Overexpression of the *YAP1* oncogene in clear cell renal cell carcinoma is associated with poor outcome

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Abstract. Clear cell renal cell carcinoma (ccRCC) is the most common subtype of RCC (70-80%). Yes-associated protein 1 (YAP1) protein is a nuclear effector of the Hippo pathway and acts as a transcriptional co-activator of genes involved in the processes of growth and development of tissues. Hippo signaling, with its key kinases, MST2 and large tumor suppressor kinase 1 (LATS1), plays a significant role in the negative regulation of the amount and activity of YAP1 protein. Components of the Hippo pathway and YAP1 levels are frequently dysregulated in a variety of tumors, suggestive of their possible involvement in carcinogenesis. Our aim was to evaluate gene and protein expression profiles of YAP1, MST2 and LATS1 and the methylation status of MST2 and LATS1 promoters in ccRCC. mRNA levels of *MST2*, *LATS1* and *YAP1* genes were assessed in the tumor and matched normal kidney tissues of 86 patients, and in 12 samples of local metastases by quantitative PCR (qPCR). Proteins were semi-quantified in 58 patient samples by western blotting. Hypermethylation of *LATS1* and *MST2* promoters was measured by methylation-specific high-resolution-melting qPCR. We found that *LATS1* promoter hypermethylation, decreased LATS1 mRNA/protein and increased YAP1 mRNA/protein levels in tumor samples were associated with higher TNM and Fuhrman's stages and patient survival. Higher *YAP1* mRNA levels were associated with poor outcome (HR=4.03, p=0.036). No changes in MST2 (promoter/mRNA/protein) were found. We propose that deregulation of *LATS1* and *YAP1* expression is associated with ccRCC progression and poor patient survival. Measurement of *YAP1* mRNA levels in paired tumor-normal kidney tissue samples may serve as a new prognostic factor in ccRCC.

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Introduction

Clear cell renal cell carcinoma (ccRCC) is the most frequent RCC subtype and is characterized by a high mortality rate of 40% within 5 years, due to late diagnosis and distant metastases found in 30 (1) to 80% (2) of RCC patients at the time of examination or within the course of the disease. Among patients who undergo radical resection of the tumor, future metastatic disease develops in 20-40% of the ccRCC cases (3). The search for new molecular targets is continuing due to the high mortality rate of advanced RCC patients (4).

The Hippo pathway is an important regulator of cell proliferation, apoptosis, stem cell functions (5,6) as well as tissue growth and regeneration. Its deregulation is commonly observed in many human cancers, suggesting that alterations of Hippo signaling may be associated with tumor initiation and/or progression (7-9). The Hippo core cassette is formed by MST2 (serine/threonine kinase 3, STK3) and large tumor suppressor kinase 1 (LATS1) kinases (10). The phosphorylation of LATS1 by MST2 (with SAV1 and MOB1A/B co-activators) inhibits its transcriptional co-activator and downstream effector - Yes-associated protein 1 (YAP1) (11) via its phosphorylation, sequestration to the cytoplasm followed by YAP1 degradation (4,12). When YAP1 is located in the nucleus, it interacts with several transcriptional factors including TEA domain transcription factor 1-4 (TEAD1-4), OCT4, TP73 and ZEB1 (13). Increased expression of the YAP1 protein is associated with tissue regeneration or carcinogenesis (11,14,15). Moreover, the deregulation of the Hippo pathway components and/or YAP1 expression is frequently associated with the progression of various malignancies. Decreased expression of LATS1 gene and protein was observed in breast (16), colorectal (17) and non-small cell lung cancers (18), whereas lower MST2 mRNA and protein levels were reported in hepatocellular carcinoma (19) and malignant mesothelioma (20). Furthermore, the overexpression of YAP1 protein was observed in many cancer types, including lung (21), prostate (22), breast (23), and gallbladder cancers (24) and glioma (25). Since to date no quantitative analyses of the expression of the Hippo pathway effector, YAP1, and its key components, MST2 and LATS1 kinases, have been assessed in ccRCC, we decided to compare their mRNA and protein levels in tumor and normal kidney tissues, and in metastases of ccRCC. We also analyzed

Table I. Clinicopathological features of the ccRCC patients and the association between *YAP1*, *LATS1* and *MST2* mRNA levels and clinical data.

Patient characteristics (n=86)	YAP1 qPCR results, n (%)			LATS1 qPCR results, n (%)			MST2 qPCR results n, (%)		
	Low (≤0.382)	High (>0.382)	P-value (low vs. high)[a]	Low (≤1.982)	High (>1.982)	P-value (low vs. high)[a]	Low (≤0.539)	High (>0.539)	P-value (low vs. high)[a]
Age (years)									
Mean: 62.16±11.24									
Range: 33-83									
≤62	15 (17)	30 (35)	0.64	39 (45)	6 (7)	1.00	24 (28)	21 (24)	0.66
>62	11 (13)	30 (35)		36 (42)	5 (6)		19 (22)	22 (26)	
Sex									
Female (n=38)	10 (12)	28 (32)	0.64	29 (31)	9 (10)	0.43	16 (19)	22 (26)	0.28
Male (n=48)	16 (19)	32 (37)		46 (49)	2 (10)		27 (31)	21 (24)	
Tumor size (cm)									
≤7 (n=50	12 (14)	24 (28)	0.64	39 (45)	6 (7)	1.00	23 (27)	13 (15)	**0.048**
>7 (n=36)	14 (16)	36 (42)		36 (52)	5 (6)		20 (23)	30 (35)	
Fuhrman's histological grade									
1+2 (n=35)	14 (16)	21 (24)	0.08	27 (32)	8 (9)	**0.04**	17 (20)	18 (21)	1.00
3+4 (n=51)	12 (14)	39 (45)		48 (56)	3 (3)		26 (30)	25 (29)	
TNM stage									
Non-metastatic									
T1-2N0M0	21 (24)	16 (19)	**<0.0001**	29 (34)	8 (9)	**0.04**	18 (21)	19 (22)	1.00
Metastatic									
T1-2N1M0	5 (6)	44 (51)		46 (53)	3 (3)		25 (29)	24 (28)	
T3N0-1M0									
T4N0-2M0									
T1-4N2M0									
T1-4N0-2M1									

[a]P-values were calculated by Fisher's 2x2 test. ccRCC, clear cell renal cell carcinoma; YAP1, Yes-associated protein 1; LATS1, large tumor suppressor kinase 1; MST2, serine/threonine kinase 3. Bold indicates statistical significance.

the methylation status of *LATS1* and *MST2* gene promoters by methylation-specific high-resolution-melting quantitative PCR (MS-HRM-qPCR), a novel quantitative technique.

Materials and methods

Patients and samples. Tissue samples were collected from 86 ccRCC patients who underwent radical nephrectomy at the Department of Urology, Medical University of Gdansk, Poland, between January 2011 and September 2013. The clinical data of patients are presented in Table I. The study was approved by the local Ethics Committee; written consent was obtained before surgery from each patient.

