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PIDDosome expression and the role of caspase-2 activation for chemotherapy-induced apoptosis in RCCs

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Abstract. *Background*: The importance of caspase-2 activation for mediating apoptosis in cancer is not clear and seems to differ between different tumour types. Furthermore, only few data have been obtained concerning the expression of caspase-2, which can be alternatively spliced into caspase-2L and caspase-2S, and the other PIDDosome members PIDD and RAIDD in human tumours *in vivo*. We, therefore, investigated their expression in renal cell carcinomas (RCCs) of the clear cell type *in vivo* and analysed the role of caspase-2 in chemotherapy-induced apoptosis in RCCs *in vitro*.

Methods: The analyses were performed by semiquantitative real-time PCR, Western Blot and Caspase-2 Assay.

Results: Our *in vivo* results showed an overall decrease in proapoptotic caspase-2L expression during tumour progression due to an increase in the relative share of caspase-2S mRNA in total caspase-2 mRNA expression. Furthermore, an increase in the expression of PIDD and RAIDD could be observed. In contrast, antiapoptotic BCL-2 expression increased only during early tumour stages, whereas expression decreased in pT3 RCCs. *In vitro*, caspase-2 activation in RCC cell lines coincidenced with sensitivity of tumour cells towards Topotecan-induced apoptosis. However, inhibition of caspase-2 could not prevent Topotecan-induced apoptosis. Interestingly, Topotecan-resistance could be overcome by the apoptosis-sensitizing drug HA14-1.

Conclusion: Our study confirms the concept of a shift towards a more antiapoptotic transcriptional context during tumour progression in RCCs. Furthermore, it shows that caspase-2 participates in chemotherapy-induced apoptosis in RCCs although it is not mandatory for it. Additionally, inhibition of antiapoptotic BCL-2 family members might provide a possible way to overcome chemotherapy resistance of RCCs.

Keywords: Renal cell carcinoma, caspase-2, PIDD, RAIDD, PIDDosome, BCL-2, HA14-1

1. Introduction

The broad resistance of renal cell carcinomas (RCCs) towards chemo- and radiotherapy is a major difficulty in treatment of advanced tumour stages [1,2]. Until recently, these patients had few treatment options beyond cytokine therapy (interleukin-2 and interferon-α (IFN-α)), but new targeted therapies with Sunitinib, Bevacizumab and Temsirolimus in combination with IFN-α lately revealed improved progression free survival and response rates compared to INF-α alone. However, data on overall survival improvement by these therapies are sparse. Only Temsirolimus has been shown to improve overall survival by 3.6 months until now [3]. Therefore, identification and understanding of the cellular mechanisms responsible for the broad chemotherapy resistance of RCCs remains a major challenge and is necessary for further improvement of therapy strategies against this type of cancer.

Defects in the apoptotic signalling pathways due to dysregulation of pro- and antiapoptotic genes have been recognized as one of these resistance mechanisms [4]. Thus, apoptosis susceptibility is thought to depend on the delicate balance of pro- and antiapoptotic genes and a shift of this balance towards an antiapoptotic context is assumed to be important for tumour progression.

The main apoptotic pathway consists of the cascade-like activation of caspases [5]. One interesting caspase is caspase-2 participating not only in the intrinsic but also in the extrinsic apoptotic pathway [6]: in the intrinsic pathway, caspase-2 acts as an initiator caspase and forms the so called PIDDosome by associ-

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ation with PIDD and RAIDD [7]. The PIDDosome transfers the DNA-damage induced apoptotic signal to downstream apoptotic pathways. Thus, caspase-2 activation has been reported upstream of caspase-8 cleavage in Ceramide and Etoposide-induced apoptosis [8] as well as upstream of mitochondrial activation [9]. In the extrinsic apoptosis pathway, caspase-2 is involved in processing of caspase-8 upon binding of death ligands to their receptors [10]. However, the putative role of caspase-2 as an initiator caspase is still under debate: Samraj and coworkers could show that in lymphoma cell lines caspase-9 precedes caspase-2 activation and was located downstream of the mitochondria, playing the role not of an initiator but of an executioner caspase [11].

In vivo, caspase-2 expression has been examined in many cancers like follicle B-cell neoplasms [12], acute myelogenous leukaemia [13], squamous cell carcinoma of the vulva [14], gastric cancer [15], acute lymphoblastic leukaemia, [16] and metastatic brain cancer [17]. However, data on the expression of caspase-2 in RCCs are missing.

The other proapoptotic members of the PIDDosome, PIDD and RAIDD, have rarely been investigated *in vivo*. Besides playing an important role in apoptosis, PIDD also marks a bifurcation between cell survival and cell death [18,19] and thus has not exclusively proapoptotic functions. However, there is only one *in vivo* study on PIDD expression in oral squamous cell carcinomas [20]. Similarly, the expression of RAIDD has only been examined in mantle cell lymphomas [21].

