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High nuclear Livin expression is a favourable prognostic indicator in renal cell carcinoma

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OBJECTIVES

To assess the protein expression of Livin, an apoptosis inhibitor, in renal cell carcinoma (RCC) and to determine its prognostic relevance.

PATIENTS AND METHODS

Immunohistochemical staining for Livin was performed in tissue microarrays (TMAs), including tumour tissue cores, from patients with RCC who had undergone renal surgery. In 682 TMAs cytoplasmatic staining intensity and nuclear staining quantity were evaluated, and the association of Livin expression with progression-free survival (PFS) and cancer-specific survival (CSS) was analysed with a multivariate Cox regression model.

RESULTS

Over a median (range) follow-up of 5.2 (0–16.1) years, 204 patients (28%) had died from their disease. The CSS rates at 1 and 5 years for the entire cohort was 88% and 71%. Cytoplasmatic Livin staining was absent in 516 (76%) specimens; staining was positive in 166 (24%) specimens. Weak nuclear Livin staining (≤25%) was present in 571 (84%) specimens, strong nuclear staining (26–100%) in 111 (16%). In multivariate analysis, high (>25%) nuclear Livin expression was a favourable independent predictor of PFS and CSS even after adjusting for tumour stage, Fuhrman grade, age, sex and Karnofsky severity rating. Cytoplasmatic Livin expression did not offer additional prognostic information.

CONCLUSION

High nuclear Livin expression is a favourable independent predictor of PFS and CSS in patients with RCC.

KEYWORDS

renal cell carcinoma, Livin, apoptosis inhibitor, long-term survival

INTRODUCTION

Metastatic RCC is one of the most treatment-resistant malignancies. Even after complete resection of the primary tumour, relapse occurs in 20–30% of cases. The overall 5-year survival rate is 60%; in those who present with metastases, the median survival is ≈13 months, with a 5-year survival of <10% [1,2].

To date only cytokine therapy and novel targeted therapies have been of limited benefit [1,3–7]. In a meta-analysis of >3600 patients with metastatic RCC, different chemotherapeutic agents evoked disappointing response rates: the highest objective remission rates were for vinblastine, 5-fluorouracil, floxuridine (6.67%, 6.57%, and 9.66%, respectively) [8].

Chemotherapeutic agents exhibit their antitumour effects by different mechanisms, one of the most important being induction of apoptosis [9]. The ability of cells to evade apoptosis plays a crucial role during development and progression of cancer and is considered to be a major cause of therapeutic resistance to cytotoxic therapies [10,11].

The molecular pathways leading to apoptosis are evolutionarily conserved and controlled by proteins that either promote or inhibit the activation of a cascade of intracellular caspases [11,12]. Caspase activity has been shown to be inhibited by proteins of the inhibitors of apoptosis proteins (IAPs) family. First identified in baculoviruses, in which they function to prevent the death of the infected host cells, IAPs contain tandem repeats of ≈70 amino acids, termed baculovirus IAP repeat (BIR) domains and frequently also possess a C-terminal Really Interesting New Gene (RING) zinc finger domain [13]. To date, eight human IAPs have been identified [11,13], two of which have been shown to be independent unfavourable prognostic factors in RCC: XIAP [14] and Survivin [15]. Livin, another member of the IAP family also called melanoma-linked IAP, has also been linked to RCC. Our research group was able to show Livin mRNA and protein expression in RCC and adjacent normal tissue, with significantly higher mRNA levels in tumour tissues [16]. Kitamura *et al.* [17] reported immunohistochemical Livin staining in 26 of 45 tumour specimens and Kempkensteffen *et al.* [18] found Livin mRNA expression in 59 of 152 RCC specimens. Neither study identified Livin as an independent prognostic indicator, perhaps limited by their relatively low numbers.

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The aim of the present investigation was to explore the relevance of Livin protein expression for tumour progression and prognosis in a large cohort of RCCs.