Sample acquisition. Samples were obtained according to our previous reports (26,27). In short, dissected tissue samples of primary ccRCC tumors (n=86, named T), normal kidney (n=86, named C as control) and adrenal gland or the whole lymph node (n=12, named M), were collected in the operating theatre (by J.K.) and placed immediately in approximately five volumes of RNAlater (Ambion Inc., Austin, TX, USA).

Assessment of MST2, LATS1 and YAP1 mRNA expression. RNA isolation and cDNA synthesis were performed as previously described (26,27). Briefly, ExtractMe RNA kit (DNAGdansk, Gdansk, Poland) was used for RNA extraction. Two micrograms of total RNA was reverse transcribed with the use of RevertAid Reverse Transcriptase (Fermentas-Thermo Fischer Scientific, Fitchburg, WI, USA). qPCR details are presented in Table II. All reactions were run in duplicate. Based on the results of our previous study on the choice of suitable qPCR reference gene in ccRCC (27), we chose the assessment of *GUSB* gene expression to normalize the mRNA levels in the samples with the use of Schmittgen and Livak's ΔΔCt equation (28).

DNA extraction, bisulfite modification, acquisition of control DNA and MS-HRM-qPCR. The methodology has been previously described (26). In short, DNA was isolated to a total volume of 20 μl followed by bisulfide modification (DNA Methylation-Direct™ kit; Zymo Research, Irvine, CA, USA). For the generation of a dilution series of control DNA standards, fully methylated (named MD) and unmethylated (UMD) human genomic DNAs (Zymo) were used.

Table II. Details of qPCR assays.

Assay	Primer sequences	Amplicon size/CpGs in product	qPCR efficiency	qPCR reaction conditions	qPCR reaction content
LATS1 promoter methylation	5'-AAGTATTTTTGGTGGGGTAGAG 5'-AAAAAAAACCAAATCCTCAC	96 bp/9 nt	93%	95˚C, 5 min; 42x (95˚C, 5 sec; 56˚C, 10 sec; 72˚C, 10 sec; 73 ˚C, 10 sec - sample reading).	5 μl SensiFast HRM (with Eva-Green fluorophone) (BioLine, London, UK), 300 nM each primer, Σ 10 μl
MST2 promoter methylation	5'-TTTGGAAAAAGATAAGGTTTTTAT 5'-CCTAAAATTAACTCTCTCAACCTCTC	118 bp/7 nt	91%	Melting curve: 95˚C, 15 sec; 60˚C, 1 min; 60˚C → 95˚C reading every 0.1˚C	
LATS1 mRNA level measurment	5'-TGCACTGGCTTCAGATGGACAC 5'-ATGTGCTAGACATCGCTGGTGC	177 bp	93.4%	95˚C, 3 min; 37x (95˚C, 5 sec; 58˚C, 10 sec; 72˚C, 10 sec; 75 ˚C, 10 sec - sample reading).	5 μl SensiFast NoRox SYBR-Green (BioLine, London, UK), 200 nM each primer, Σ 10 μl
MST2 mRNA level measurment	5'-CTATAACTGTGTGGCCGACATCTGG 5'-TTGTGTTGCAGTAGCTCTCTGCTC	223 bp	90.3%	Melting curve: 95˚C, 15 sec; 60˚C, 1 min; 60˚C → 95˚C reading every 0.3˚C	
YAP1 mRNA level measurment	5'-TCAGACAACAACATGGCAGGACC 5'-TCTCTGGTTCATGGCAAAACGAGG	235 bp	101.3%		
GUSB mRNA level measurment for qPCR normalization	5'-ATGCAGGTGATGGAAGAAGTGGTG 5'-AGAGTTGCTCACAAAGGTCACAGG	177 bp	99.6%	95˚C, 3 min; 35x (95˚C, 5 sec; 57˚C, 10 sec; 72˚C, 10 sec; 75 ˚C, 10 sec - sample reading) Melting curve: 95˚C, 15 sec; 60˚C, 1 min; 60˚C → 95˚C reading every 0.3˚C. Melting curve: 95˚C, 15 sec; 60˚C, 1 min; 60˚C → 95˚C reading every 0.3˚	

Methylation was assessed in the samples with the use of the MS-HRM-qPCR method (29). Reactions were set on the Step-One Plus apparatus, then post-PCR products were analyzed with the use of HRM software ver. 3.1 (both from Life Technologies, Grand Island, NY, USA). For each run, matched DNA from T, C and M samples were set; standard dilutions of control MD and UMD were made to 100, 50, 25, 10 and 0 of MD in UMD and used in the same PCR plate as well as the no template control.

Western blot analysis. Protein lysates were prepared with Mammalian Cell Extraction kit (BioVision, Milpitas, CA, USA). The lysates (10 μg) were loaded onto a 10% Mini-Protean TGX gel (Bio-Rad, Hercules, CA, USA), resolved by SDS-PAGE, and transferred to a PVDF membrane using the Trans-Blot Turbo system (Bio-Rad). Membranes were stained with 0.1% Ponceau S to ensure equal loading after transfer, and subsequently blocked with 5% albumin fraction V in TBS buffer with 0.1% Tween-20 (TBST) for 1 h at room temperature (RT). After washing with TBST, the membranes were incubated (overnight, 4˚C) with specific primary antibodies in 2% albumin/TBS: rabbit anti-LATS1 (1:2,000, Bioss, Woburn, MA, USA), rabbit polyclonal anti-YAP1 (1:1,000), and rabbit monoclonal anti-MST2(STK3) (1:2,000) (both from Abcam, Cambridge, UK) and anti-GAPDH peroxidase-conjugated IgM (1:50,000; Sigma-Aldrich, St. Louis, MO, USA). After triple washing with TBST, the blots were incubated for 2 h at RT with horseradish peroxidase-conjugated secondary antibodies: anti-rabbit IgG or anti-mouse IgG (1:15,000; Sigma-Aldrich). Following triple washing with TBST, immunoreactive bands were detected on medical X-ray film (Agfa HealthCare, Mortsel, Belgium) using chemiluminescent peroxidase substrate (Sigma-Aldrich). Densitometric analysis of immunoreactive protein bands was performed with Quantity One software (Bio-Rad) and calculated as units = intensity/mm^2. After normalization to GAPDH protein units for each sample, the semi-quantitative results for either tumor or metastasized samples were obtained as a ratio: mean units$_{T/M}$/mean units$_C$ for MST2, LATS1 or YAP1 proteins.

Statistical analysis. Statistical analysis was performed with the use of the GraphPad Prism ver. 6.05 software (GraphPad Software, San Diego, CA, USA). The following statistical tests were used: non-parametric Mann-Whitney U, Kruskal-Wallis ANOVA, Fisher's 2x2 exact test, multivariate regression, and Cox-Mantel proportional hazard regression model. Survival relationships were presented as hazard ratios (HR) with their 95 confidence interval (CI) and p-values (30) using Cox and Kaplan-Meier estimations. Rates of overall survival (OS) and progression-free survival (PFS) were calculated separately. In all analyses, a two-sided $p<0.05$ was considered as statistically significant with a 95% CI.

