Cell. Mol. Life Sci. 64 (2007) 1137 – 1144
1420-682X/07/091137-8
DOI 10.1007/s00018-007-6510-7

Cellular and Molecular Life Sciences

Research Article

Targeted inhibition of Livin resensitizes renal cancer cells towards apoptosis

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Received 30 November 2006; received after revision 22 February 2007; accepted 20 March 2007
Online First 16 April 2007

Abstract. Cancer cells are typically characterized by apoptosis deficiency. In order to investigate a possible role for the anti-apoptotic *livin* gene in renal cell cancer (RCC), we analyzed its expression in tumor tissue samples and in RCC-derived cell lines. In addition, we studied the contribution of *livin* to the apoptotic resistance of RCC cells by RNA interference (RNAi). *Livin* gene expression was detected in a significant portion of RCC tumor tissue specimens (13/14, 92.9 %) and tumor-derived cell lines (12/15, 80.0 %). Moreover, targeted inhibition of *livin* by RNAi markedly sensitized RCC cells towards pro-apoptotic stimuli, such as UV irradiation or the chemotherapeutic drugs etoposide, 5-fluorouracil, and vinblastine. These effects were specific for *livin*-expressing tumor cells. We conclude that *livin* can contribute significantly to the apoptosis resistance of RCC cells. Targeted inhibition of *livin* could represent a novel therapeutic strategy to increase the sensitivity of renal cancers towards pro-apoptotic agents.

Keywords. Apoptosis regulation, Livin/ML-IAP, inhibitor of apoptosis proteins, renal cell cancer, RNA interference.

Introduction

Renal cell cancer (RCC) accounts for 3 % of all adult malignancies and is the most lethal urological cancer [1]. RCCs typically exhibit high intrinsic resistance towards chemotherapeutic drugs, and the prognosis of metastatic RCC is very poor, with 5-year survival rates of less than 10 % [2]. In recent years, it became increasingly clear that apoptosis deficiency is a major cause of the therapeutic resistance of solid tumors, since many of the anticancer agents used in the clinic act through induction of apoptosis [3].

The inhibitor of apoptosis proteins (IAPs) comprise a family of structurally related cellular factors that suppress apoptosis induced by a variety of stimuli. There is evidence that inadequate expression or functional overactivity of IAPs can contribute both to the development and to the therapeutic resistance of cancers [4]. Livin (alternatively termed ML-IAP) is an IAP that was initially linked to malignant melanoma [5]. More recently, however, *livin* expression was detected in additional cancers, including leukemias [6], bladder cancer [7], lung cancer [8], and neuroblastoma [9].

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A possible role for *livin* in RCC has not been investigated so far. We examined primary tumor tissue samples and RCC-derived cell lines for *livin* expression. In addition, we inhibited endogenous *livin* expression in RCC cell lines by RNA interference (RNAi) [10, 11]. We show that targeted silencing of the *livin* gene efficiently sensitized RCC cells towards different pro-apoptotic stimuli, including chemotherapeutic drugs. These data indicate that *livin* plays a role in the apoptosis-resistant phenotype of RCC cells and that the targeted inhibition of *livin* could represent a novel strategy to improve the therapeutic response of RCC.

Materials and methods

Tissue samples. Samples of tumor tissue from patients with localized RCC (n=14) were obtained from the tissue bank of the National Center for Tumor Diseases (NCT) Heidelberg. Samples were immediately snap-frozen in liquid nitrogen after resection. Histological diagnosis was established from formalin-fixed tissue sections that were located immediately adjacent to the section used for RNA extraction. Hematoxylin and eosin-stained sections were prepared from fresh-frozen tissue for the assessment of the percentage of tumor cells, and only samples with a tumor cell content higher than 60% were selected for further analysis. The post-surgical stage of each tumor was classified according to the tumor-node-metastasis (TNM) staging system of the Union Internationale Contre le Cancer (UICC) (edition 6, 2002) [12]. This work was approved by the ethical committee of the University of Heidelberg (No. 206/2005). Informed consent was obtained from each patient.

