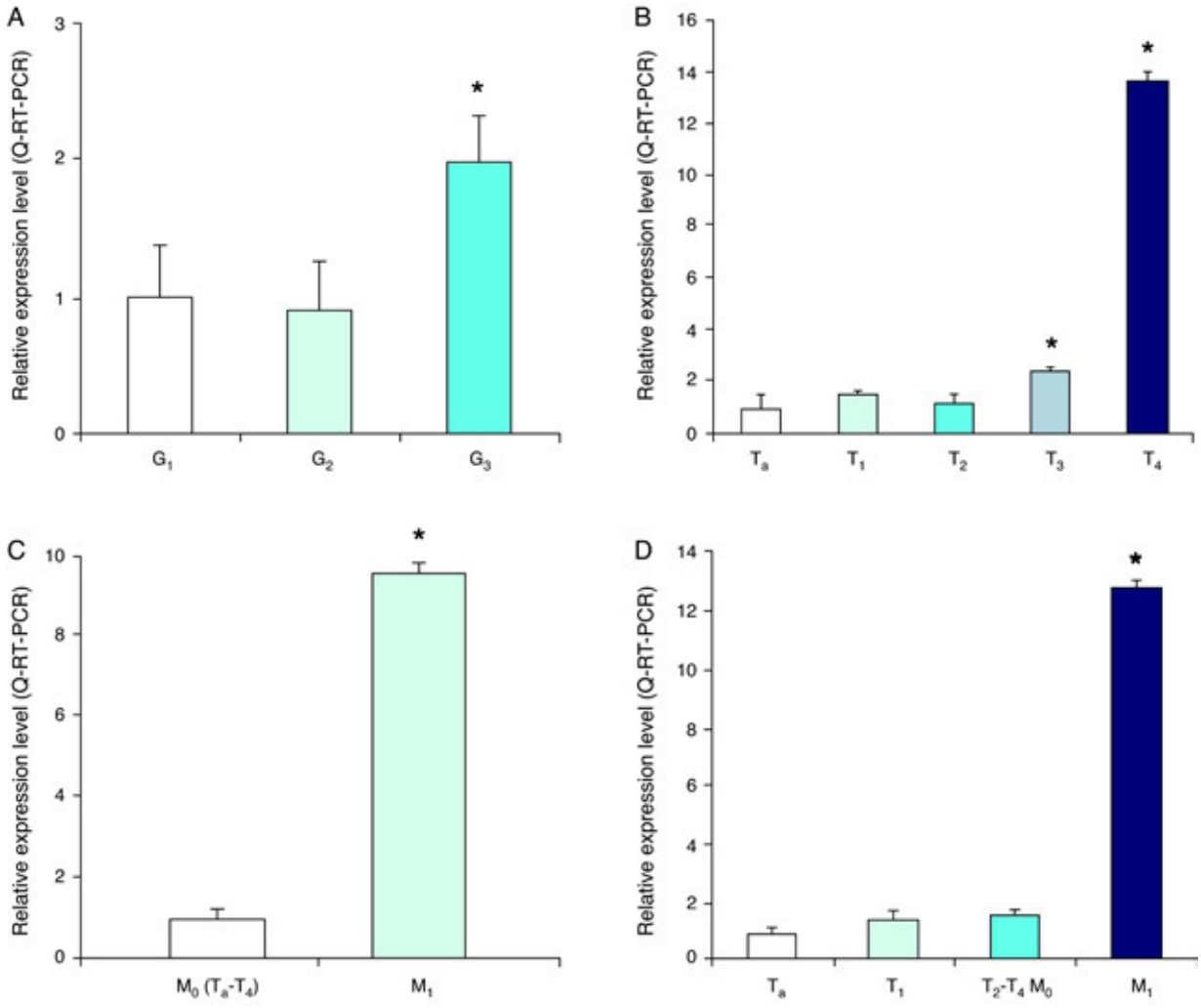

Figure 3 A. Expression of MMP-2 mRNA in peripheral blood cells of patients with bladder cancer of different histological grade. B. Clinical classification T. C. Metastatic state. D. Tumour stage. The results are expressed as relative expression levels (arbitrary units) versus reference sample A (*$p<0.05$).

metastatic disease ($M_{1)}$ with non-metastatic disease (M_0) (figs. 2-4).