Results

Clinicopathological characteristics of the patients. Of the 86 ccRCC patients (62.1±11.2 years, mean age ± SD) (Table I), 37 were diagnosed as stage I (T1-2N0M0), 8 as stage II (T2N0M0), 12 as stage III (T1-2N1M0 or T3N0-1M0) and 29 as stage IV (T4N0-2M0 or T1-4N2M0 or T1-4N0-2M1). TNM stages of the kidney cancer are as follows: stage I, tumor

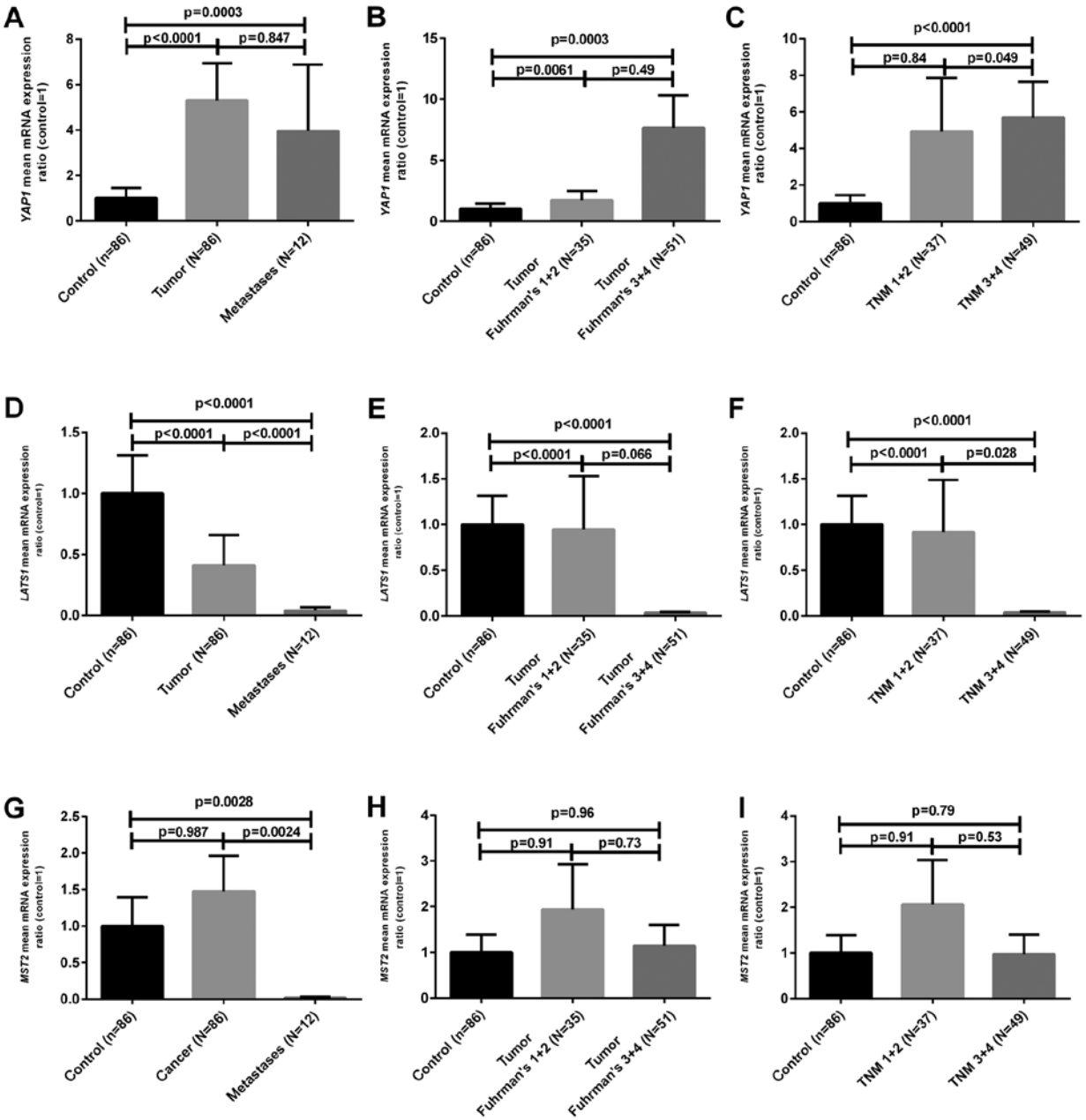

Figure 1. *YAP1*, *LATS1* and *MST2* gene expression in ccRCC. *YAP1* (A), *LATS1* (D) and *MST2* (G) mRNA levels in tissue samples of ccRCC patients were assessed by qPCR as described in Materials and methods. Plots show gene expression in tumor samples related to Fuhrman's grading (B, E and H) and TNM (C, F and I). Bars and whiskers represent mean ± SEM normalized to control kidney samples. P-values between groups (Mann-Whitney U test) are noted. ccRCC, clear-cell renal cell carcinoma; qPCR, quantitative PCR; LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1.

≤7 cm and limited to the kidney; stage II, tumor 7-10 cm, limited to the kidney; stage III, tumor extends into major veins or perinephric tissues but not into the ipsilateral adrenal gland and not beyond Gerota fascia or T1-T3 with metastasis in a single regional lymph node; stage IV, metastasis in more than one regional lymph node or distant metastasis (31). At the time of surgery, 47.7% of the ccRCC patients were diagnosed with local or distant metastases. Histological nuclear staging in renal cancer is based on the Fuhrman grading; grade 1: small, round, uniform nuclei (10 microns), inconspicuous nucleoli; grade 2: slightly irregular nuclei, nuclear diameter 15 microns, open chromatin; grade 3: visible nucleoli, nuclei very irregular, diameter 20 microns, open chromatin (32). According to Fuhrman's division 4 patients were grade 1, 32 grade 2, 23 grade 3 and 26 were grade 4. None of the patients underwent chemotherapy or radiotherapy before surgery. The mean follow-up period was 21 months (range, 3-48). To date, 45 patients were alive (52%); all deaths (except for one patient) were related to ccRCC progression. Median OS rate was 12 months. During follow-up, metastases occurred in 38 (44%) patients while the median PFS rate was 6 months.