RNA extraction and real-time RT-PCR. For RNA extraction from tissue samples, 10–20 sections, each with a thickness of 10 µm, were cut from snap-frozen specimens and homogenized. Total RNA was isolated from tissues or from cultured RCC cells by phenol/chloroform extraction, followed by precipitation with isopropanol. RNA was resuspended in RNase free water and stored at –70°C. The amount of RNA was quantified spectrophotometrically (GeneQuant, Amersham Biosciences, Piscataway, NJ, USA) at 260 nm, and RNA integrity was verified by agarose gel electrophoresis. DNA was digested with DNase I Amplification Grade (Invitrogen, Karlsruhe, Germany). Reverse transcription of 1 µg RNA was performed using the oligo-dT primer and SuperScriptIII First-Strand kit (Invitrogen) according to the manufacturer's protocol. Expression of *livin*, *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), and *hypoxanthine phosphoribosyl-transferase 1* (*HPRT1*) was determined by real-time PCR with a 7300 Real-Time PCR System detector (Applied Biosystems, Forster City, CA, USA) using SYBR green PCR Master Mix (Applied Biosystems) supplemented with 500 nM of each forward and reverse primer. The following primers were used: for *livin*, forward primer (Livin-for5): 5'-GTTCCCCAGCTGTCAGTTC-3' (nt 614–632; AF311388), reverse primer (Livin-rev1): 5'-CGTCTTCCGGTTCTTCCCA-3' (nt 711–729); for *GAPDH*, GAPDH-for: 5'-GAAGGTGAAGGTCGGAGTC-3' (nt 108–126; NM_002046), GAPDH-rev: 5'-GAAGATGGTGATGGGATTTC-3' (nt 314–333); and for *HPRT1,* HPRT-For2: 5'-ACGAAGTGTTGGATATAAGCCA-3' (nt 592–613; NM_000194), HPRT-Rev2: 5'-TGATAATTTTACTGGCGATGTC-3' (nt 786–807). The cycling conditions comprised an initial incubation period of 2 min at 50°C, subsequent polymerase activation for 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing and extension for 1 min at 60°C. Controls without template as well as calibrator samples were included in each experiment. Each measurement was performed in duplicate. The sizes of the PCR products were initially analyzed by agarose gel electrophoresis and subsequently checked by melting point analysis after each reaction. Sequence Detection Software (version 1.2, Applied Biosystems) results were exported as tab-delimited text files and imported into Microsoft Excel for further analysis.

Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method [13]. Data are presented as the fold difference in gene expression normalized to a housekeeping gene index, as an endogenous reference, and relative to a calibrator sample. The housekeeping gene index was calculated from the geometric mean of the expression levels of two housekeeping genes, *GAPDH* and *HPRT1*. These genes were chosen among several tested housekeeping genes for normalization of gene expression, since they exhibited amplification efficiencies equal to our gene of interest. The RNA from HeLa cervical cancer cells was chosen as a calibrator due to its readily detectable *livin* mRNA signal in Northern blot assays [10]. A validation assay with serial dilutions of the calibrator was performed and the results plotted with the log input concentration on the x-axis and the difference in Ct on the y-axis. The absolute value of the slope was less than 0.1 in the range between 5- to 0.0005-fold of the calibrator sample (data not shown). On this basis, our method is suitable for the relative quantification of samples with values within this range [13].

Protein extraction and Western blot analyses. Proteins were extracted from fresh-frozen tissue specimens, essentially as described [14]. Briefly, ten freshly prepared tissue sections were homogenized in 100 µl lysis buffer (80 mM Tris-HCl pH 6.8, 2% SDS, 0.5 M 2-mercaptoethanol, 10% glycerol) supplemented with a cocktail of protease inhibitors P8340 (Sigma, Taufkirchen, Germany) and Pefabloc (Biomol, Hamburg, Germany). The lysates were centrifuged at 100 000 × g for 30 min at 4°C, and supernatants were collected for further analysis. Protein extracts from cell lines were prepared as detailed elsewhere [10].

Proteins were separated by 12.5% SDS polyacrylamide gel electrophoresis, transferred to an Immobilon-P membrane (Millipore, Bedford, USA), and detected with a monoclonal anti-Tubulin antibody (Oncogene, Boston, USA) or monoclonal anti-Livin antibody IMG-347 (Imgenex, San Diego, USA), employing enhanced chemiluminescence (Amersham, Braunschweig, Germany).

