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Loss of expression of TIMP3 in clear cell renal cell carcinoma

Damien Masson [a,b], *Nathalie Rioux-Leclercq* [a], *Patricia Fergelot* [a], *Florence Jouan* [a], *Stéphanie Mottier* [a], *Sandrine Théoleyre* [a], *Kalyane Bach-Ngohou* [b], *Jean-Jacques Patard* [a], *Marc G. Denis* [a,*]

[a] *CNRS UMR 6061, Institut de Génétique et Développement, Université Rennes 1, 35043 Rennes, France*
[b] *INSERM U913, Institut des Maladies de l'appareil digestif, CHU Nantes, 44035 Nantes, France*

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ABSTRACT

Aims: In clear cell renal cell carcinoma (CCRCC), vascular endothelial growth factor (VEGF) represents the central positive mediator of tumour angiogenesis while VEGF receptor (VEGFR) is the primary target of anti-angiogenic therapies. TIMP3 is a physiological VEGFR-2 antagonist and thus could be considered as an anti-angiogenic factor. We therefore determined the status of this physiological inhibitor in CCRCC.

Patients and methods: Archival tumour from 105 patients was studied. TIMP3 expression was analysed using immuno-histochemistry and real-time RT-PCR. Results were correlated with clinicopathological variables. To analyse the mechanisms of gene silencing involved, we performed Multiplex Ligation-dependent Probe Amplification (MLPA) and methylation-specific MLPA (MS-MLPA). At last, we evaluated the main upstream pathway described implicating TGFβRII, which induces TIMP3 expression.

Results: A down-expression of TIMP3, determined by immunohistochemistry, affected 100/105 renal cancers (95.2%). TIMP3 mRNA levels were significantly lower in high-grade tumours. Loss of heterozygosity of the *TIMP3* gene was observed in 8 tumours (7.6%) and the 5′CpG island of the *TIMP3* promoter was found to be methylated in 25 tumours (23.8%). A down-expression of TGFβRII was found in 85/105 CCRCCs (80.9%). A significant correlation was found between TIMP3 expression and TGFβRII expression.

Conclusions: This is the first demonstration that the loss of TIMP3 expression is observed in almost all CCRCCs. This loss of expression is a common molecular event in CCRCC. It may be an important initiation step for tumour development in a complex process implicating loss of heterozygosity on chromosome 22q, promoter hyper-methylation and inactivation of the TGFβRII pathway.

1. Introduction

Clear cell renal cell carcinoma (CCRCC) accounts for ~85% of renal cancers.[1] The loss of function of the von Hippel–Lindau (*VHL*) tumour suppressor gene[2–4] is involved in ~70% of sporadic CCRCCs.[5–7] The biallelic disruption of the *VHL* gene leads to dysregulation of the direct downstream Hypoxia Inductible Factor (HIF) target genes,[8] and among the growth factors involved in the VHL pathway, vascular endothelial growth factor (VEGF) seems to be the central positive mediator of tumour angiogenesis. For this reason, VEGF has recently become the primary target of novel anti-angiogenic treatments.[9,10]

* *Corresponding author:* Address: INSERM U913, Faculté de Médecine, 1 rue Gaston Veil, 44035 Nantes cedex, France. Tel.: +33 240 41 29 59; fax: +33 240 08 39 91.
E-mail address: marc.denis@univ-nantes.fr (M.G. Denis).
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VEGF exerts its activity via two distinct tyrosine kinase receptors: VEGFR-1 and VEGFR-2.[11,12] The only physiological antagonist of VEGFR-2 identified so far is the Tissue Inhibitor of Metalloproteinase-3 (TIMP3).[13] TIMP3 is a member of a family of endogenous matrix metalloproteinases (MMP) inhibitors.[14] TIMP3 contributes to VEGF-mediated angiogenesis regulation by binding directly to VEGFR-2 and inhibiting the downstream signalling pathways required for cell stimulation. While VEGF signalling is already strongly enhanced in most CCRCC, loss of TIMP3 might have an additional impact on the activation of this pathway. Indeed, recent studies on methylation-associated silencing of *TIMP3* suggested a tumour suppressor role in different cancers and the loss of *TIMP3* has been related to the acquisition of tumourigenesis.[15–18] In the area of new anti-angiogenic drugs targeting VEGFR, it is of importance to analyse the expression of this physiological anti-angiogenic factor and its relationship with usual clinical and pathologic prognostic factors. In this work, we addressed these important issues by combining several approaches, i.e. immuno-histochemistry, real-time RT-PCR and MLPA analysis in a series of CCRCCs. Using archived tumour and normal renal tissue samples and long-term follow-up on a cohort of 105 CCRCC patients, we evaluated the association of TIMP3 expression with pathologic features and cancer-specific survival. Our findings indicate that the loss of TIMP3 expression is observed in almost all CCRCCs. Despite TIMP3 expression is not a significant prognostic factor, it may be an important initiation step for tumour development.