Expression of the YAP1, LATS1 and MST2 genes at the mRNA level. As shown in Fig. 1A, *YAP1* mRNA levels in T (tumor) and M (metastatic) samples were ~5 and 4 times higher when compared to the C (control tissue) samples, respectively ($p<0.01$). When the samples were divided according to median mRNA values in the C samples, we found that 60 (70%) out

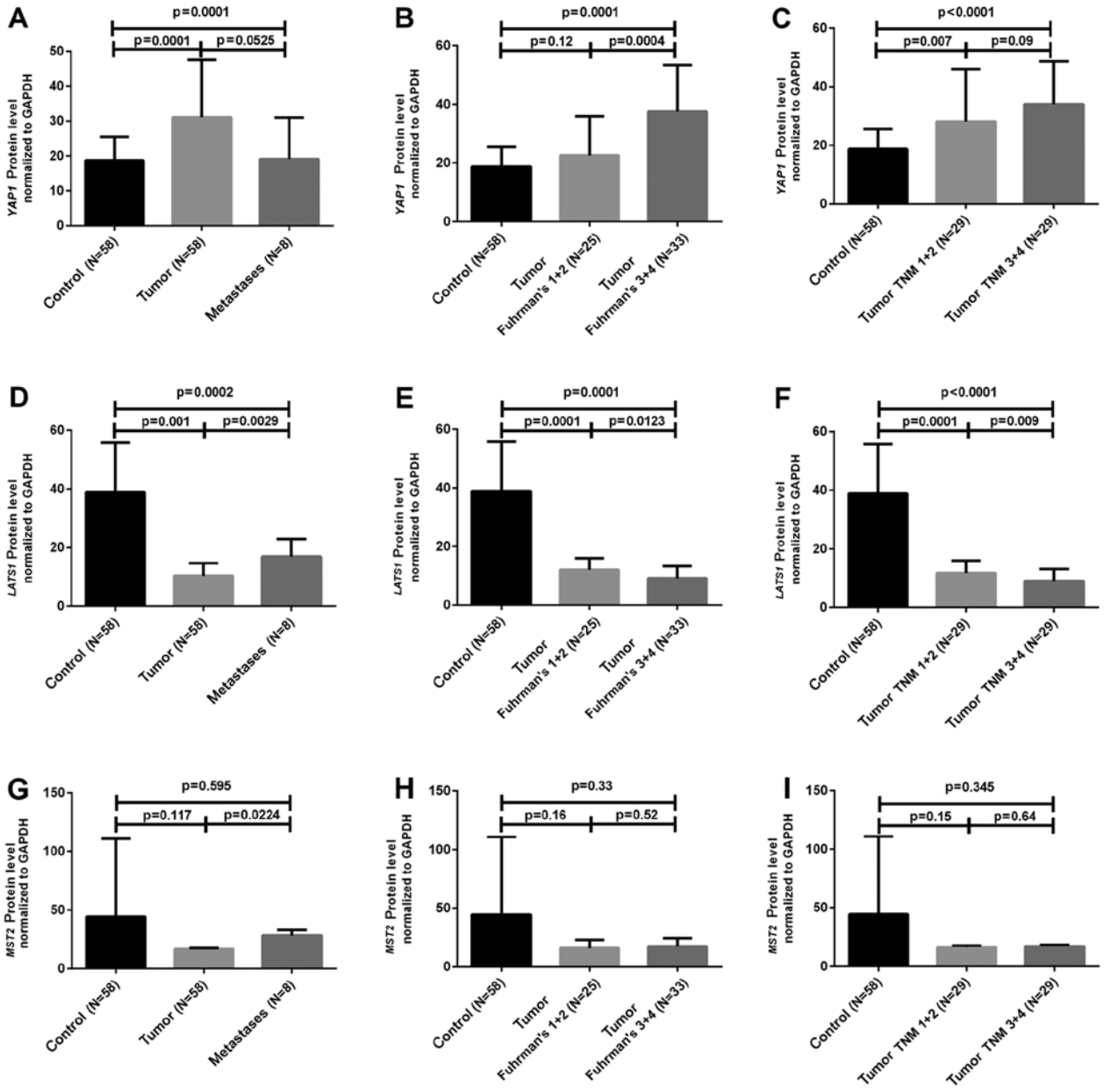

Figure 2. Analysis of YAP1, LATS1 and MST2 proteins in ccRCC by western blot analysis. Semi-quantitative analysis of YAP1 (A), LATS1 (D) and MST2 (G) in samples. Plots show protein expression in tumor samples related to Fuhrman's grading (B, E and H) and TNM (C, F and I). Bars and whiskers represent mean ± SEM normalized to GAPDH level in each sample. P-values between groups (Mann-Whitney U test) are noted. LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1.

of 86 tumor samples contained an increased *YAP1* mRNA level (Table I). The mRNA levels of *LATS1* were ~3 and 25 times lower in the T and M samples ($p<0.05$) (Fig. 1D) and lower *LATS1* mRNA content was observed in 75/86 (87%) tumor samples (Table I). On the contrary, the expression of *MST2* at the mRNA level was decreased only in M samples, showing a statistically not significantly increased ratio in 43/86 (50%) T samples as compared to the level in the control tissue (Fig. 1G and Table I). The comparison of the clinicopathological data with mRNA levels revealed that poorly developed ccRCC tumors (Fuhrman's grades 3 and 4) were characterized by decreased *LATS1* and increased *YAP1* mRNA levels (Table I and Fig. 1B and E) as compared to the control samples. In addition, we found either lower *LATS1* mRNA level or higher *YAP1* mRNA ratio in tumor ccRCC cases which were diagnosed with local (N1-2) or distant metastasis (M1) as shown in Table I and Fig. 1C and F. No statistically significant relationships between *MST2* mRNA ratios and clinical data were observed except for the higher content of *MST2* transcript in samples obtained from larger tumors (Table I).

Expression of YAP1, LATS1 and MST2 proteins. The semi-quantification of the studied proteins normalized to GAPDH protein was performed in paired samples of 58 ccRCC cases as well as in 8 M cases. As presented in Fig. 2A and Table III, the YAP1 protein level was ~2 times higher in 47 (81%) of the 58 analyzed T samples, whereas LATS1 protein ratio was ~4 times lower in 42 (72%) T samples when compared to the C samples (Fig. 2D). The relationship between

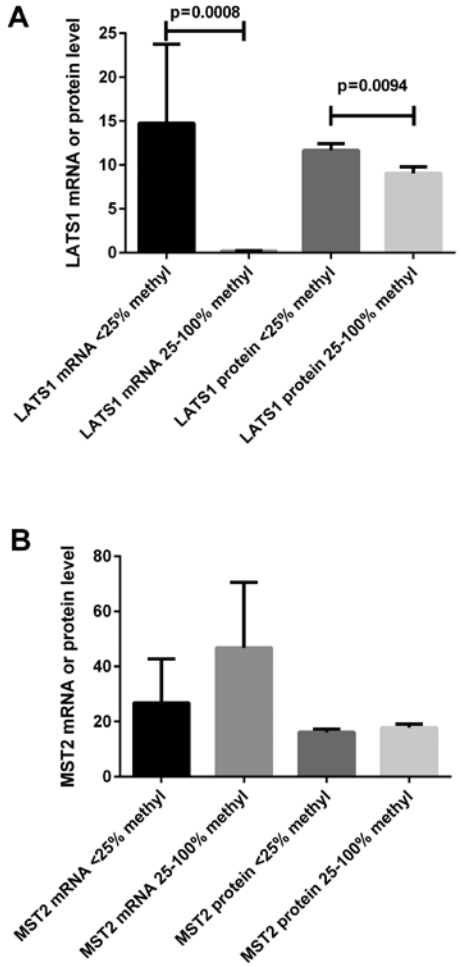

Figure 3. Quantitative comparison between LATS1 and MST2 mRNA and protein levels in tumor samples divided according to the methylation of LATS1 and MST2 gene promoters. qPCR and western blot results of either LATS1 (A) or MST2 (B) mRNA and protein levels in samples divided according to DNA methylation; 25% DNA methylation was treated as the threshold. Bars and whiskers represent mean ± SEM of either mRNA normalized to *GUSB1* mRNA level or protein normalized to GAPDH protein level in each sample. Mann-Whitney U test was applied: P-values <0.05 are noted. LATS1, large tumor suppressor kinase 1.