Cells, synthetic small interfering RNAs (siRNAs), and transfections. The following RCC cell lines were investigated: 769-P, 786-O, A-498, A-704, ACHN, Caki-1, Caki-2, KTCTL-1M, KTCTL-2, KTCTL-11, KTCTL-18, KTCTL-26, KTCTL-30, KTCTL-53, and KTCTL-84. All cells were maintained in RPMI medium supplemented with 10% fetal calf serum (FCS) except for 769-P and 786-O cells, which were grown in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% FCS.

siRNAs were chemically synthesized (Dharmacon Research, Lafayette, USA). siLivin-2, the isoform-specific siRNAs siLivin-A and siLivin-B, and their respective target sequences within *livin* mRNA have been described in detail elsewhere [10, 11]. siRNA "siControl", 5'-UAGCGACUAAACACAUCAA-3' (Dharmacon Research), bears at least four mismatches to all known human genes. Synthetic siRNAs were transfected with Oligofectamine (Invitrogen), as previously described [11]. In brief, 8 µl Oligofectamine and 200 nM of the individual siRNAs were diluted in Opti-MEM I reduced serum medium (Invitrogen) and mixed in a final volume of 400 µl transfection solution. Cells, plated on 6-cm dishes at 30–50% confluency, were transfected in 1.6 ml Opti-MEM I and 4 h later supplemented with 1 ml D-MEM containing 30% FCS.

Treatment with pro-apoptotic agents and TUNEL analysis. For treatment with pro-apoptotic agents, cell lines were exposed 24–48 h post-transfection to a single dose of 50 J/m^2 UV irradiation (Stratalinker 2400, Stratagene), to 50 µg/ml etoposide, to 100 µg/ml 5-fluorouracil (5-FU), or to 0.1 µg/ml vinblastine sulphate (Sigma) and harvested 16–48 h later. TdT-mediated dUTP nick end labeling (TUNEL) analyses for detection of apoptosis were performed using the *in situ* cell death detection kit (Roche Molecular Biochemicals, Mannheim, Germany). Nuclei were stained with

4',6-diamidino-2-phenylindole (DAPI) (Roche Molecular Biochemicals). Apoptotic strand breaks and total DNA were visualized by epifluorescence microscopy.

Results

Livin is expressed in RCC tumor tissue and in RCC cell lines. Expression of the *livin* gene in RCC tumor tissue was quantified by real time RT-PCR. *Livin* mRNA was detected in 13 of 14 tumor specimens (92.9 %) (Fig. 1). Expression levels of *livin* differed greatly between positive samples, with no obvious link to tumor stage or tumor grade (data not shown) for the limited number of tissue samples investigated here. In addition, 12 of 15 (80.0 %) tumor-derived RCC cell lines scored positive for *livin* mRNA expression, although levels were generally much lower than in primary tissues (Fig. 2). This was also reflected on the protein level, where 10/11 (90.9 %) tissue samples expressed Livin protein, as assessed by immunoblotting (Fig. 3a), whereas we did not detect Livin protein in any of 11 analyzed RCC cell lines within the detection limits of this method (Fig. 3b).

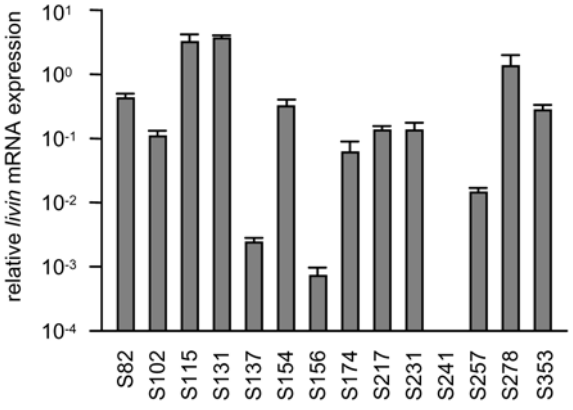

Figure 1. *Livin* mRNA expression in RCC tissue specimens. *Livin* mRNA was measured by real-time RT-PCR analysis. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, and data are presented as fold differences in gene expression, normalized to a housekeeping gene index as an endogenous reference and relative to the calibrator sample (HeLa cervical carcinoma cells). Standard deviations are indicated.