2. Patients and methods

2.1. Patient population

The study was based on the analysis of 105 consecutive patients who underwent radical or partial nephrectomy for CCRCC from 2002 to 2005. The human Ethics Committee of Rennes University Medical School and Hospital approved this prospective study and informed consent was obtained from all patients. The clinicopathological data of the patients are summarised in Table 1. Survival information, overall and cancer specific, was available for all patients. Mean follow-up was 32 ± 16 months. During this period, 34 patients (32.4%) died, including 29 (27.6%) from a cancer-related cause.

2.2. Tumour samples and immunostaining procedure

Formalin-fixed paraffin sections were stained with hematoxylin and eosin–safran for light microscopy. Histological parameters including nuclear Fuhrman grade and tumour stage were noted.

For each case of CCRCC, a representative slide of the tumour with the highest nuclear Fuhrman grade and the corresponding paraffin block were selected for TIMP3 and TGFβRII immunostaining with the monoclonal anti-TIMP3 (1/100, clone 136-13H4, Calbiochem, Darmstadt, Germany) or the monoclonal anti-TGFβRII (1/100, clone sc-400, Santa Cruz Biotechnology, Santa Cruz, USA). A biotin–streptavidin detection system (Dako, Glostrup, Denmark) was then used with diaminobenzidine as the chromogen (Sigma–Aldrich, Lyon, France).

Table 1 – Clinicopathologic characteristics of the 105 CCRCC patients.

Age (years)	64 (21–83)
Sex (M/F)	59/46
Tumour size (cm)	7 (1.5–18)
Fuhrman grade	
1	2 (1.9%)
2	38 (36.2%)
3	37 (35.2%)
4	28 (26.7%)
Tumour stage	
1	41 (39%)
2	19 (18.1%)
3	41 (39%)
4	4 (3.8%)
Lymph node status	
0	93 (88.6%)
1	8 (7.6%)
2	4 (3.8%)
Metastasis status	
0	76 (72.4%)
1	29 (27.6%)

Values are presented as median (minimum–maximum) for continuous variables and number of patients (%) for categorical variables.

Negative control was performed by omitting the primary antibody. The intensity of TIMP3 or TGFβRII reactivity of tumour cells was scored using a four-scale model. Intense membranous and cytoplasmic staining was considered as 3+; staining with a lower intensity was considered as 2+; faint or light brown membranous or cytoplasmic staining was scored as 1+; and no staining was scored as 0. For each sample, the proportion of positive tumoural cells was determined. For statistical analysis, a TIMP3 and a TGBβRII score (= intensity × percent of tumoural cells) were attributed to each tumour.

2.3. RNA samples and real-time RT-PCR analysis

Total RNA was extracted from snap-frozen tumours using the Qiamp total RNA kit (QIAGEN, Hilden, Germany) after histological control. Real-time PCR was performed according to previous reports with some modifications.[19] The amplification conditions of the TIMP3, TGFβRII, and β-actin templates were optimised for the Rotorgene 3000 instrument (Corbett Research, Biolabo, Archamps, France). Primers were designed from the sequence of the human cDNAs. The sequences of these primers were as follows: TCTGCAACTCCGACATCGT (forward) and TTGGTGAAGCCTCGGTACAT (reverse) for TIMP3, TCCTTCAAGCAGACCGATGT (forward) and CACCTTGGAACCAAATGGAG (reverse) for TGFβRII and CCTTCCTGGGCATGGAGTCCTG (forward) and GGAGCAATGATCTTGATCTTC (reverse) for β-actin. PCR amplifications were performed using SYBR Green I master mix (Roche Diagnostics, Mannheim, Germany). To exclude primer–dimer artefacts, fluorescence was measured at a temperature above the melting point of primer–dimer and below the melting point of the specific PCR product (85° for β-actin, 83 °C for TIMP3 and 79 °C for TGFβRII).

External standard curves were generated with serial 5-fold dilutions of cDNA samples prepared with RNA extracted from the RCC4 CCRCC cell line. The relative amount of TIMP3, TGFβRII and β-actin transcripts was calculated from these standard curves using the RotorGene software. For each sample, the ratio between the relative amount of each specific transcript and β-actin was then calculated to compensate for variations in the quantity and quality of starting mRNA.