PATIENTS AND METHODS

Our protocol was approved by the Institutional Review Board of the University of Heidelberg (Ethics Committee, no. 206/2005). In all, 724 patients with RCC who underwent radical nephrectomy or nephron-sparing surgery at the Department of Urology, University of Heidelberg, between 1990 and 2005, and had no other malignant tumour before or within 1 month after surgery were entered into a prospective database. The clinicopathological characteristics of the study population are presented in Table 1. Owing to the few Fuhrman grade 4 specimens, these were grouped with grade 3 for further evaluation.

The patients were prospectively evaluated every 3 months for the first 2 years after surgery, every 6 months for the next 3 years, and yearly thereafter (chest X-ray or thoracic CT, abdominal sonography or CT or MRI, serum chemistry). Data analysis commenced in June 2006.

No adjuvant therapy was administered after radical surgery. Patients with metastases, a Karnofsky severity rating of ≥80, and no medical contraindications received palliative interferon-α- and interleukin-2-based immunotherapy. No tyrosine-kinase inhibitors were given.

For the immunohistochemistry, formalin-fixed paraffin-embedded tumour tissue samples from all patients were available for constructing tissue microarrays (TMAs). Morphologically representative areas of the primary tumour were marked on haematoxylin-stained sections. Areas of necrosis or severe leukocyte infiltration were avoided. With the Tissue Arrayer MTA-1 (AlphaMetrix, Rödermark, Germany), two cores of 0.6 mm diameter were taken from the marked areas of the corresponding tissue block and placed in predefined array locations in a recipient blank paraffin block. The blocks were then placed in an oven at 37 °C for 4 h to attach the cores to the surrounding paraffin. In all, 19 arrays were constructed, each containing two cores from up to 50 tumour specimens.

Variable	*N* (%)
Study population	724
Sex	
Male	451 (62.3)
Female	273 (37.7)
Age at surgery, years (median 61.6; range 14.6–89)	
<65	421 (58.1)
≥65 years	303 (41.9)
Karnofsky severity rating, %	
≥80	664 (91.7)
<80	60 (8.3)
Tumour extent:	
Stage I	425 (58.7)
Stage II	58 (8.0)
Stage III	114 (15.8)
Stage IV	127 (17.5)
Lymph node involvement	
N0	665 (91.9)
N+	59 (8.1)
Metastases at surgery	
M0	601 (83.0)
M1	123 (17.0)
Fuhrman grade	
G1	174 (24.0)
G2	415 (57.3)
G3	128 (17.7)
G4	2 (0.3)
Not classified by the pathologist	5 (0.7)
Histopathological subtype	
Clear cell RCC	608 (84.0)
Chromophile/papillary	67 (9.3)
Chromophobe	29 (4.0)
Collecting Duct	3 (0.4)
Not classified by the pathologist	17 (2.3)

TABLE 1 Clinicopathological characteristics of the study population

The TMA paraffin blocks were cut at 2 μm, placed on slides and dried for 24 h at 37 °C. Sections were dewaxed, rehydrated with xylol and descending series of ethanol, and immersed in 3% H_2O_2 for 5 min to block endogenous peroxidases. After washing with Tris-buffered saline-Tween (50 mM Tris, 300 mM NaCl pH 7.6, 0.1% Tween), unspecific antibody binding sites were blocked with protein-block solution provided by the Catalysed Signal Amplification (CSA) II System (DAKO, Carpinteria, CA, USA). Sections were incubated for 30 min at room temperature with a monoclonal anti-human Livin mouse antibody (Active Motif, San Diego, CA, USA) at a dilution of 1:400. Sections were immersed in horseradish peroxidase-conjugated anti-mouse antibody for 15 min at room temperature, followed by an incubation with fluorescyl-tyramide H_2O_2 for 15 min to intensify staining. Thereafter, sections were incubated with anti-fluorescein antibody conjugated to horseradish peroxidase and exposed to 3,3′-diaminobenzidine tetrahydrochloride solution for 2 min. Counterstaining of cell nuclei was done by immersing the section in haemalaun. Sections were thoroughly washed, glass-covered, and analysed by light microscopy at ×400 (Olympus Vanox-T, Hamburg, Germany).