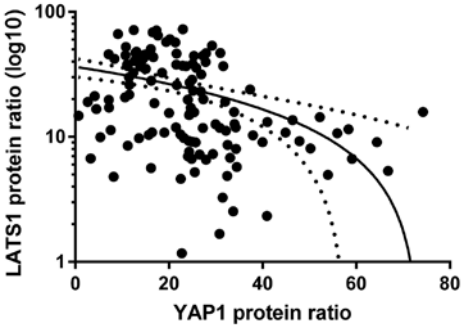

Figure 4. Correlation between LATS1 and YAP1 protein levels in all samples. The semi-quantitative results of protein assessment are presented as a plot; results for LATS1 protein are shown in log10 scale due to the high dispersion of data. Solid line represents linear regression curve with 95% confidence band (dots); rs=-0.51; p<0.05, Spearman's test. LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1.

clinicopathological data and LATS1 and YAP1 protein expression was noted. Poorly developed (high Fuhrman's grades) T cases were characterized by increased YAP1 and decreased LATS1 protein ratios (Table III and Fig. 2B and E). The difference in protein expression of either LATS1 or YAP1 between non-metastatic vs. metastatic tumor ccRCC cases was observed (Table III and Fig. 2C and F). Semi-quantification of MST2 protein did not show any differences either between tumor and normal kidney samples or between cancer samples classified according to clinicopathological status (Table III an d Fig. 2G H and I).

LATS1 and MST2 promoter methylation status. Methylation analysis was carried out in 58 tumor and 10 control samples. According to the analysis of MD/UMD standards, the results of MS-HRM-qPCR were qualified into four grades: 1, 0-10% methylation; 2, 10-25%; 3, 25-50%; 4, 50-100%. Based on the results of 10 control samples and our previous results (26), we set the value >25% methylation as the hypermethylation status for either LATS1 or MST2 promoters. We found that *LATS1* or *MST2* hypermethylation was observed in 28 (48%) or 22 (38%) of 58 tumor ccRCC samples, respectively (Table III). The hypermethylation of *LATS1* promoter was associated with higher Fuhrman's grades (3 and 4 vs. 1 and 2) as well as the presence of local and/or distant metastasis (Table III). Since the same T samples were analyzed for either *LATS1* or *MST2* methylation and mRNA and protein content, we ascertained whether the hypermethylation of the gene promoter region was associated with its mRNA/protein content. As shown in Fig. 3, the mRNA and protein expression of *LATS1* gene was related to the hypermethylation status of this gene; such observation was not proven for the *MST2* gene.

Relationships between LATS1, MST2 and YAP1 proteins. We checked possible correlations between mRNA-mRNA, mRNA-protein, protein-mRNA and protein-protein levels of LATS1-YAP1, LATS1-MST2 and MST2-YAP1. We found a negative correlation between LATS1 protein and YAP1 protein levels when all paired samples of 58 patients were taken into consideration (rs=-0.51; p<0.05, Spearman's test; Fig. 4).

Association between molecular findings and clinicopathological parameters and patient outcome. As presented in Figs. 5A and B and 6A and B, OS as well as PFS were strongly associated with a higher TNM and Fuhrman's grading in the patients. The molecular data revealed that increased YAP1 expression levels either at the mRNA or protein levels as well as the hypermethylation of *LATS1* promoter were related to both PFS and OS (Figs. 5C-E and 6C-E). The increased level of *MST2* mRNA was associated with shorter OS (Fig. 5F).

Cox proportional hazard model with multivariate analyses revealed that the *YAP1* mRNA level was an independent predictor of OS in ccRCC patients when assessed by Fuhrman's histological grade (Table IV). There was no association between molecular data and hazard ratio when the PFS rate was checked (Table V).

Discussion

The Hippo pathway is an important regulator of cell proliferation, tissue homeostasis, organ size and stem cell func-

Table III. Comparison between YAP1, LATS1 and MST2 protein levels, *LATS1* and *MST2* methylation status and clinical data of the 58 ccRCC patients.

	YAP1 protein assessment (AU) (%)			*LATS1* methylation			LATS1 protein assessment (AU) (%)			*MST2* methylation			MST2 protein assessment (AU) (%)		
Patients (n=86)	Low (≤17.636)	High (>17.636)	P-value (low vs. high)[a]	<25%	25-100%	P-value (low vs. high)[a]	Low (≤12.667)	High (>12.669)	P-value (low vs. high)[a]	<25%	25-100%	P-value (low vs. high)[a]	Low (≤19.31)	High (>19.31)	P-value (low vs. high)[a]
Age (years)															
Median: 62.5															
Mean: 62.82±11.94															
Range: 33-83															
≤62	7 (12)	22 (38)	0.50	14 (24)	15 (25)	0.79	19 (33)	10 (17)	0.38	19 (33)	10 (17)	0.59	19 (33)	10 (17)	0.78
>62	4 (7)	25 (43)		16 (28)	13 (22)		23 (40)	6 (10)		16 (28)	13 (22)		17 (29)	12 (21)	
Sex															
Female (n=28)	4 (7)	24 (41)	0.51	18 (31)	10 (16)	0.07	21 (36)	7 (12)	0.77	14 (24)	14 (24)	0.18	14 (24)	14 (24)	0.06
Male (n=30)	7 (12)	23 (40)		12 (21)	18 (31)		21 (36)	9 (16)		21 (36)	9 (16)		23 (40)	7 (12)	
Tumor size (cm)															
≤7 (n=24)	5 (9)	19 (33)	0.75	12 (21)	12 (21)	1.00	18 (31)	6 (10)	0.47	14 (24)	10 (17)	1.00	13 (22)	11 (19)	0.41
>7 (n=34)	6 (10)	28 (48)		18 (31)	16 (27)		24 (41)	10 (17)		21 (36)	13 (22)		23 (40)	11 (19)	
Fuhrman's histological grade															
1+2 (n=25)	8 (14)	17 (29)	**0.04**	21 (36)	4 (7)	**<0.0001**	15 (26)	10 (17)	**0.03**	13 (22)	12 (21)	0.29	16 (28)	9 (16)	1.00
3+4 (n=33)	3 (5)	30 (52)		9 (15)	24 (41)		28 (48)	5 (9)		22 (38)	11 (19)		20 (34)	13 (22)	
TNM stage															
Non-metastatic T1-2N0M0	8 (11)	16 (22)	**0.02**	22 (38)	7 (12)	**0.0005**	18 (31)	11 (19)	0.14	17 (29)	12 (21)	1.00	19 (33)	10 (17)	0.78
Metastatic															
T1-2N1M0	5 (7)	44 (60)		8 (14)	21 (36)		24 (41)	5 (9)		18 (31)	11 (19)		17 (29)	12 (21)	
T3N0-1M0															
T4N0-2M0															
T1-4N2M0															
T1-4N0-2M1															

[a]P-values were calculated by Fisher's 2x2 test. ccRCC, clear cell renal cell carcinoma; LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1. Bold indicates statistical significance.