2.4. DNA sample and MLPA analysis

DNA was extracted from snap-frozen tumours using the iPrep gDNA Tissue kit (Invitrogen, Carlsbad, CA, USA), after histological control. The Multiplex Ligation-dependent Probe Amplification (MLPA) method and the methylation-specific MLPA (MS-MLPA) method were performed as described by Schouten and colleagues[20] using the probe-mixture included in the SALSA MS-MLPA kit ME001B Tumour suppressor-1 (MRC-Holland, Amsterdam, the Netherlands). Details on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com.

The SALSA MS-MLPA kit ME001B Tumour suppressor-1 allows to detect deletions or duplications in the *TIMP3* gene. The TIMP3-probes of the kit target the promoter region of the *TIMP3* gene. Genomic DNA (50–200 ng) was denatured and the probes were allowed to hybridise (16 h at 60 °C). PCR was performed according to the manufacturers' instructions. Fragments were separated by electrophoresis on an Applied 3130XL capillary sequencer and quantified using the GeneMarker version 1.6 software (SoftGenetics LLC). For copy number detection, normal control DNA samples were included in each set of MLPA experiments. Interpretation was based on the comparison of peak heights between the control DNA and the tumour sample. Cut-off levels for loss of relative copy number were set at 0.75.

The SALSA MS-MLPA kit ME001B Tumour suppressor-1 allows detecting aberrant methylation of CpG-Islands located in the promoter region of the *TIMP3* gene. The TIMP3 probes used in this study for methylation quantification analysis contained two HhaI restriction site in the target recognition sequence. Following probe hybridisation, the samples were divided in two and one half of the samples was ligated, whereas for the other part of the sample ligation was combined with Hha1. This digestion resulted in ligation of only the methylated sequences. PCR was performed on both parts of the samples and analysed by electrophoresis as described above. Reference unmethylated DNAs, isolated from blood from healthy volunteers, were included in each set of MLPA experiments. The unmethylated DNA will not generate a signal, and a normal probe signal will be detected if the site is methylated. Methylation positive (HT29-Cl.16E cell line) and negative (Caco2 cell line) controls were included in the MS-MLPA analysis.

2.5. Statistical analysis

Analyses were performed using the software Statistical Package for Social Science, version 15.0 (SPSS, Chicago, IL). The non parametric Kruskal–Wallis test followed by Dunn's post-test were used to evaluate the association between TIMP3 and TGFβRII expression, between TIMP3 or TGFβRII expression and the clinicopathological parameters. The Mann–Whitney test was used to compare TIMP3 or TGFβR2 expression with tumour stage or size. The Wilcoxon test was used to compare TIMP3 expression in tumour and matched normal renal tissue. Disease-specific survival was defined as the interval between primary surgery and the last follow-up visit or disease-related death. Survival curves were plotted using the Kaplan–Meier method, and statistical significance was assessed using the log-rank test. A p value less than 0.05 was considered as significant.

3. Results

3.1. TIMP3 expression

TIMP3 expression was first assessed by immuno-histochemistry. Tumour samples of 105 patients with CCRCC were analysed. In normal renal tissues, TIMP3 exhibited strong cytoplasmic and membranous staining in normal tubular epithelial cells and in lymphocytes (Fig. 1A). A weak TIMP3 staining was observed in the stroma and blood vessels of normal renal tissues. In CCRCC tissues, TIMP3 exhibited absent to strong staining in tumour cells, depending on the tumours (Fig. 1B–D). The majority of the tested tumours (62.9%; 66/105) showed no (0; $n = 41$) or weak (1+ in less than 60% of tumour cells; $n = 25$) cytoplasmic and membranous staining for TIMP3. Other tumours (37.1%; 39/105) revealed moderate (2+ in 5–100% of tumour cells; $n = 32$) or strong (3+ in 10–100% of tumour cells; $n = 7$) TIMP3 expression. Altogether, a reduced TIMP3 expression in the tumour as compared to normal renal tissue was observed in 100 out of the 105 tested patients. The remaining 5 tumours showed moderate (2+) or strong expression (3+) of TIMP3 in 100% of tumour cells. The statistical analysis revealed that loss of TIMP3 expression did not correlate with any histological parameters including tumour size, nuclear Fuhrman grade and tumour stage or clinicopathological data such as the metastasis status. Cumulative survival curves were calculated. Again, no statistical difference was obtained from Kaplan–Meier survival curve analysis with regard to protein TIMP3 expression (not shown).