Silencing of endogenous *livin* gene expression by RNAi sensitizes RCC cells towards pro-apoptotic stimuli. To assess the functional significance of *livin* for the apoptosis resistance of RCC cells, we chose the strategy to silence endogenous *livin* gene expression by RNAi and to subsequently investigate the apoptotic response towards different pro-apoptotic stimuli.

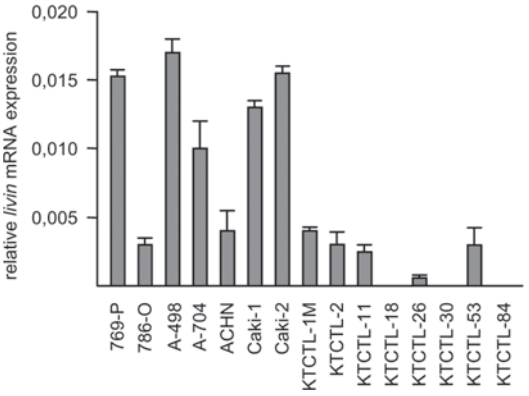

Figure 2. *Livin* mRNA expression in RCC cell lines. *Livin* mRNA was measured using real-time RT-PCR analysis. Relative quantification was performed using the comparative Ct ($2^{-\Delta\Delta Ct}$) method, and data are presented as fold differences in gene expression, normalized to a housekeeping gene index as an endogenous reference and relative to the calibrator sample (HeLa cells).

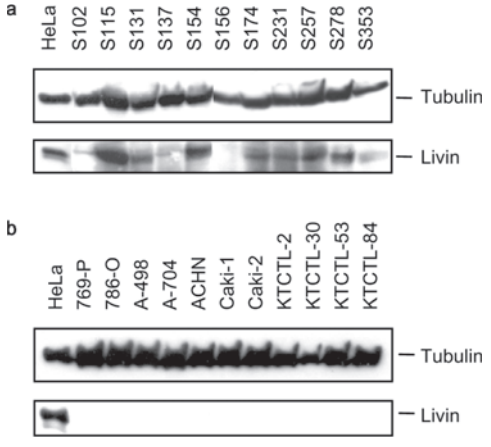

Figure 3. Immunoblot analysis of Livin protein expression in (*a*) RCC tumor specimens and (*b*) RCC cell lines (HeLa cells, positive control; Tubulin: α-tubulin protein, loading control).

First, we transfected a panel of RCC cell lines with siLivin-2, a synthetic siRNA that acts as a specific and efficient inhibitor of *livin* expression [10, 11]. Real-time RT-PCR analyses performed 48 h post-transfection showed that *livin* mRNA levels were significantly reduced by siLivin-2 in all *livin*-expressing RCC cell lines when compared with control transfections (Fig. 4). Residual *livin* expression levels varied from 50 % (KTCTL-2) to less than 15 % (Caki-1) of the respective controls and generally correlated with the transfection efficiency for the individual cell lines, as assessed by transfection of a green fluorescent protein (GFP)-producing vector (data not shown).

Next, siLivin-2- or control siRNA-transfected cells were treated with different pro-apoptotic stimuli. We employed UV irradiation, a DNA-damaging agent, and three different chemotherapeutic drugs: the topoisomerase II inhibitor etoposide, the antimetabolite

5-fluorouracil (5-FU), and the microtubule disruptor vinblastine. Apoptotic cells were identified by their nuclear morphology (hyperdense chromatin in DAPI staining) as well as by a positive TUNEL assay.
Representative results for *livin*-expressing RCC cell lines are depicted in Fig. 5a, showing that siRNA-mediated silencing of *livin* efficiently sensitized Caki-1 cells towards UV irradiation or 769-P cells towards 5-FU. This is visualized by the strong increase in the number of apoptotic cells compared to control transfectants (Fig. 5a). In contrast, KTCTL-30 cells, which lack detectable *livin* expression (Fig. 2), were not affected by these treatments (Fig. 5a). Notably, each *livin*-expressing RCC cell line (livin+) showed a clear pro-apoptotic sensitization upon inhibition of endogenous *livin* expression in response to all tested pro-apoptotic stimuli. These results are summarized in Fig. 5b–e. In contrast, both RCC cell lines scoring negative for *livin* gene expression (livin–) were not sensitized towards any of the pro-apoptotic stimuli, underlining the specificity of the siLivin-2 effect (Fig. 5a–e). As previously observed [10, 11], RNAi-mediated inhibition of *livin* gene expression did not affect – or affected at most marginally – apoptosis in the absence of additional pro-apoptotic stimuli (not shown). In conclusion, the targeted inhibition of *livin* expression by RNAi markedly increased the sensitivity of RCC cells towards pro-apoptotic agents in a *livin*-dependent manner.