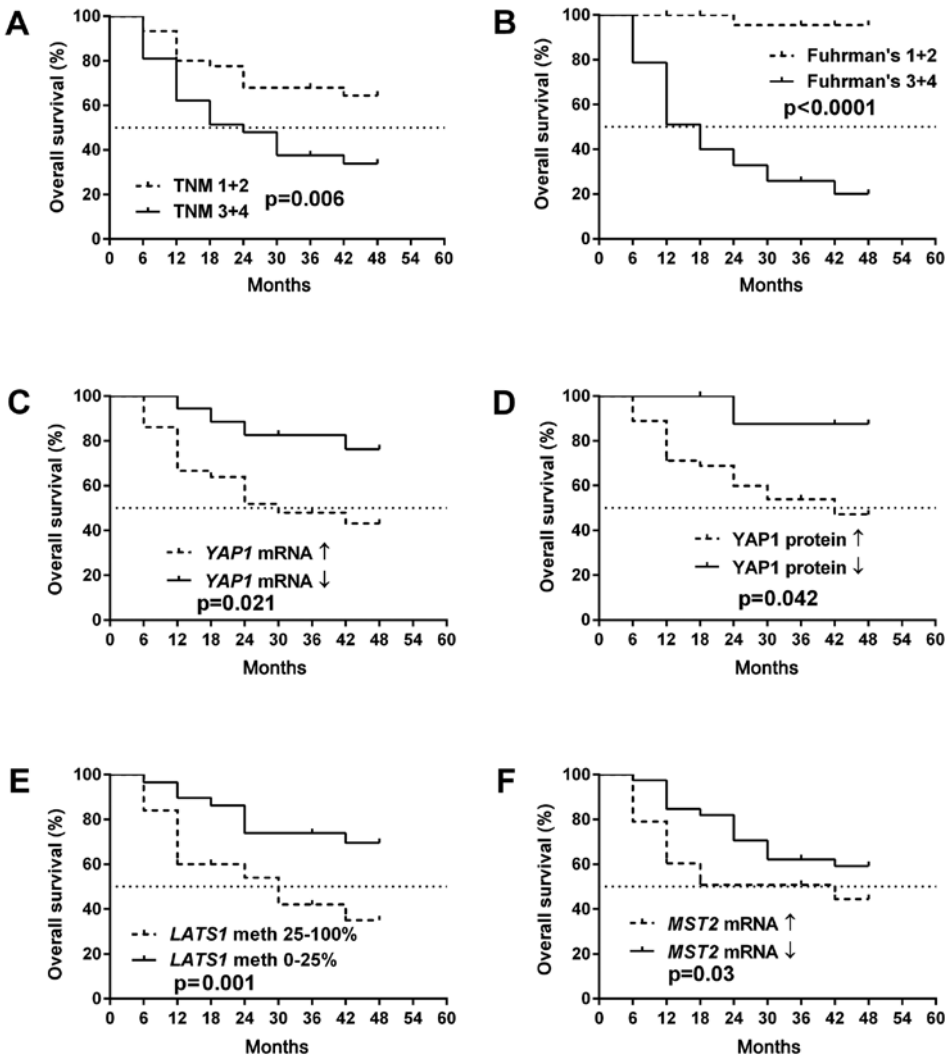

Figure 5. Kaplan-Meier's overall survival analysis of ccRCC patients related to clinicopathological and molecular data. Overall survival plots for 86 (A-C and F) or 58 (D and E) ccRCC patients. ccRCC, clear cell renal cell carcinoma. LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1.

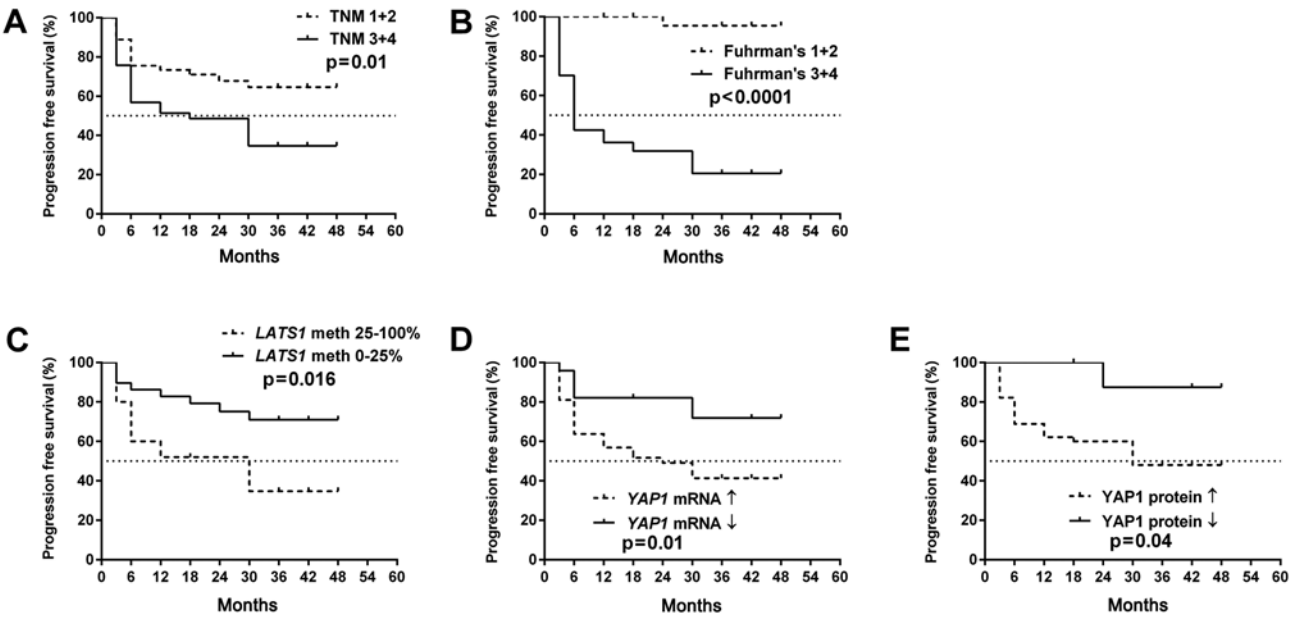

Figure 6. Kaplan-Meier's progression-free survival analysis of ccRCC patients related to clinicopathological and molecular data. Progression-free survival plots for 86 (A, B and D) or 58 (C and E) ccRCC patients. ccRCC, clear cell renal cell carcinoma. LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1.