This decreased expression of TIMP3 was confirmed at the mRNA level by analysing an independent series of 26 CCRCC. In 21 of 26 cases (80.8%), TIMP3 mRNA level was significantly lower ($p < 0.001$) in tumours than in matched normal renal tissue (Fig. 2A).

We then analysed TIMP3 expression at the mRNA level in relation to clinicopathological parameters in 61 (out of 105) CCRCCs by quantitative RT-PCR, when adequate RNA material was available. The relative TIMP3 mRNA expression did not correlate with histological parameters including tumour stage or clinicopathological data such as metastasis status. However, this analysis revealed that TIMP3 mRNA levels were significantly lower in Fuhrman 4 grade (4-fold decrease; $p < 0.05$) (Fig. 2B). Cumulative survival curves were calculated according to the Kaplan–Meier method. We compared the survival data of 61 patients with CCRCC in relation to TIMP3 mRNA levels. It appeared that although not statistically significant there was a trend towards better survival in CCRCC patients exhibiting high mRNA TIMP3 expression (not shown) ($p = 0.089$).

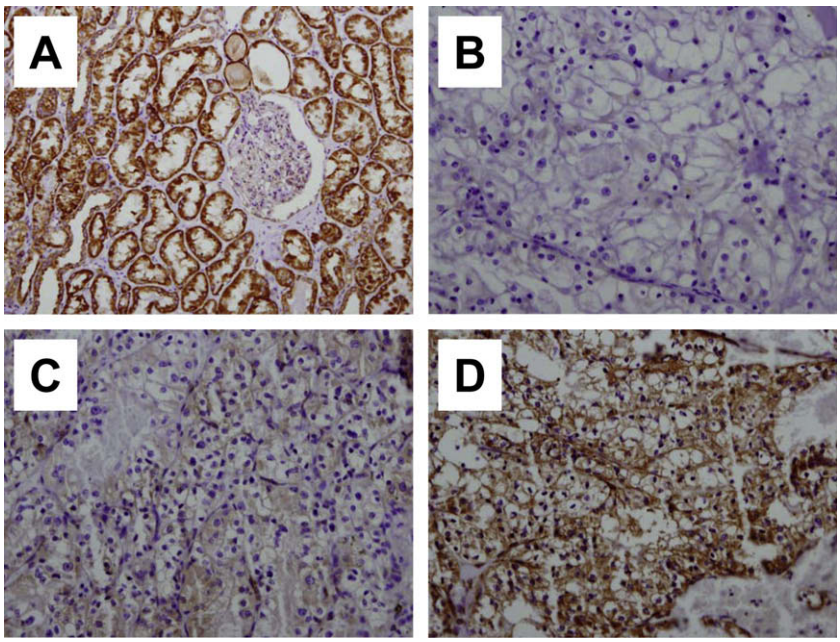

Fig. 1 – TIMP3 immunohistochemistry in CCRCC. (A) TIMP3 expression in normal renal tissue. (B–D) Representative immunostaining for TIMP3 in CCRCC: (B) no expression, (C) weak expression, (D) high expression. Original magnification was ×40 for (A) and ×100 for (B–D).

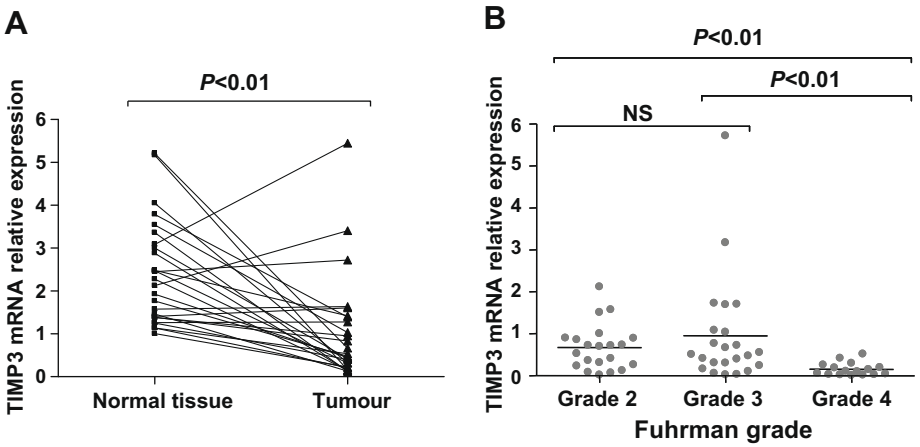

Fig. 2 – Relative expression of TIMP3 in 60 CCRCC, according to Fuhrman grade (grade 2: *n* = 21; grade 3: *n* = 23; grade 4: *n* = 16). TIMP3 expression was measured by real time PCR and normalised to β-actin. The grade 1 tumour was not represented. Horizontal lines represent mean values. Kruskal–Wallis tests were performed and the corresponding *p* values are presented.