For practical reasons, 4 different levels can be defined from a clinical perspective:

1. T_a (non-invasive).
2. T_1 lamina propria invasive (microinvasive)
3. T_{2-4} M_0 muscle invasive or beyond (deeply invasive) non-metastatic.
4. T_{2-4} M_1 (metastatic).

The ability of mRNA levels to distinguish between these 4 different levels of tumor invasion has also been investigated. MMP-9 is significantly higher in T_{2-4} M_1 (12.72±0.2 times), but not in T_{2-4} M_0 patients (1.5±0.21 times) and T_1 (1,43±0.27 times) when compared with T_a non-invasive (fig. 2). MMP-2 is significantly higher in T_{2-4} M_1 (12.62±0.25 times), T_{2-4} M_0 (4.44±0.26 times) and T_1 (2.21±0.34 times) with respect to T_a (fig. 3). TIMP-2 level is significantly higher in T_{2-4} M_1 (1.8±0.22 times) compared with T_{2-4} M_0 disease. However, there is no difference between levels of TIMP-2 T_{2-4} M_0, T_1 or T_a disease (fig. 4).

Index proposal to increase molecular staging capacity

An index based on the additive effect of gelatinases MMP-9 and MMP-2 and the inhibitory effect of TIMP-2 can be proposed, which counteracts the activity of MMP-2 (MMP-9 + MMP-2), TIMP-2 / (MMP-9 + MMP-2). Of course, each individual gene expression must be corrected in relation to the expression of housekeeping gene, 18S. Using this relationship, the relative combined gene expression is significantly higher in tumor than in controls (4.95±0.21 times, $p<0.05$).

In addition, relative quantification using the comparative CT method for this index increases the ability to segregate patients with tumors of different grades and affecting all