Both Livin isoforms contribute to the apoptotic resistance of RCC cells. The *livin* gene codes for two splice variants, designated Livin α and Livin β [5, 15]. In order to evaluate the individual contributions of these splice forms to the apoptotic resistance of RCC cells, we employed siRNAs that specifically target *livin α* (siLivin-A) or *livin β* (siLivin-B) mRNA [11]. As exemplified by the TUNEL analysis of 769-P cells (Fig. 6a), both siRNAs led to a clear increase of the apoptosis rate in RCC cells that scored positive for *livin* expression upon treatment with UV irradiation (Fig. 6b). In contrast, the apoptosis rate of KTCTL-30 cells, scoring negative for *livin* expression, was not affected by any of the *livin*-targeting siRNAs (Fig. 6a, b). Corresponding findings were obtained upon treatment with etoposide (Fig. 6c). These observations indicate that both *livin* splice forms can contribute to the apoptosis resistance of RCC cells.

Discussion

Tumor cells have evolved molecular strategies to resist apoptosis, including the up-regulation of anti-apoptotic proteins or the down-regulation of pro-apoptotic proteins [3]. This enables their survival under abnormal growth stimulation and is linked to increased resistance towards different forms of cellular stress, such as DNA damage, hypoxia, or nutrient deprivation. In addition, apoptosis deficiency is considered to be a major determinant for the therapeutic resistance of tumors towards chemotherapy and radiotherapy [3].

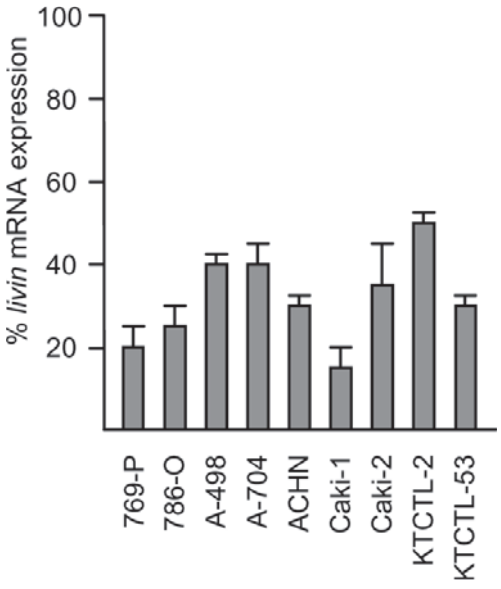

Figure 4. Interference with *livin* mRNA expression in RCC cell lines by RNAi. *Livin* mRNA was quantitated 48 h after transfection of the cells with either siControl or siLivin-2. *Livin* expression levels of control transfections with siControl were set at 100 % for each cell line. Standard deviations are indicated.

RCC represents a tumor entity that is typically highly resistant to chemotherapy. In order to develop new and rational strategies for improved therapy, it will be important to identify the molecular determinants that mediate the apoptotic resistance of RCC cells. We show here, for the first time, that the *livin* gene is expressed in primary RCC tissues and in RCC-derived tumor cell lines. Notably, *livin* gene expression appeared to be generally higher in RCC tissues than in RCC-derived cell lines. Although speculative at the moment, this could indicate that RCC cells are under increased selection pressure to express higher levels of *livin*, at *in vivo* conditions. The possibility that IAP expression can be further increased as a protective mechanism of tumor cells *in vivo* is supported by results from a metastatic nude mouse model for prostate cancer. Here, circulating metastatic cells exhibited increased XIAP gene expression when compared to the parental tumor cell line *in vitro* [16].
Nevertheless, despite its low expression level, *livin* exerted measurable anti-apoptotic activity in RCC cells, since the targeted interference with *livin* ex-