3.2. *Mechanisms underlying TIMP3 inactivation: loss of heterozygosity and promoter hypermethylation*

To detect copy number changes in the TIMP3 gene, MLPA analysis was carried out on 105 CCRCCs. LOH of the TIMP3 gene on chromosome 22q was observed in 8 tumours (Fig. 3B). To analyse the mechanism of gene silencing by promoter hypermethylation of TIMP3, we performed MS-MLPA analysis in 105 CCRCC. The 5′CpG island of TIMP3 was found to be methylated in 25 of 105 tumours (Fig. 3D). Altogether, 33 epigenetic or genetic alterations were detected in the 100 tumours exhibiting loss of TIMP3 expression. In contrast to other genes, TIMP3 promoter hypermethylation or LOH on chromosome 22q does not explain the loss of expression of the TIMP3 gene in the overwhelming majority of the CCRCC analysed.

3.3. *Mechanism underlying down-expression of TIMP3: TGFβRII expression*

TGF-β1 has been shown to induce TIMP3 expression partly through the PI3K/Akt and Smad pathways in human chondrocytes.[21] We therefore evaluated the association between TGFβRII and TIMP3 expression both at the protein and the mRNA levels in our CCRCC specimens.

TGFβRII expression was first assessed by immunohistochemistry. In normal renal tissues, TGFβRII exhibited cytoplasmic and membranous staining in normal tubular

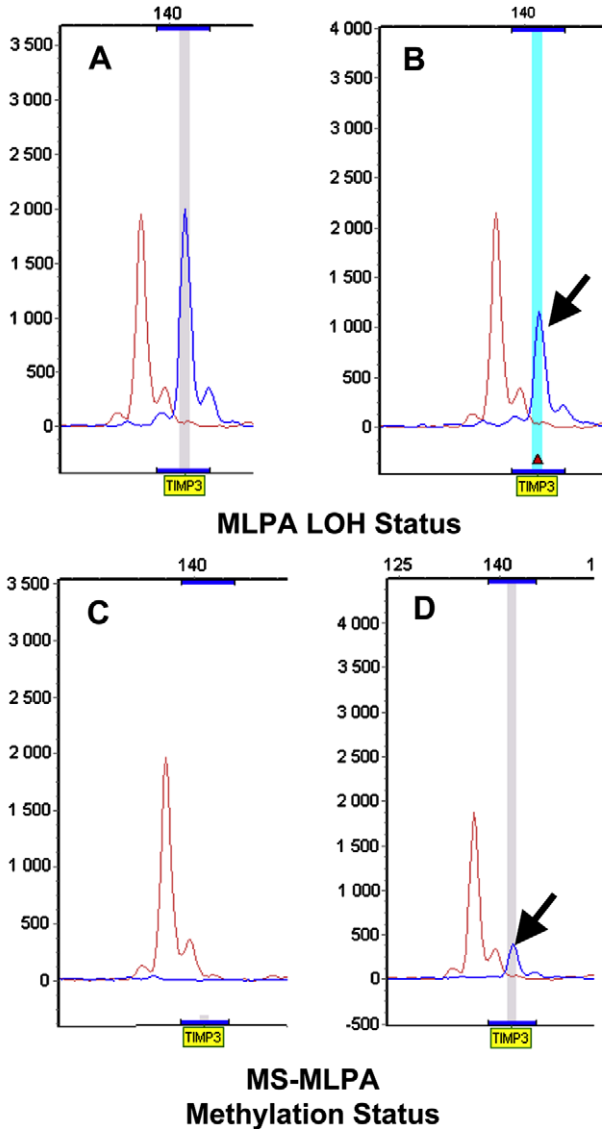

Fig. 3 – Representative results of detection of LOH and methylation status in CCRCC. LOH status on TIMP3 gene promoter: the red line corresponds to normal DNA. The blue line corresponds to tumoural DNA. (A) Illustration of a tumour carrying no deletion. (B) Tumour presenting a deletion. Methylation status: The red line corresponds to undigested DNA. The blue line corresponds to the same sample but digested with Hha1. (C) Pattern generated from unmethylated tumour. Following MS-MLPA, no signal is generated from the probe. (D) Pattern generated by MS-MLPA by methylated tumour. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

epithelial cells (Fig. 4A). In CCRCC, TGFβRII expression was heterogeneous (Fig. 4B–D). This analysis showed that 32.4% (34/105) of the CCRCC cases presented no (0; $n = 2$) or weak (1+ in 100% of tumour cells; $n = 32$) cytoplasmic and membranous staining for TGFβRII. 67.6% (71/105) of the cases revealed a moderate (2+ in 20–100% of tumour cells; $n = 33$) to strong (3+ in 20–100% of tumour cells; $n = 38$) TGFβRII expression. Altogether, 85/105 renal cancers presented a decrease in staining intensity or/and percentage of TGFβRII expressing cells. In contrast, 20 of 105 renal cancers showed moderate (2+) or strong expression (3+) of TGFβRII in 100% of tumour cells.