Tissue samples from palatine tonsil, melanoma and neuroblastoma were used as positive controls. As negative controls, sections were incubated with non-immune serum instead of the primary antibody, followed by the detection method as described above.

The tissue specimens were examined in a random order for the presence or absence of cytoplasmatic and nuclear Livin staining by

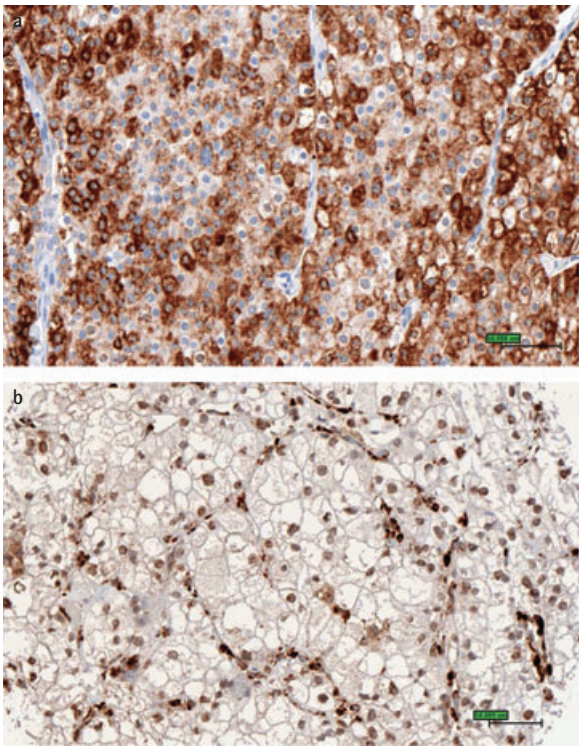


FIG. 1.
*Immunohistochemical Livin expression: clear cell RCC, Fuhrman grade 2, with (**a**) positive cytoplasmatic Livin staining (score 2) and (**b**) no cytoplasmatic staining, but >50% Livin-stained nuclei.*

two independent examiners unaware of the clinicopathological data. In each core the staining intensity for cytoplasmatic Livin was scored on a four-point scale (0, negative; 1, weakly positive; 2, moderately positive; 3, strongly positive). Semiquantitative nuclear Livin staining was also scored on a four-point scale (0, none up to 2% of tumour cell nuclei stained; 1, 3–25%, 2, 26–50%, and 3, >50% of tumour cell nuclei stained).

The patients' progression-free survival (PFS) and cancer-specific survival (CSS) was calculated from the date of renal surgery. The survival endpoint was the date of last follow-up or progression and death. Kaplan–Meier estimates were used to describe survival rates, including pointwise asymptotic 95% CIs. Data from patients with proven tumour-independent death were censored. Furthermore, assuming independence of RCC and other tumours in an individual patient, patient observation time was censored at the time of the occurrence of a second malignancy.

The following clinical and pathological features were studied for their prognostic relevance on long-term survival in RCC: age (≥65 vs <65 years), sex, performance status (Karnofsky severity rating <80 vs ≥80), tumour stage (stage II, III, IV vs I), Fuhrman grade (grade 2, 3/4 vs 1), cytoplasmatic livin expression (combined groups 1/2/3 vs 0) and nuclear livin expression (scores 0/1 vs 2/3).

The Cox proportional hazards model was used for univariate and multivariate analyses of prognostic factors. For each prognostic factor the hazard ratio (HR) in the univariate analysis and the adjusted HR in the multivariate analysis are given, including the 95% CI. A $P < 0.05$ was considered to indicate statistical significant.

RESULTS

The median (range) follow-up of patients alive at data analysis was 5.2 (0–16.1) years. By June 2006, 204 patients (28.2%) had died from their disease. The PFS rates for the entire cohort at 1 and 5 years after surgery were 82.9% (95% CI 79.9–85.5%) and 66.8% (95% CI 62.9–70.4%), respectively; the CSS rates were 88.4% (95% CI 85.9–90.6%) and 71.2% (95% CI 67.3–74.7%).