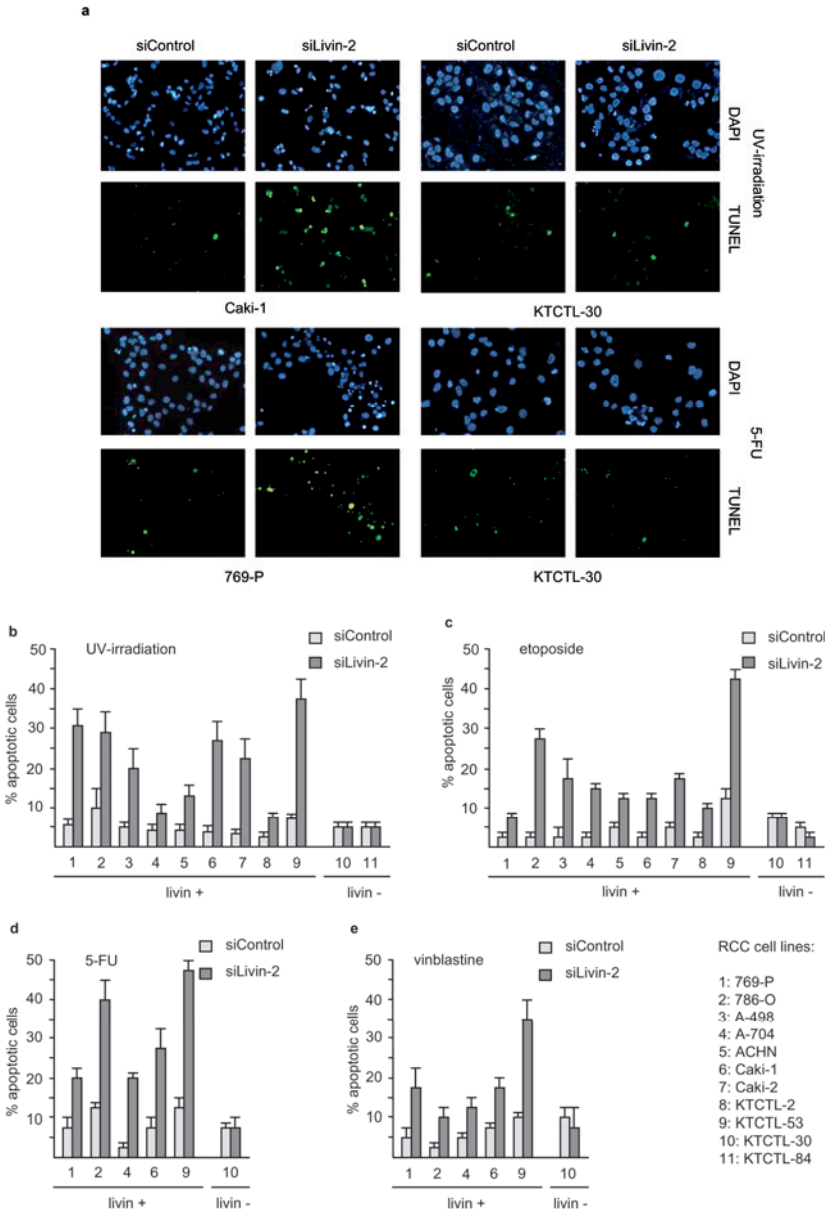

Figure 5. Inhibition of *livin* expression sensitizes RCC cells towards different pro-apoptotic stimuli. Cells were transfected with siRNAs, as indicated, and subsequently exposed to UV irradiation, etoposide, 5-FU, or vinblastine. (*a*) Detection of apoptotic cells after UV irradiation and 5-FU treatment of Caki-1 and 769-P cell lines scoring positive (livin+), and KTCTL-30 cells scoring negative (livin–) for *livin* expression. Nuclei were stained with DAPI, and cells undergoing apoptosis were visualized by TUNEL. (*b–e*) Percentage of apoptotic cells after treatment of RCC cell lines with (*b*) UV irradiation, (*c*) etoposide, (*d*) 5-FU, and (*e*) vinblastine (see lower right corner of the panel for numbering of individual cell lines). Standard deviations are indicated.