The loss of TGFβRII expression did not correlate with any histological parameter including tumour size, nuclear Fuhrman grade and tumour stage or clinicopathological data such as the presence of distant metastases. No statistical difference was obtained from Kaplan–Meier survival curve analysis with regard to protein TGFβRII expression (not shown).

We then quantified TGFβRII mRNA transcripts by qRT-PCR in the 61 CCRCCs tested for TIMP3 expression. The statistical analysis revealed that TGFβRII mRNA levels were significantly lower in grade 4 tumours (3-fold decrease; $p < 0.001$) as compared to Fuhrman 2 and 3 tumours (Fig. 5). Cumulative survival curves were calculated according to the Kaplan–Meier method. When comparing survival data of 61 CCRCCs patients in relation to TGFβRII mRNA levels, there was no significant relationship between TGFβRII mRNA expression and survival (not shown).

3.4. *Relation between TIMP3 and TGFβRII expression*

Regression analysis was carried out to analyse the relationship between TIMP3 and TGFβRII expression both at the protein and at the mRNA levels. A significant correlation was found between TIMP3 protein expression and TGFβRII protein expression determined by immuno-histochemistry ($r = 0.40$, $p < 0.001$). Based on this result, the cohort was split into three groups according to the TGFβRII score (low score: 0–99; medium score: 100–199; high score; 200–300). High TGFβRII score tumours had a higher TIMP3 protein expression than medium or low TGFβRII score tumours (Fig. 6A). We also identified a strong correlation between TIMP3 and TGFβRII expression at the mRNA level ($r = 0.81$, $p < 0.001$) (Fig. 6B).

4. Discussion

In CCRCC, tumour angiogenesis is an important and complex process that is regulated by a balance between pro-angiogenic and anti-angiogenic factors.[22] Most of these activators are ligands of kinase receptors, such as VEGF, and the only physiological antagonist of VEGFR-2 identified so far is TIMP3 which could be considered as an anti-angiogenic factor. In the present study, we observed a significant down-regulation of TIMP3 expression in 95% of CCRCCs compared to normal renal tissues which is the tissue that normally expresses the highest TIMP3 levels,[23] suggesting that the loss of TIMP3 is an important event in pathogenesis and progression of renal cancer. We then investigated the relationship between loss of TIMP3 expression and survival in patient with CCRCC. Unfortunately, in our cohort, the loss of TIMP3 protein expression did not correlate with any histological parameters. However, at the transcriptional level, TIMP3 was significantly lower in Fuhrman 4 grade tumours. Although loss of expression was not associated with a decreased disease-specific survival after radical nephrectomy, there was a trend towards an association between low TIMP3 expression and early tumour progression.