All the TMAs could be stained specifically for Livin with the CSA II System combined with a counterstaining for muscle cell nuclei. No immunoreactions were detected in the negative controls.

On the TMAs, cores of 682 patients were included in the analysis. Cytoplasmatic Livin expression was identically scored in both evaluated cores of each patient in 91.8% and nuclear Livin expression in 80.9%. There was no cytoplasmatic Livin staining (Score 0) in 516 (75.7%) specimens, weakly positive staining (Score 1) in 161 (23.6%), moderately positive (Score 2) in four (0.6%), and strongly positive staining (Score 3) in one (0.2%). For statistical evaluations the scores 1, 2 and 3 were combined owing to the few specimens with scores of 2 and 3. Figure 1a shows a tissue core positively stained for cytoplasmatic Livin (Score 2).

The scores of nuclei staining positively for livin were as follows: Score 0 (≤2%), 507 (74.3%); Score 1 (3–25%), 64 (9.4%); Score 2 (26–50%), 36 (5.3%); and Score 3 (>50%), 75 (11%). Figure 1b shows a tissue core with >50% Livin-stained nuclei.

In our analysis of nuclear Livin expression, we compared the combined Scores 0 and 1 (≤25%) with the combined Scores 2 and 3 (>25%). The distribution of nuclear Livin expression had suggested a threshold either between Scores 0 and 1,2,3 or between Scores 0,1 and 2,3. The latter was chosen because of the better statistical discrimination between the low- (0–25% livin-positive nuclei) and high- (26–100%) level groups on univariate Cox regression analysis. Figures 2 and 3 present the Kaplan–Maier PFS and CSS curves of patients with different cytoplasmatic and nuclear Livin stainings.

Patients with a good performance status (Karnofsky severity rating of ≥80%), localized (stage I) and low-grade (G1) disease had a high rate of Livin-positive nuclei (>25%), significantly more often than patients with a Karnofsky severity rating of <80%, advanced disease and/or high-grade tumours (Table 2).

On univariate analyses, there was no difference in the PFS and CSS rates between patients with or with no cytoplasmatic Livin expression. By contrast, the risk of progression or death from RCC in patients with numerous Livin-positive nuclei (26–100%) was about one-third that of patients with few Livin-positive nuclei (0–25%).

In univariate analyses, the following clinical and pathological features had a statistically significant affect on PFS and CSS in patients with RCC: sex, Karnofsky severity rating, tumour stage, and Fuhrman grade.

In our multivariate model, we included these clinical and pathological prognostic factors, age at the time of surgery, and cytoplasmatic and nuclear Livin expression.

Sex, Karnofsky severity rating, tumour stage, and Fuhrman grade 3 again had a statistically significant prognostic influence on progression or death from RCC in our multivariate model. As in the univariate analysis, cytoplasmatic Livin staining had no prognostic influence. Again, nuclear Livin staining showed itself to be a prognostic variable even after adjustment for the above clinical and pathological factors. Numerous Livin-positive nuclei (26–100%) were associated with higher long-term survival than limited nuclear Livin staining (0–25%), be it PFS or CSS.

The HRs, CIs and *P*-values of the uni- and multivariate analyses of PFS and CSS are given in Table 3.

DISCUSSION

In the present analysis of 682 TMAs, high nuclear Livin staining (>25%) was identified as a favourable independent prognostic factor in patients with RCC.

At first glance, this result is unexpected, as Livin is a protein that belongs to the family of IAPs and would be expected to be associated with poor survival. However, the prognostic role of Livin varies among different tumour entities. In nasopharyngeal carcinoma the Livin expression level was unrelated to patient survival [19]. In patients with neuroblastoma, Livin expression alone similarly had no effect on survival, although the combination of high Livin expression and amplified *MYCN* significantly decreased survival [20]. In Dukes B colorectal cancer, Livin was overexpressed and associated with poor prognosis, and in superficial bladder carcinoma Livin α-isoform expression was associated in tumour progression and used as a marker for early recurrence [21,22]. In contrast, a favourable prognosis was reported in patients with Livin-positive malignant pleural mesotheliomas [23] and in childhood acute lymphoblastic leukaemia (ALL) [23,24]. In the former group, Livin gene expression was associated with longer CSS and in the latter with relapse-free survival.