pression markedly increased the apoptotic sensitivity of all *livin*-expressing RCC cell lines under investigation. This effect was observed for four pro-apoptotic stimuli with different biochemical activities, including the chemotherapeutic drugs 5-FU and vinblastine, which are used for the therapy of advanced RCC [17]. Furthermore, isoform-specific silencing of *livin* α and *livin* β indicated that both splice forms contribute to apoptosis resistance of RCC cells. This differs from findings for HeLa cervical cancer cells, in which apoptosis inhibition was primarily linked to the *livin* β form [11], indicating that the functional significance of individual Livin isoforms is dependent on the tumor cell background. The specificity of the RNAi approach chosen here is underlined by the observation that RCC cells scoring negative for *livin* expression did not exhibit a pro-apoptotic sensitization upon treatment with *livin*-specific siRNAs. Taken together, these findings indicate that the *livin* gene contributes to the anti-apoptotic resistance of RCC cells.

Several other members of the IAP family have been previously implicated to play a role in RCC. An inverse correlation between XIAP expression and RCC aggressiveness has been reported [18]. High levels of Survivin correlated with poor survival in RCC patients [19], and siRNAs targeting *survivin* sensitized renal cancer cells towards chemotherapeutic agents and inhibited cell proliferation [20]. The expression of Smac, an endogenous inhibitor of IAPs, was down-regulated in RCC and inversely correlated