Besides these proapoptotic genes enhancing the proapoptotic function of caspase-2, caspase-2 is under tight control of several antiapoptotic genes on the mRNA and protein level: antiapoptotic caspase-2S, a truncated form of caspase-2, results from alternative splicing of the caspase-2 pre-mRNA [22,23]. It is transcribed on the expense of proapoptotic caspase-2L mRNA and thus, increased mRNA expression of caspase-2S is able to inhibit caspase-2L on the mRNA level [24]. Furthermore, caspase-2S protein has been shown to inhibit caspase-2L *in vitro* when overexpressed [25]. Although revealing a poor mRNA stability [26], caspase-2S *in vivo* protein expression could be demonstrated in macrophage derived foam cells [27]. Other *in vivo* studies concerning expression of caspase-2S, especially in tumour tissues, are missing. Additionally, on the protein level BCL-2, another important antiapoptotic gene which is mainly responsible for the integrity of the mitochondrial membrane [28], has been reported to inhibit caspase-2 activation [29]. BCL-2 expression has extensively been examined in RCCs with divergent results: whereas some studies found an increased survival in patients with RCCs revealing high expression levels of BCL-2 [30,31] others could not confirm this correlation [32–34].

Additionally, we tested apoptosis induction by Topotecan – as an example of classical DNA-damaging drugs – in combination with HA14-1 in RCC cell lines in this study: we chose Topotecan on the one hand, since induction of cell death by this Topoisomerase I inhibitor has extensively been examined in multiple RCC cell lines [35,36]. HA14-1, on the other hand, is a putative inhibitor of antiapoptotic BCL-2 family members, which could strongly induce apoptosis in RCC cell lines in a previous study and, furthermore, had a synergistic effect on TRAIL-induced apoptosis in RCCs [37].

The aim of our study was to analyse expression of the PIDDosome components caspase-2, PIDD and RAIDD and of the important caspase-2 inhibiting factors caspase-2S as well as BCL-2 in RCCs *in vivo* to determine their meaning for tumour progression. Furthermore, we analysed the relevance of caspase-2 in chemotherapy-induced apoptosis in RCC cell lines *in vitro* and identify a potential mechanism for overcoming chemotherapy resistance in RCCs.

2. Material and methods

2.1. Patients and specimen

Tissue samples of 36 RCCs of the clear cell type of different tumour stages and grades as well as 36 samples of adjacent non-neoplastic renal tissues were obtained from patients who had undergone nephrectomy at the University Hospital of Duesseldorf (Table 1). No preoperative chemotherapy had been performed. The tissue was macrodissected and histologic examination of tumour tissue revealed a tumour cell content of more than 80%. The tumour specimens were immediately flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Tumour staging was performed according to the World Health Organization and the International Union Against Cancer: pT1 comprised tumours $\leqslant$7 cm confined to the kidney, pT2 comprised tumours $>$7 cm confined to the kidney and pT3 comprised tumours with invasion of larger blood vessels, of the adrenal gland or of perirenal tissue.

Table 1
Stage, number and differentiation grade of the RCCs investigated as well as gender of the patients

Stage	G2	G3	Male	Female
pT1	11	1	8	4
pT2	10	2	9	3
pT3	8	4	7	5

Notes: Our investigation comprised 36 tumours, of which 12 were pT1, 12 were pT2 and 12 were pT3 tumours. The pT1 tumours contained 11 grade 2 and 1 grade 3 RCC; 8 were gained from male and 4 from female patients. The pT2 tumours covered 10 grade 2 and 2 grade 3 RCCs; 9 were obtained from male and 3 from female patients. The pT3 tumours comprised 8 grade 2 and 4 grade 3 RCCs; 7 were removed from male and 5 from female patients. Tumour staging was performed according to the World Health Organization and the International Union against Cancer. Tumour grading was performed according to the Fuhrmann nuclear grading system.

Tumour grading was performed according to the Fuhrmann nuclear grading system: In grade 2 tumours the nuclear size was approximately 15 µm, the nuclear shape was round, the chromatin was finely granular and the nucleoli were small and not visible in the 10× objective. In grade 3 tumours the nuclear size was approximately 20 µm, the nuclear shape was round to oval, the chromatin was coarsly granular and the nucleoli were prominent in the 10× objective [38].

2.2. Cells and culture

The permanent cell lines clearCa-2, clearCa-6 and clearCa-17 were derived from typical representatives of the clear cell type of RCCs [39,40]. These cell lines were maintained with Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin, aspartat/asparagin and HEPES buffer (standard growth medium) and cultivated at 37°C in an atmosphere with 5% CO_2.