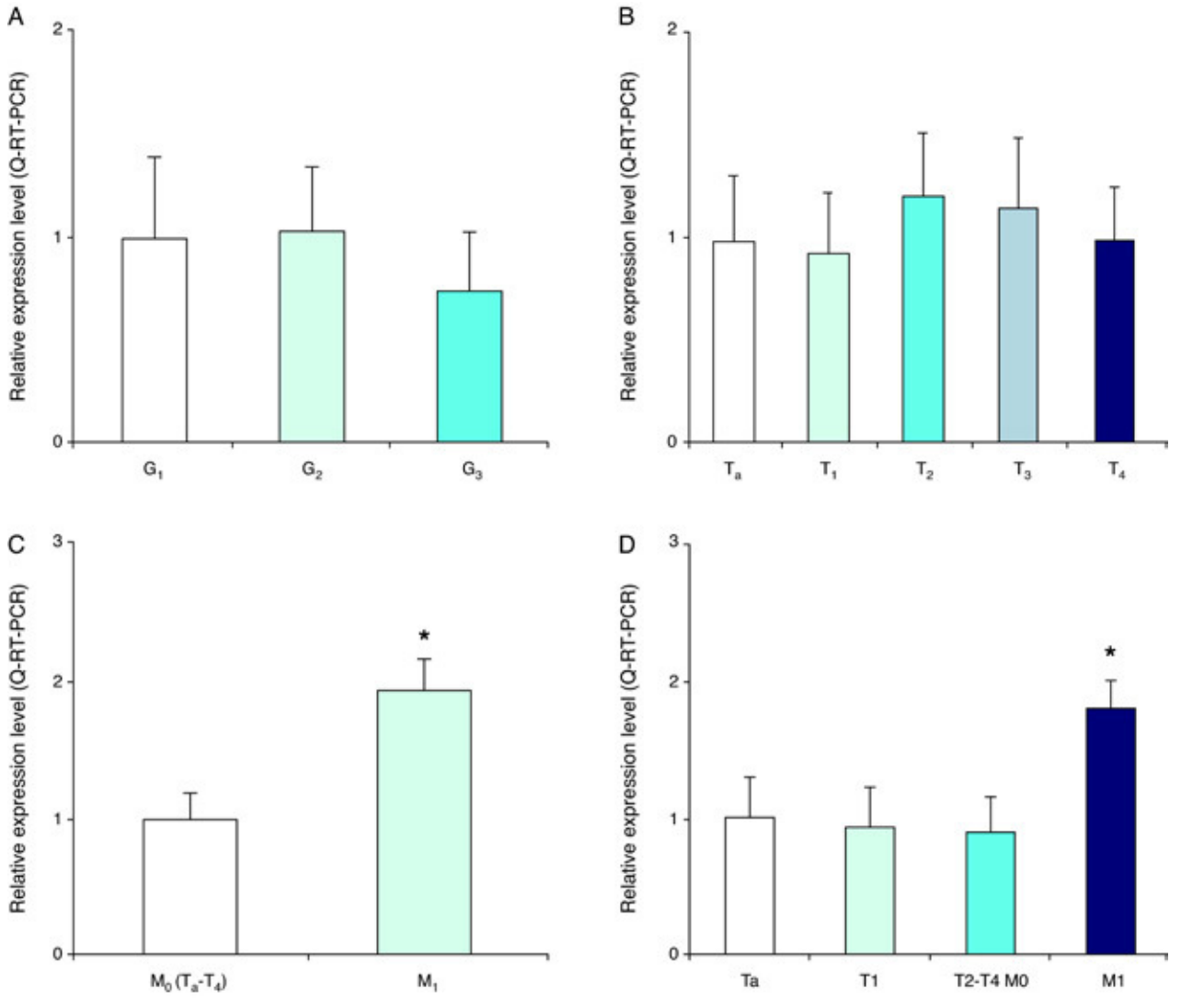

Figure 4 A. Expression of TIMP-2 mRNA in peripheral blood cells of patients with bladder cancer of different histological grade. B. Clinical classification T. C. Metastatic state. D. Tumour stage. The results are expressed as relative expression levels (arbitrary units) versus the reference sample (*$p<0.05$).

levels of mural invasion, and also to distinguish metastatic disease. The index also correlates with tumor grade, and it is higher in both grade 3 tumors (25.2±0.26 times) and grade 2 (2.98±0.18 times) compared to grade 1 (fig. 5). The depth of invasion is significantly higher in T_4 (120.1±0.36 times), T_3 (18.12±0.1 times), T_2 (5.14±0.36 times) and T_1 (3.1±0.07 times) than T_a (fig. 5), and the difference is even greater in the presence of metastatic disease (51.7±0.24 times) compared to non-metastatic disease (M_0) (fig. 5). In short, the rate is significantly higher in $T_{2\text{-}4}$ M_1 (166±0.15 times), $T_{2\text{-}4}$ M_0 (6.76±0.36 times) and T_1 (3.06±0.08 times) than T_a (fig. 5).

Discussion

Several authors have studied the role of MMP and more specifically of gelatinases and TIMP-2 in bladder cancer.[2-24] Different approaches have been applied to study the activity of these enzymes in ECM degradation. They have often been sought in cancerous tissues of the bladder, sometimes in barbotage urine samples, and more recently in the serum of patients. To our knowledge, this is the first study that analyzes the expression of metalloproteinase mRNA in peripheral blood of patients with and without transitional cell carcinoma of the bladder.

Marguiles et al were pioneers in suggesting that bladder cancer is correlated with increased collagenase activity.[2] Later, Davies et al reported the expression pro-MMP-9 and pro-MMP-2 significantly correlated with the degree of bladder tumor using quantitative gelatin zymography and *in situ* hybridization.[3] They also found that MMP-9 levels were significantly higher in bladder cancer than in controls. We collaborated to carry out the first immunohistochemical analysis of gelatinases in bladder cancer and we observed immunostaining in approximately 3 or 4 invasive bladder tumors.[5] They were located predominantly but not exclusively in cancer cells. In fact, weak diffuse staining