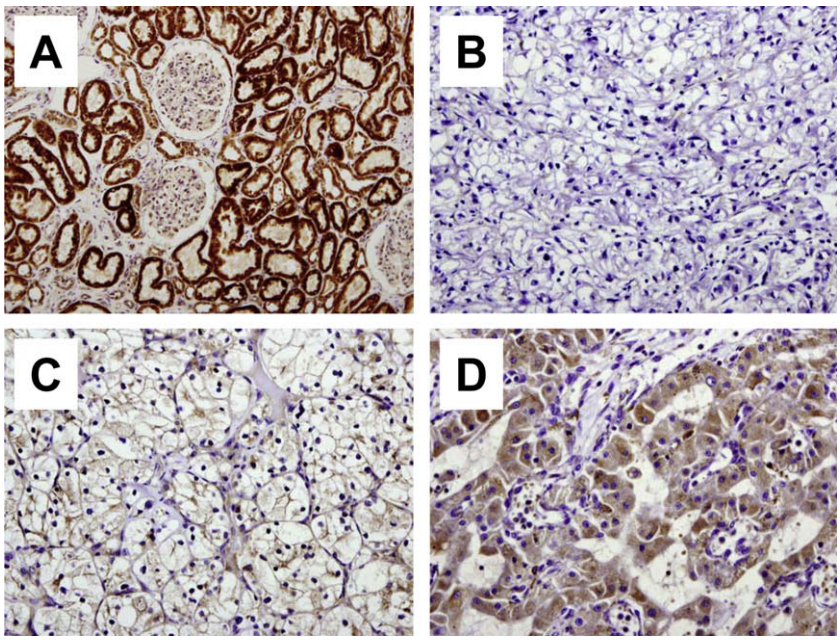

Fig. 4 – TGFβRII immunohistochemistry in CCRCC. (A) TGFβRII expression in normal renal tissue. (B–D) Representative immunostaining for TGFβRII in CCRCC: (B) no expression, (C) weak, expression, (D) high expression. Original magnification was ×40 for (A), ×100 for (B–C) and ×200 for (D).

It has been suggested that biallelic inactivation of the *TIMP3* gene may play a role in CCRCC carcinogenesis.[23] Thus, cumulative effects of LOH, *TIMP3* promoter hyper-methylation or down regulation of TGFβRII could affect TIMP3 expression. In our study, LOH of the *TIMP3* gene on chromosome 22q was observed in 7.6% of the CCRCC analysed. Most CCRCCs have deletions or translocations involving the short arm of chromosome 3, but all autosomes were involved in structural rearrangements. Recently, a study described the most frequently altered chromosomes in CCRCC. LOH on chromosome 22 did not appear to be common in CCRCC but the frequency of patients with chromosome 22 loss was 12.5%.[24] Furthermore, numerous studies demonstrated that loss of TIMP3 expression is associated with dense methylation at the 5′CpG-island in primary cancers such as colon, breast and lung carcinoma.[15–23] In our work, methylation of one 5′CpG-island of *TIMP3* promoter was examined using a sensitive MS-MLPA approach. The frequency of *TIMP3* promoter methylation was 24%. Previously, Bachman and colleagues[23] showed that methylation-associated silencing of TIMP3 is tumour specific and particularly frequent (78%) in tumours of 36 patients with renal cancer. They explored the sequences densely methylated flanking the transcription start site but they did not examine the clinical implications of the loss of TIMP3. We also evaluated the main upstream pathway described implicating TGFβRII which induces TIMP3 expression and the association between TGFβRII and TIMP3 expression both at protein and mRNA levels. The relation between TGFβRII pathway and TIMP3 expression demonstrated in human chondrocytes[21] remains to be investigated in human renal cancer cell lines. We found a strong association between TGFβRII and TIMP3 at both levels. The statistically significant association between mRNA and protein levels of TIMP3 and TGFβRII is in agreement with the dependency of TIMP3 expression on TGFβRII signalling. The loss of TGFβRII signalling pathway has been shown to play an important role in renal carcinogenesis and in the development of aggressive cancer phenotypes as well.[25] The TGFβ receptors, primarily TGFβRII, are known to be inactivated by somatic mutation, gene deletion or epigenetic silencing in several different types of human malignancies.[26–28] In the present study, by assessing TGFβRII expression by

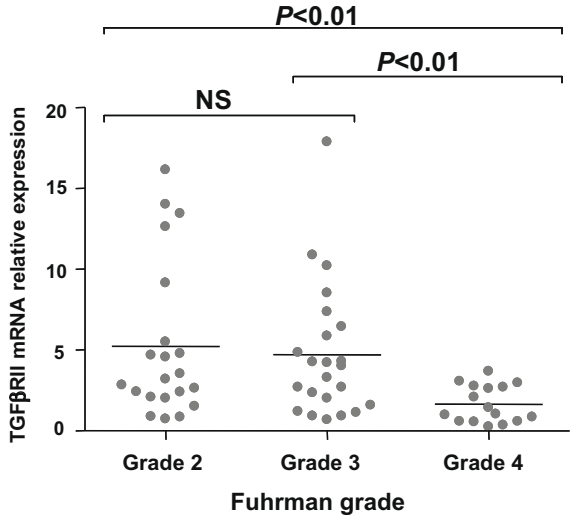

Fig. 5 – Relative expression of TGFβRII in 60 CCRCC according to Fuhrman grade. (grade 2: *n* = 21; grade 3: *n* = 23; grade 4: *n* = 16). TGFβRII expression was measured by real time PCR and normalised to β-actin. The grade 1 tumour was not represented. Horizontal lines represent mean values. Kruskal–wallis tests were performed and the corresponding *p* values are presented.