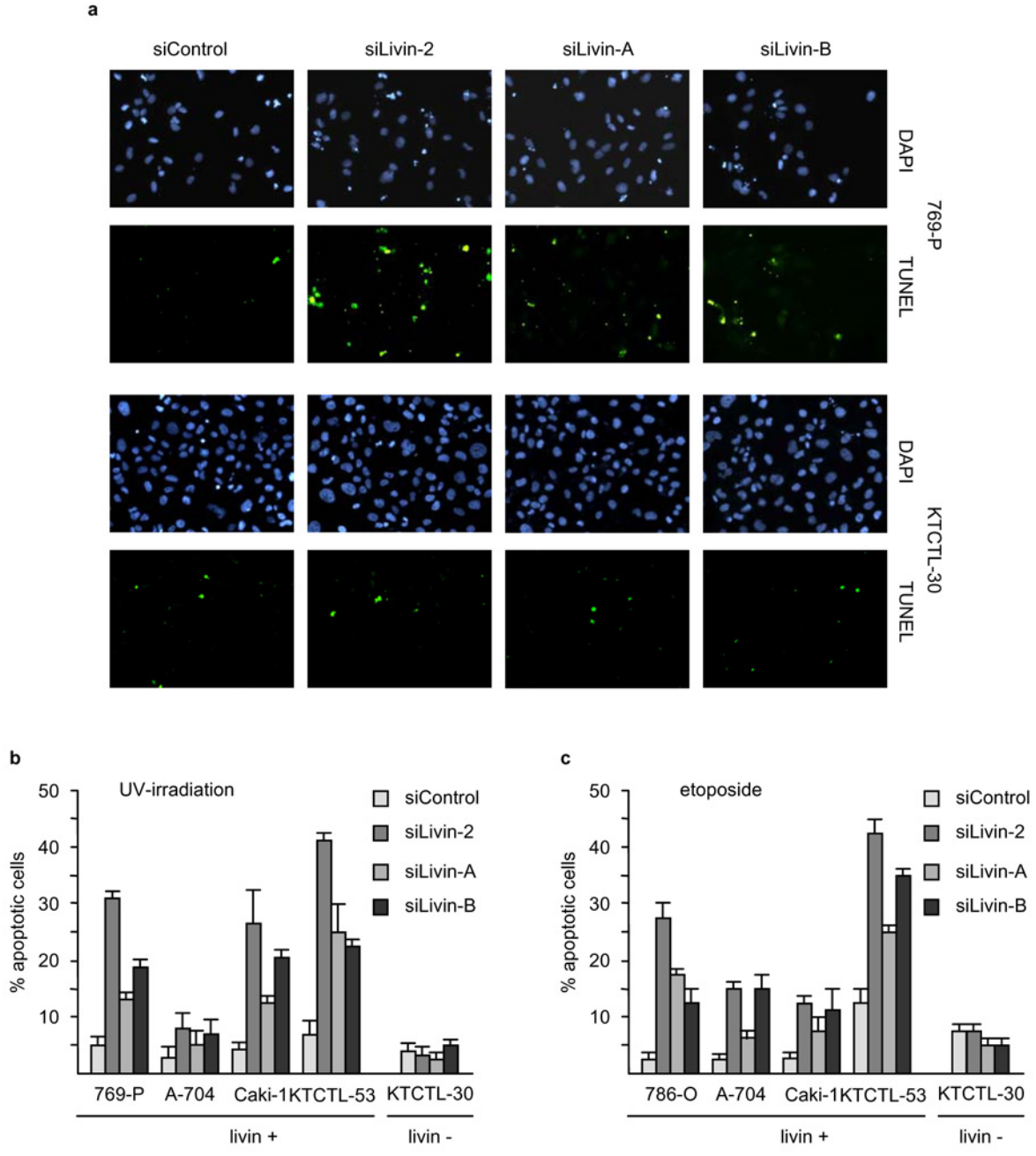

Figure 6. Both Livin isoforms contribute to the apoptotic resistance of RCC cells. (*a*) Cells were transfected with siRNAs specific for the *livin α* (siLivin-A) or *livin β* (siLivin-B) isoform [11] or with siLivin-2, which concomitantly inhibits of both isoforms, followed by exposure to UV irradiation. Cell nuclei were visualized by DAPI staining, and apoptotic cells were detected by TUNEL. (*b*) Percentage of apoptotic cells upon treatment with UV irradiation. (*c*) Percentage of apoptotic cells upon treatment with etoposide. Standard deviations are indicated.

with tumor stage and grade. Additionally, RCC cells were sensitized to pro-apoptotic stimuli after transfection of Smac cDNA [21]. On the other hand, experiments utilizing a Smac-derived peptide gave no indication that endogenous IAPs exert an inhibiting effect in primary RCC cells [22]. Our results indicate that *livin* extends the range of IAP genes that increase the apoptotic resistance of RCC cells. Conceivably, the targeted inhibition of functionally relevant IAPs (*e.g.* by siRNAs or by small molecule inhibitors) may provide a novel rational strategy to lower the apoptotic threshold of RCC cells and to increase the therapeutic efficiency of pro-apoptotic chemotherapeutics.

Although the number of primary RCC tissues analyzed here is limited, the percentage scoring positive for *livin* expression (roughly 90%) is similar to previously reported data for other tumor types, such as melanoma (70.6%) [23], lung cancer (76.3%) [8], and neuroblastoma (79.5%) [9]. Notably, studies indicate that *livin* expression might be useful as a marker of early recurrence in superficial bladder cancer [7] and, in combination with other biological features, can identify a subset of neuroblastoma patients with a particularly poor prognosis [9]. Furthermore, a positive correlation has been found between *livin* expression in melanoma, increased *in vitro* drug resistance, and poor clinical response of the patients [24]. Additional studies involving larger numbers of RCC patients and longer follow-up periods are required to evaluate whether *livin* expression has the potential to serve as a novel diagnostic or prognostic marker for RCC.

In conclusion, this work shows that the *livin* gene is expressed in RCC and contributes to the apoptotic resistance of RCC cells. These findings indicate that *livin* could represent a novel molecular target for RCC therapy. Much effort is being currently undertaken to develop therapeutically useful inhibitors of IAP proteins [25]. If successful, one could envision the combination of specific inhibitors of Livin with pro-apoptotic agents as a future therapeutic strategy to increase the apoptotic sensitivity of RCC.

Acknowledgements. We thank Dr. Elisabeth Schwarz and Dr. Ingrid Herr for RCC cell lines and Willi Albert (Homburg) for logistics. N. W. is financed by a "Gerok" scholarship from the German Cancer Research Center. The study was supported by a grant from the Deutsche Krebshilfe (106213).

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