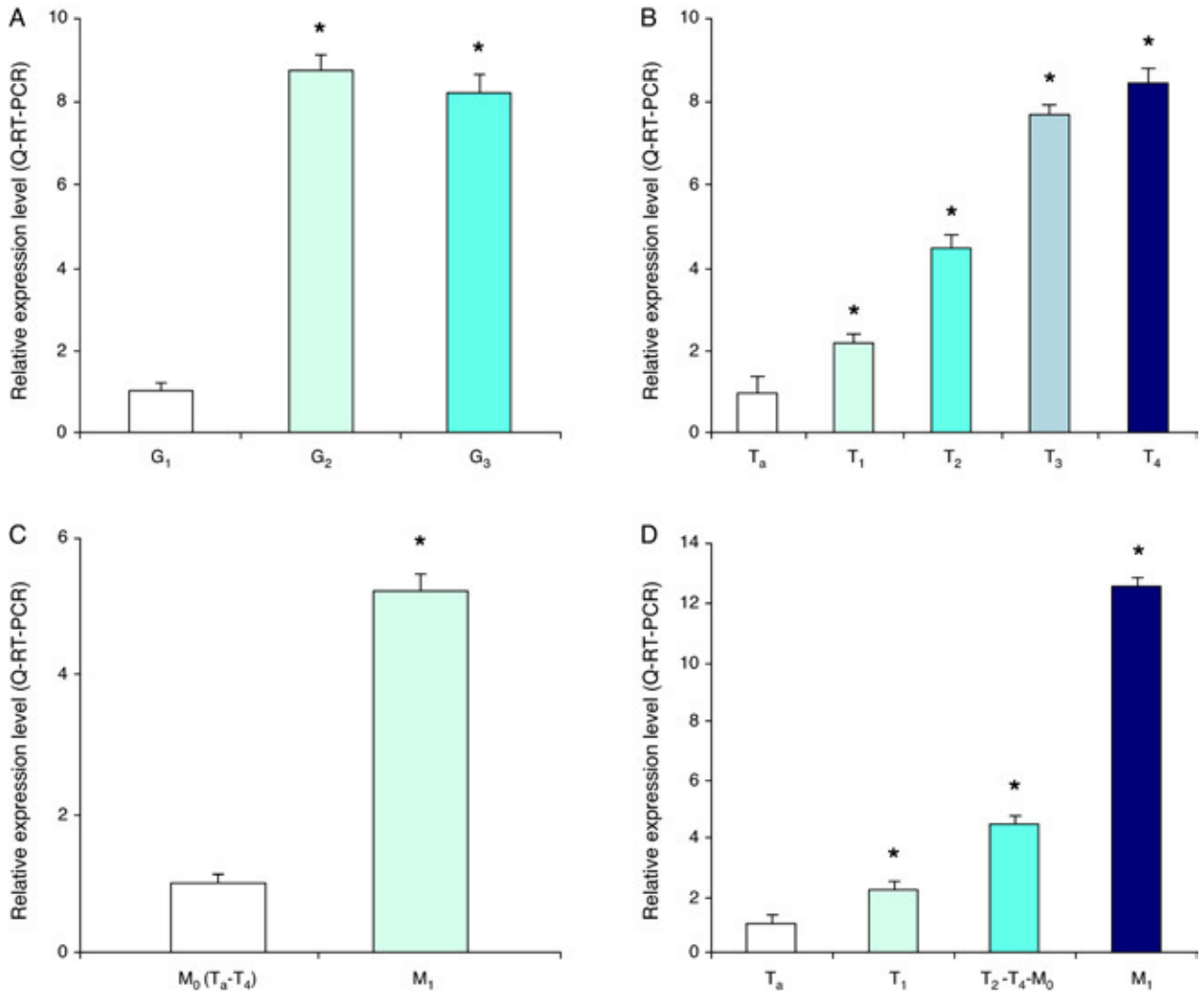

Figure 5 A. Relative quantification using the ratio [(MMP-9 + MMP-2), TIMP-2 / (MMP-9 + MMP-2)] mRNA in peripheral blood cells of patients with bladder cancer of different histological grade. B. Clinical classification T. C. Metastatic state. D. Tumour stage. The results are expressed as relative expression levels (arbitrary units) versus the reference sample (*$p<0.05$).