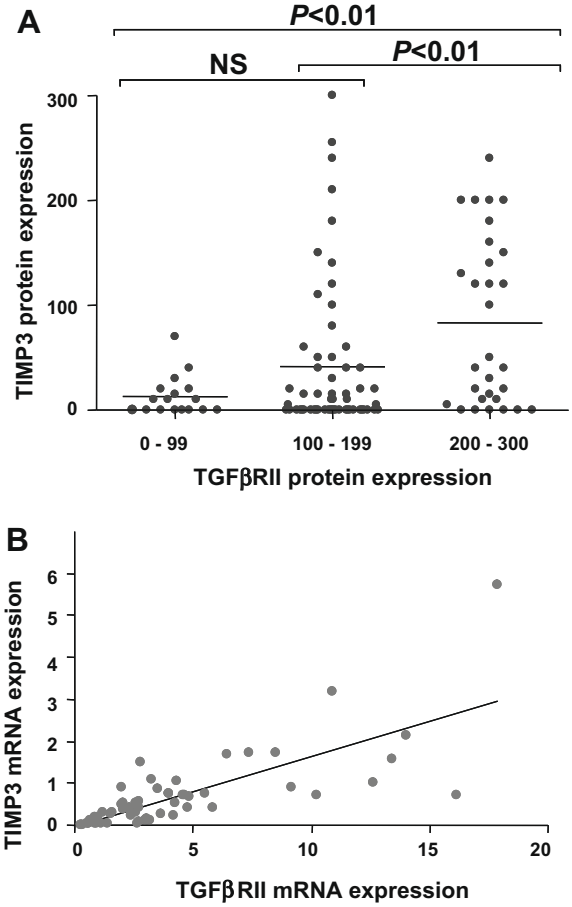

Fig. 6 – Relationship between TIMP3 and TGFβRII expression determined by immuno-histochemistry and qRT-PCR. (A) TIMP3 protein expression according to TGFβRII score (Low: 0–99; Medium: 100–199; High: 200–300). Kruskal–Wallis tests were performed and the corresponding *p* values are presented. (B) Positive correlation between TIMP3 mRNA expression and TGFβRII mRNA expression.

immuno-histochemistry, we demonstrated a TGFβRII down-expression in 76% of the tested CCRCCs. We did not find any significant association between TGFβRII protein expression and histological or clinicopathological data including tumour size, nuclear Fuhrman grade, tumour stage or metastasis status. At the mRNA level, TGFβRII was significantly less expressed in high Fuhrman 4 grade tumours. Recently, Miyajima and colleagues[29] and Parker and colleagues[30] examined the clinical implication of loss of TGFβRII expression in 2 cohorts of CCRCC patients with opposite conclusions. The two studies showed a loss of expression of TGFβRII in 50% and 43.2% of the patients, respectively. Miyajima and colleagues[29] reported that the loss of TGFβRII expression might be a tumour aggressiveness marker while associated with a decreased disease-specific survival. Conversely, Parker and colleagues[30] reported that lower TGFβRII expression levels were associated with less aggressive tumour phenotype. We present here the first report showing that the loss of TIMP3 expression is a common molecular event, suggesting that it may play a key role in renal carcinogenesis. However, the exact mechanism by which TIMP3 influences malignant progression in CCRCC is not known. TIMP3 is a secreted 24-kDa protein that, (1) inhibits the proteolytic activity of MMPs, (2) inhibits angiogenesis by attenuating the binding of VEGF to VEGFR-2 and (3) is also capable of inhibiting members of the ADAM family and thus inhibit the shedding of growth factors. Thus, alteration of TIMP3 may be a critical step during malignant progression. Furthermore, numerous studies demonstrated in several human tumour cell lines that TIMP3 induces growth arrest,[31] or promotes apoptosis[32] but it has not yet been demonstrated in human CCRCC cells lines. It is plausible that in CCRCC, loss of TIMP3 may abrogate normal apoptotic programs, enhance primary tumour growth, angiogenesis, invasiveness, the secondary growth of metastases; and therefore, potentially contribute to all stages of malignant progression. Further work is required to address these issues in human renal cancer cells lines.

In conclusion, these data represent an important progress in our understanding of CCRCC progression. Loss of TIMP3 observed in 95% of the CCRCC may be an important initiation step for tumour development. It is a complex process implicating inactivation of *TIMP3* by loss of heterozygosity on chromosome 22q, promoter hyper-methylation, or/and down-expression of TIMP3 by inactivation of an upstream pathway implicating the TGFβRII. TIMP3 expression is not a significant prognostic factor in patients with CCRCC but further large studies will be necessary for identifying potential clinical implications or better defining patient subgroups which may benefit from specific anti-angiogenic therapies.

Conflict of interest statement

None declared.

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