Table IV. Univariable and multivariable Cox regression analysis of the overall survival rate of the ccRCC patients.

	Univariable analysis		Multivariable analysis	
Parameters	P-value	HR (95% CI)	P-value	HR (95% CI)
Sex				
Female vs. Male	0.064	2.28 (0.95-5.46)		
Age (years)				
>62 vs. ≤62	0.56	0.78 (0.34-1.786)		
Tumor size (cm)				
>7 vs. ≤7	0.29	0.64 (0.285-1.47)		
Tumor grade				
T3+4 vs. T1+2	**0.001**	**4.72 (1.85-12.06)**	0.91	0.93 (0.28-3.15)
Histological grade				
F3+4 vs. F1+2	**0.0005**	**8.61 (2.54-29.18)**	**0.019**	6.04 (1.34-27.26)
LATS1 mRNA levels				
↓ (≤1.982) vs. ↑ (>1.982)	0.58	1.35 (0.46-3.99)		
LATS1 methylation				
↑ (>25%) vs. ↓ (≤25%)	**0.01**	**3.06 (1.29-7.29)**	0.52	1.35 (0.52-3.51)
LATS1 protein levels				
↓ (≤12.669) vs. ↑ (>12.669)	0.33	0.58 (0.19-1.72)		
YAP1 mRNA levels				
↑ (>0.328) vs. ↓ (≤0.328)	**0.01**	**4.87 (1.43- 16.52)**	**0.036**	**4.03 (0.96-16.79)**
YAP1 protein levels				
↑ (>17.363) vs. ↓ (≤17.363)	**0.047**	**6.11 (0.82-45.48)**	0.67	1.60 (0.17-14.43)
MST2 mRNA levels				
↓ (≤0.539) vs. ↑ (>0.539)	0.72	1.16 (0.49-2.71)		
MST2 methylation				
↑ (>25%) vs. ↓ (≤25%)	0.52	0.75 (0.32-1.78)		
MST2 protein levels				
↓ (≤10.09) vs. ↑ (>10.09)	0.82	0.91 (0.39-2.11)		

ccRCC, clear cell renal cell carcinoma; HR, hazard ratio; LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1; CI, confidence interval. Bold indicates statistical significance.

tions (6). Its deregulation is frequently observed in many types of malignancies, suggesting that alterations of this signaling are connected with cancer progression and patient survival (7-9,21,33,34). The core components of this pathway include MST1/2, SAV1, LATS1/2 and MOB1 proteins (10,12,15). When the Hippo signaling is active, LATS1/2 kinases phosphorylate two major downstream effectors, YAP1 or its paralog, TAZ, resulting in their ubiquitination and proteolytic degradation (35,36). In contrast, deregulation of the pathway components, the consequent Hippo silencing, increases the YAP1 protein level in the cell as well as augments the nuclear localization of YAP1 (37). In turn, YAP1 nuclear accumulation triggers the upregulation of target genes (e.g., *CTFG* and *CYR61*), which are associated with processes such as cell migration, proliferation and angiogenesis (37).

The recent results of *in vitro* studies show that the inhibition of LATS1 kinase is strongly connected with the upregulation of YAP1 resulting in the increased metastatic potential of cancer cells (35,38). Mei *et al* showed that direct interaction between small ubiquitin-like modifier (SUMO) and LATS1 protein in L02 (normal human hepatic) and HepG2 (hepatocellular carcinoma) cells resulted in the attenuation of LATS1 kinase activity and inhibition of the Hippo pathway. As a consequence, the levels of YAP1, CTFG and CYR61 proteins were increased in SUMOtylated-LATS1 cells (35). Our results based on clinical samples of ccRCC showed a direct association between the presence of LATS1 and YAP1 in kidney tissues; a decreased LATS1 protein level was correlated with increased ratio of YAP1 protein in both tumor and matched normal kidney tissue samples. Another recent study on LATS1-YAP1 interaction in cancer (38) was performed in MDA-MB-231 and MCF7 breast cancer cell lines. Nokin *et al* found that methylglyoxal, a glycolysis side-product, indirectly targets inactivation of LATS1 in cells. As a result, increased levels of YAP1 protein and its co-effectors were observed which corresponded with the increased metastatic potential of cancer cells in a mouse xenograft model (38). The results of our study indicate that the decreased expression of LATS1

Table V. Univariable and multivariable Cox regression analysis of progression-free survival rate of ccRCC patients.

	Univariable analysis		Multivariable analysis	
Parameters	P-value	HR (95% CI)	P-value	HR (95% CI)
Sex				
Female vs. Male	0.11	3.12 (1.29-7.59)		
Age (years)				
>62 vs. ≤62	0.44	0.78 (0.34-1.786)		
Tumor size (cm)				
>7 vs. ≤7	0.43	0.72 (0.32-1.62)		
Tumor grade				
T3+4 vs. T1+2	**0.002**	**3.84 (1.58-9.31)**	0.77	0.84 (0.26-2.66)
Histological grade				
F3+4 vs. F1+2	**0.0002**	**15.01 (3.51-64.19)**	**0.001**	13.68 (2.73-68.34)
LATS1 mRNA levels				
↓(≤ 1.982) vs. ↑ (>1.982)	0.54	0.61 (0.12-2.95)		
LATS1 methylation				
↑(> 25%) vs. ↓ (≤25%)	0.65	1.27 (0.43-3.77)		
LATS1 protein levels				
↓(≤ 12.669) vs. ↑ (>12.669)	0.49	0.70 (0.26-1.89)		
YAP1 mRNA levels				
↑ (> 0.328) vs. ↓ (≤0.328)	**0.008**	**2.39 (0.88- 6.45)**	0.09	1.84 (0.45-6.23)
YAP1 protein levels				
↑ (>17.363)vs. ↓ (≤17.363)	**0.007**	**6.21 (0.83-46.07)**	0.12	1.72 (0.19-14.91)
MST2 mRNA levels				
↓ (≤ 0.539) vs. ↑ (>0.539)	0.61	1.23 (0.54-2.79)		
MST2 methylation				
↑ (> 25%) vs. ↓ (≤25%)	0.18	0.55 (0.22-1.32)		
MST2 protein levels				
↓ (≤ 10.09) vs. ↑ (>10.09)	0.52	0.76 (0.34-1.72)		

ccRCC, clear-cell renal cell carcinoma; HR, hazard ratio; LATS1, large tumor suppressor kinase 1; YAP1, Yes-associated protein 1; CI, confidence interval. Bold indicates statistical significance.

and increased YAP1 either at the mRNA or protein levels are highly associated with renal cancer progression.