A possible explanation for the unexpected prognostic effect repoted by Choi *et al.* [24] in childhood ALL is age. Indeed, apoptosis regulation in children may be different from that in adults. For example, the overexpression of Bcl-2, a well known anti-apoptotic protein, has been reported to be significantly associated with a better prognosis in childhood ALL, whereas it was not associated with distinct clinical or biological characteristics in adult ALL [25,26].

FIG. 2. Kaplan–Meier survival curves of PFS in: ***a****, patients with presence (Score 1,2,3) or absence (Score 0) of cytoplasmatic Livin staining;* ***b****, patients with different quantities of Livin-stained nuclei (0–2% vs 3–25% vs 26–50% vs >50%);* ***c****, patients with high (>25%) and low (0–25%) nuclear Livin staining.*

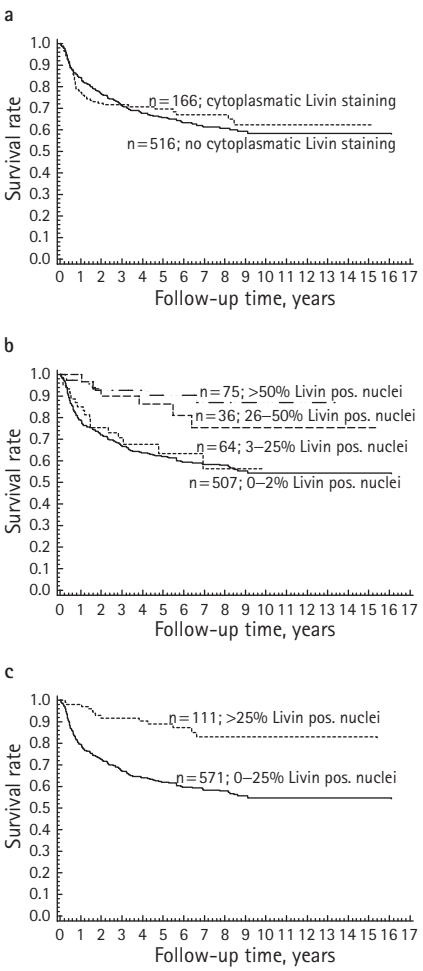
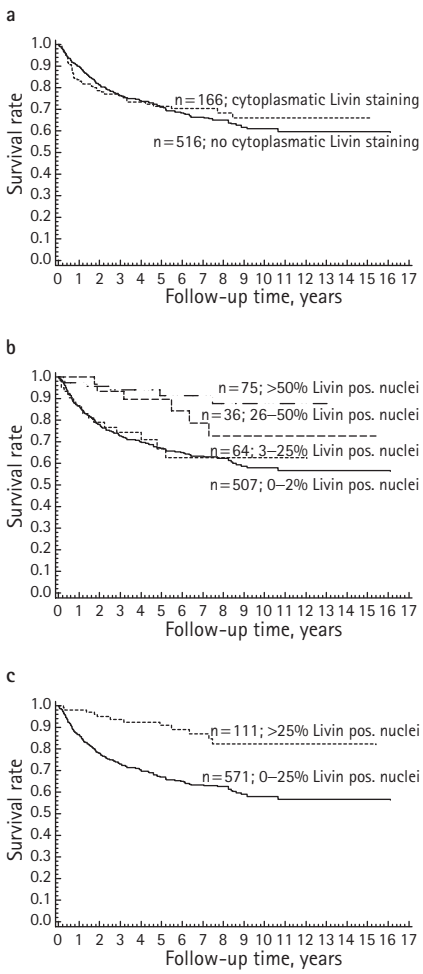


FIG. 3. Kaplan–Meier survival curves of CSS in: ***a****, patients with presence (Score 1,2,3) or absence (Score 0) of cytoplasmatic Livin staining;* ***b****, patients with different quantities of Livin-stained nuclei (0–2% vs 3–25% vs 26–50% vs >50%);* ***c****, patients with high (>25%) and low (0–25%) nuclear Livin staining.*



In the present study, patient age cannot be the reason for the favourable prognosis of high nuclear Livin expression in RCC because 99% of our patients were aged >18 years.