appeared in the stroma. We could not identify a correlation between MMP expression and stage-2 or MMP-9. However, the lesions were uniformly high-grade muscle-invasive carcinomas. Moreover, the expression of gelatinase could not be correlated with survival. TIMP-2 was detected in both neoplasic cells in the stroma, and associated with the absence of immunostaining for type IV collagen and with significantly worse survival.[5] The high level of stromal TIMP-2 correlated with poor survival and was associated with extensive loss of basement membrane. Many other studies have evaluated the prognostic implications of the immunohistochemical determination of ECM components in bladder cancer, and basically confirmed the findings of Grignon et al.[12,17-20] The increased expression of MMP-2 and TIMP-2 may determine prognosis.[3-5,9,13,17,19,20] In fact, a study analyzing 1,176 cancer-related genes by RT-PCR in cancerous bladder tissue showed that MMP-2 and TIMP-2 were expressed in 100%of patients who died of as a result of the disease.[25] Research on other MMP and matrilysin (MMP-7) also appear promising for predicting the outcome of patients with bladder cancer,[26] although the same cannot be said for collagenase 3 (MMP-13).[27]

Bianco et al measured the expression of gelatinases in exfoliated bladder cells and found a significant correlation between expression of pro-MMP-9 and the presence of malignancy.[7] Other authors have investigated MMP-2 and MMP-9 as markers of bladder cancer, either using gelatin zymography, immunoblotting or ELISA techniques in urine.[2,6,12,18] MMP serological studies have also been carried out. Naur et al found that patients with advanced or metastatic tumors were almost double that of TIMP-1 compared with controls.[28] Gohji et al found no difference in circulating levels of MMP-2 between superficial tumors and controls.[4] Subsequently, this group reported a higher risk of recurrence and progression when the MMP-2/ TIMP-2 ratio was high.[8]

There is no doubt that there is a regulatory process between metalloproteinases and tissue inhibitors in tumor progression and metastasis development. Numerous reports show that the gene expression of MMP and activation are related to the potential for invasion of cancer cells. Initially it was believed that MMPs were produced exclusively by tumor cells. This concept was soon replaced by the more widespread belief that some MMPs are produced primarily by the stroma, such as stromelysin (MMP-3) produced by tumor fibroblasts. Similarly, TIMP-1 and TIMP-2 appear to be produced in response to the receptor, counteracting the activity of collagenase and gelatinase, and have been detected in both neoplastic and stromal cells. The expression of TIMP-2 seems to have contradictory functions as a growth factor. On the one hand, it can facilitate the development of metastasis, yet on the other hand, it may inhibit angiogenesis as a homeostatic response to high MMP-2 expression, thereby limiting the development of metastasis. Today, it is increasingly evident that both MMP-2 and TIMP-2 are produced not only in intratumoral stromal cells, but also in the peritumoral stromal cells and neoplastic tissues. In fact, the role of MMPs in non-neoplasic conditions with greater angiogenesis such as pregnancy, wound healing and inflammatory diseases has been well-documented.[29]

Of course, discrepancies between MMP-2 levels in steady state mRNA and enzyme activity can be explained by different regulation at the posttranscriptional level. The expression of both gelatinase mRNA in peripheral blood confirmed healthy controls suggests non-neoplasic production. Several types of cells may act as sources for the production of MMP, not only lymphocytes, and possibly tumor-associated lymphocytes, but monocytes and tissue macrophages. The local inflammatory reaction that is often associated with advanced neoplasia may also stimulate the overexpression of MMP-2, MMP-9 and TIMP-2 mRNA. There are concerns that the presence of micrometastases or circulating tumor cells may contribute to increased expression of gelatinase in advanced cases, however, that fact can not be expected in healthy controls or non-invasive intraepithelial neoplasia bladder.

The RT-PCR expression of mRNA in peripheral blood of MMP should be investigated in a clinical setting. Our results suggest that it can be used as a marker of ECM remodeling activity associated with the presence of bladder cancer, and can be correlated with tumor grade, depth of invasion and metastasis. The increased gelatinase activity may contribute to tumor aggressiveness, not necessarily through direct tumor invasiveness, but by other effects such as neoangiogenesis.[30] The prognostic implications of this technique for molecular staging in bladder cancer seem to be very promising.

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Conflict of interest

The authors declare that they have no conflict of interest

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