Our data corroborate the findings of Chen *et al* (39) in an RCC cell line (786-O) as well as in tissue samples. In paired tumor and normal kidney samples of 30 ccRCC patients they observed decreased LATS1 mRNA and protein levels in tumor samples; ccRCC progression was associated with lower LATS1 content (39). Our data obtained on a much larger group of ccRCC patients extend these observations suggestive of the roles of LATS1 and YAP1 in ccRCC development since we found that the patients with deregulated LATS1 or YAP1 mRNA and protein levels share poorer clinical outcome. Thus, our and Chen *et al* (39) findings suggest that measurements of *YAP1* mRNA content in ccRCC tumor samples could serve as a potential survival marker together with high Fuhrman's grades. In contrast to a previous study (37) we used quantitative techniques (qPCR vs. RT-PCR and MS-HRM-qPCR vs. bisulfide sequencing PCR) to assess a much larger group of ccRCC patients (86 vs. 30). Although we did not focus on the expression of Hippo pathway components in renal cancer cell lines, Chen *et al* showed in 786-O and HEK293 kidney cell lines that the decreased expression of *LATS1* was associated with promoter hypermethylation (39) which was found by us in ccRCC clinical samples. Moreover, we observed that *LATS1* hypermethylation in tumor samples was characteristic of ccRCC patients with earlier occurrence of either metastasis or death. Chen *et al* also found that the controlled decrease in LATS1 protein level resulted in an increased YAP1 protein level. Furthermore, they observed that overexpression of LATS1 downregulated the YAP1 protein level, inhibited cell proliferation, induced cell apoptosis and cell cycle arrest in 786-O cells (39). LATS1 downregulation and its contribution to cancer progression has been observed in other malignances such as glioma (40), nosopharyngeal carcinoma (41), astrocytoma (42), non-small cell lung cancer (18), breast cancer (16), colorectal cancer (17) and renal carcinoma (39). Additionally,

association between *LATS1* hypermethylation and tumor progression has been noted in lung cancer (43), schwannomas (44), oral squamous cell carcinoma (45), colorectal cancer (17) and astrocytoma (42), however, the authors did not observe the influence of *LATS1* methylation status on patient outcome. Therefore, we believe that our observations may promote studies of LATS1 gene/protein expression to assess the impact on ccRCC progression and prognosis.

Our results suggest that the second core part of Hippo signaling, MST2 protein, is neither involved in ccRCC progression nor in YAP1 regulation. Although MST1/2 kinases have been acknowledged as tumor-suppressor proteins since loss of function of MST1/2 was observed in prostate (46) and breast cancer (47), and a decreased *MST1* mRNA level was associated with node metastasis in colorectal cancer (48), however, in hepatocellular carcinoma HepG2 cells increased MST1/2 levels were reported (49). Decreased *MST1* expression was associated with promoter methylation of this gene in soft tissue sarcomas (50). Since we did not find an association between *MST1* promoter methylation and gene expression, we suggest that such a regulation of *MST1* gene expression does not occur in ccRCC. MST1 protein is the upstream regulator of YAP1 protein (10,12,13,15,48,51,52), therefore the lack of an MST1/YAP1 association as observed by us in ccRCC should be discussed. The relationship between MST1/2 protein and YAP1 level in intestinal epithelium was observed by Zhou *et al* during an *in vivo* study (53). Their study using an Mst1/2-deficient mouse model showed that MST1 and MST2 proteins are crucial in the regulation of the Yap1 protein level in normal colonic epithelium (53). On the contrary, they found that the antiproliferative role of MST1 or MST2 was overcome in colon cancer by the abundance of Yap1 protein (53). Such an observation is in line with our results, since we did not find alterations in the expression of *MST1* mRNA or protein levels in the studied samples of ccRCC. Another *in vivo* study using mouse models showed different results in regards to the Mst2/Yap1 association in cancer development (19). Zhou *et al* found that tumorigenesis of hepatocellular carcinoma was associated with loss of Mst2 and a decreased level of phosphorylated Yap1 protein (19). Such an observation could be contrary to our results, however, they observed that the deregulation of Mst1/2 protein did not change the level of Lats1/2 proteins. Based on that, we suppose that such independent regulation of MST2 and LATS1 may occur in ccRCC. However, such a conclusion should be supported by further studies. Moreover, since our study is the first to use complex *MST2* quantification in ccRCC, we propose that lack of contribution of this gene in renal cancer progression must be confirmed by independent studies.

The most significant observation revealed in our study was, in our opinion, the possibility of *YAP1* mRNA measurement as a potential prognostic factor in ccRCC. Our previous study showed a similar correlation between Hippo upstream regulator, *RASSF1A* gene, and patient outcome (26). Therefore, in this study we aimed to assess the possible role of *YAP1* in ccRCC. Although our report is not the first study of YAP1 expression in ccRCC since Cao *et al* published a similar study in 2014 (54), there are some significant differences: a larger group of patients (86 vs. 30 persons), study on metastasized samples, modern quantitative techniques (qPCR vs. classical PCR) and survival data. Despite the mentioned differences, Cao *et al* obtained comparable results since the increased YAP1 protein level was associated with higher Fuhrman's and clinical stages (54). They also performed *in vitro* studies on 786-O and HEK293 kidney cells and found that knockdown of *YAP1* inhibited expression of the *TEAD1* gene as well as suppressed cell proliferation (54). Most studies on the role of *YAP1* in other cancer types such as RCC (39), oral squamous cell carcinoma (55), ovarian cancer (56), head and neck cancer (57), colorectal cancer (58), melanoma (59), lung (18) and breast cancer (23), revealed an association between YAP1 overexpression (mostly at the protein level) and tumor progression. Furthermore, we found that increased YAP1 levels of either mRNA or protein in tumor samples were associated with poorer patient outcome (survival and occurrence of metastasis). Other authors found a similar correlation between higher YAP1 levels and patient outcome in esophageal cancer (60), gastric adenocarcinoma (61) and papillary thyroid cancer (62).

Another important aspect is the mechanism of *YAP1* mRNA regulation. Notably, we observed that only ccRCC patients with increased *YAP1* mRNA levels in tumor samples were characterized by a higher risk of death (Cox test). Recent data indicate that some microRNA molecules directly regulate the *YAP1* mRNA level. Pan *et al* found that miR-509-3p targeted YAP1 mRNA in a large group (293 cases from TCGA cohort) of ovarian cancer (63). Moreover, miR-138 was found to be a strong suppressor of YAP1 mRNA in oral squamous cell carcinoma (64) and in non-small cell lung cancer (65). The reported associations between decreased levels of either miR-509-3p or miR-138 in the studied types of cancer and poorer patient outcome (63-65), consolidating the influence of YAP1 in tumor progression. In fact, the contribution of YAP1 protein in tumor progression is so important that it was acknowledged as a pivotal molecular target in modern cancer treatment (5,34,38,51,52,66,67). Some authors found an association between YAP1 overexpression and chemoresistance of cancer cells, e.g., in head and neck cancer cases resistant to cetuximab (57), resistance to RAF- and MEK-targeted therapy (33), 5-FU chemotherapy-resistant colon cancer (68) and osteosarcoma resistance (69).

In conclusion, we suggest that dysregulation of *LATS1* and *YAP1* levels, but not *MST2*, is associated with ccRCC progression and patient survival. We propose that the assessment of *YAP1* mRNA levels in paired tumor-normal kidney tissue samples could serve as a new prognostic factor in ccRCC.

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