Another possible explanation is the pro-apoptotic potential that has been recently proposed for Livin, in addition to its anti-apoptotic function. It has been reported that effector caspases-3 and -7 can produce a truncated form (tLivin) with pro-apoptotic activity [27], which exhibits a distinct subcellular localization upon ectopic expression [28]. Whereas the full-length Livin

TABLE 2 Correlation of nuclear Livin expression with clinicopathological characteristics

Variable	Nuclear Livin, *n* (%) Low (≤25%)	High (>25%)	*P*
Sex			0.52
Male	354 (62)	65 (59)	
Female	217 (38)	46 (41)	
Age at surgery, years			0.18
<65	320 (56)	70 (63)	
≥65	251 (44)	41 (37)	
Karnofsky severity rating, %			**0.02**
≥80	518 (91)	108 (97)	
<80	53 (9)	3 (3)	
Tumour extent			**<0.001**
Stage I	309 (54)	90 (81)	
Stage II	44 (8)	9 (8)	
Stage III	102 (18)	7 (6)	
Stage IV	116 (20)	5 (5)	
Fuhrman Grade			**<0.001**
G1	119 (21)	46 (42)	
G2	334 (59)	59 (54)	
G3/4	114 (20)	5 (4)	
Histopathological subtype			0.29
Clear cell RCC	473 (86)	99 (90)	
Other types	79 (14)	11 (10)	

protein shows a diffuse cytoplasmatic localization, tLivin is found in a perinuclear distribution with marked localization to the Golgi apparatus.

Extrapolating these results for the Livin expression in the present study, the 166 patients with cytoplasmatic Livin localization (Scores 1–3) may express full-length Livin and the 175 patients with >2% Livin-positive nuclei (Scores 1–3) tLivin in the tumour cell nuclei/perinuclear region. The latter population could then acquire a more favourable prognosis owing to the pro-apoptotic potential of perinuclear tLivin. This hypothesis warrants further investigation once antibodies to detect tLivin by immunohistochemistry are available. However, clearly, looking at the present multivariate analyses, cytoplasmatic Livin had no prognostic influence on survival of patients with RCC, whereas high expression of nuclear/perinuclear Livin had a favourable influence.

Notably, the prognostic meaning of nuclear staining for Livin may be tumour-dependent, as suggested by a study of patients with high-grade osteosarcoma. For this cancer, nuclear Livin expression was significantly correlated with decreased overall survival [29].

Two other studies have evaluated Livin expression in RCC. Kitamura *et al.* [17] evaluated 45 cases of clear cell RCCs and identified positive cytoplasmatic staining of Livin in 26 (58%). They were unable to identify Livin expression (HR 1.91, 95% CI 0.47–7.25, $P = 0.347$) as an independent prognostic factor in their multivariate analysis in which other pathological factors such as clinical stage, Fuhrman grade, tumour diameter, pathological T stage, and tumour necrosis were added. These results are in accordance with the present observation that cytoplasmatic Livin expression has no prognostic influence in patients with RCC, but they must be viewed with some caution because the number of evaluated cases is very small for a multivariate analysis.

Kempkensteffen *et al.* [18] identified Livin mRNA expression in 59 (38.8%) of 152 evaluated RCC specimens. Although there was no association of Livin with any of the known prognostic parameters of RCC, Livin expression was present at early tumour stages (pT1) and well-differentiated RCC (grade 1), which may indicate that up-regulation of Livin expression occurs early in tumour development. This finding is in accordance with the present study, where high nuclear Livin expression was observed significantly more often in patients with stage I and/or low grade (grade 1) RCC.

In addition, the authors did not detect a significant difference in PFS and CSS between patients with positive vs negative Livin expression. A possible explanation for the lack of proving Livin expression as an independent prognostic factor is the different grouping in the study of Kempkensteffen *et al.* and the present study. They focused on the presence or absence of Livin expression, whereas we focused on high expression vs the combination of absent or low nuclear Livin expression. Although we clearly identified high nuclear Livin expression as a significant independent favourable prognostic marker of RCC, Kempkensteffen *et al.* only noted a strong trend ($P = 0.07$) towards a favourable recurrence-free prognosis in patients with high vs low Livin expression, possibly due to the few Livin-positive specimens ($n = 59$) in their subgroup analysis.

In addition to identifying Livin as a new prognostic factor in RCC, which can add to the development of a modified risk stratification system including clinical, pathological and molecular biological parameters, the present data suggest new therapeutic modalities. If perinuclear/nuclear Livin is verified as the truncated form with pro-apoptotic action, the promotion of Livin cleavage would provide a novel strategy to increase apoptosis in RCC cells that express Livin in a considerable percentage [28].

In conclusion, we were able to identify cytoplasmatic and nuclear Livin expression in a large cohort of RCC specimens. Although cytoplasmatic Livin expression had no prognostic implications, high nuclear Livin expression was a favourable independent prognostic factor for PFS and CSS in RCC.

CONFLICT OF INTEREST

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TABLE 3 Uni- and multivariate analyses (n = 677) of prognostic factors influencing PFS and CSS in patients with RCC undergoing surgery

	PFS				CSS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
Variable	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Stage								
II vs I	3.59 (2.12–6.07)	<0.001	2.78 (1.58–4.91)	<0.001	3.58 (1.96–6.53)	<0.001	2.69 (1.42–5.11)	0.003
III vs I	7.69 (5.24–11.28)	<0.001	5.36 (3.50–8.19)	<0.001	8.32 (5.37–12.90)	<0.001	5.42 (3.35–8.77)	<0.001
IV vs I	16.57 (11.58–23.71)	<0.001	10.32 (6.91–15.43)	<0.001	27.57 (18.29–41.55)	<0.001	17.98 (11.43–28.3)	<0.001
Grade								
2 vs 1	1.68 (1.13–2.50)	0.010	1.03 (0.68–1.58)	0.878	1.75 (1.14–2.69)	0.011	0.99 (0.62–1.56)	0.948
3/4 vs 1	7.65 (5.08–11.51)	<0.001	2.20 (1.38–3.50)	0.001	8.10 (5.22–12.56)	<0.001	2.12 (1.29–3.47)	0.003
Karnofsky								
<80 vs ≥80	2.27 (1.52–3.25)	<0.001	1.54 (1.03–2.28)	0.034	2.55 (1.73–3.77)	<0.001	1.82 (1.21–2.74)	0.004
Sex	1.42 (1.08–1.87)	0.013	1.34 (1.00–1.81)	0.052	1.55 (1.15–2.09)	0.004	1.45 (1.04–2.00)	0.026
Age, years								
<65 vs ≥65	0.98 (0.75–1.27)	0.865	0.99 (0.74–1.31)	0.914	1.07 (0.81–1.41)	0.638	1.19 (0.88–1.62)	0.266
Nuclear Livin, %								
>25 vs ≤25	0.27 (0.16–0.47)	<0.001	0.45 (0.25–0.82)	0.009	0.29 (0.16–0.53)	<0.001	0.52 (0.28–0.982)	0.044
Cytoplasmatic Livin, score								
1,2,3 vs 0	0.98 (0.71–1.34)	0.886	0.98 (0.71–1.36)	0.910	0.98 (0.70–1.37)	0.915	0.91 (0.64–1.30)	0.604

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Abbreviations: **TMA**, tissue microarray; **CSA**, Catalysed Signal Amplification (system); **PFS**, progression-free survival; **CSS**, cancer-specific survival; **IAP**, inhibitor of apoptosis protein; **HR**, hazard ratio; **ALL**, acute lymphoblastic leukaemia; **tLivin**, truncated Livin.