For treatment tumour cells in the exponential growth phase were seeded at 2.5×10^5 cells/cm^2 in standard growth medium. After 24 hours the tumour cells were exposed to Topotecan (0.1 respectively 10 µg/ml), HA14-1 (25 respectively 50 µM), pan-caspase-inhibitor Z-VAD (OMe)-FMK (Calbiochem) (50 µM) and caspase-2 inhibitor Z-VDVAD-FMK (Calbiochem) (50 µM) either alone or in combination for the indicated time periods.

2.3. RNA extraction

Total RNA was isolated by the RNeasy minikit with DNAse treatment according to the manufacturer's instructions (Qiagen, Germany) to exclude DNA contamination. The integrity of all tested total RNA samples was verified by intact 18S/28S rRNA bands in agarose gel electrophoresis.

2.4. Reverse transcription

cDNA synthesis was performed with the "Reverse transcription system" of Promega (Germany) according to the manufacturer's protocol. In short, for cDNA synthesis, 1 µg of total RNA was reverse transcribed in a final volume of 20 µl containing 0.5 µg Oligo (dt) Primer, 1 mM dNTP mix, 0.5 U of recombinant RNasin RNAse inhibitor, 5 mM $MgCl_2$ and 15 U of AMV reverse transcriptase with the corresponding 2 µl of 10× RT buffer. Reverse transcription (RT) was performed for 15 min at 42°C. Synthezised cDNA was diluted 1:5 for real-time PCR.

2.5. Quantitative real-time detection PCR

Amplification and quantification of caspase-2, caspase-2S, PIDD, RAIDD, BCL-2 and SDHA were carried out in the LightCycler (Roche Diagnostics, Germany) by using a total volume of 20 µl, including 10 µl Platinum SYBR Green qPCR SuperMix UDG (Roche Diagnostics, Germany), 4 µl 5× diluted cDNA, 1 µl Bovine Serum Albumin (1 mg/ml stock solution) and 1 µM each sense and antisense primers (caspase-2 forward primer: 5′-CCT TTT CCG GTG TGT GA-3′, caspase-2 reverse primer: 5′-TCT CCA GTG AAG TGC ACA-3′; caspase-2S forward primer 5′-CAC CTC CTT CTG TTC ACT GCT-3′, caspase-2S reverse primer 5′-GCA TAG CCG GAT ATC ATG TCT-3′; PIDD forward primer 5′-GGA TGC TGT CCT GGT ACT TGC-3′, PIDD reverse primer 5′-CTG GAT GAG CAG ATC CGT CAC-3′; RAIDD forward primer 5′-CTG GGT GCA GAG GTA TTG GT-3′, RAIDD reverse primer 5′-TTG CCT TCT TCA GCT TCT CC-3′; BCL-2 forward primer 5′-ATG TGT GTG GAG AGC GTC AA-3′, BCL-2 reverse primer 5′-ACA GTT CCA CAA AGG CAT CC-3′; SDHA forward primer 5′-TGG GAA CAA GAG GGC ATC TG-3′, SDHA reverse primer 5′-CCA CCA CTG CAT CAA ATT CAT G-3′). Real-time PCR was carried out in light cycler capillaries with an initial incubation step at 50°C for 3 min for reduction of dUTP-containing amplificats, followed by a denaturation step of less than 1 s at 95°C, followed by 50 cycles for 5 s at 94°C, annealing for 10 s at 60°C for caspase-2, RAIDD and SDHA, 58°C for caspase-2S as well as

54°C for BCL-2 followed by extension for 20 s at 72°C. Concerning PIDD amplification, following the initial denaturation step, 70 cycles for 15 s at 94°C, annealing for 30 s at 58°C and extension for 60 s at 72°C were performed [19]. Melting curves were directly drawn after amplification.

PCR products were additionally checked by electrophoresis on 2% agarose gels containing ethidium bromide and visualised under UV transillumination. PCR products were confirmed by DNA sequencing (data not shown).

To normalize caspase-2, caspase-2S, PIDD, RAIDD and BCL-2 expression, the housekeeping gene SDHA was used as an external standard. Statistical analysis was performed by the Mann–Whitney- and Wilcoxon-test (non-neoplastic renal tissues and primary tumour tissues) respectively the Student's t-test (cell lines). p-values < 0.05 were regarded as statistically significant.

2.6. *Quantification of caspase-2, caspase-2S, PIDD, RAIDD and BCL-2 expression*

mRNA expression of caspase-2, caspase-2S, PIDD, RAIDD and BCL-2 as well as the housekeeping gene SDHA could be detected at PCR cycles 32, 28, 25, 25, 24 and 21 respectively.

PCR analysis on serial DNA dilution series (1:5–1:5000) generated a standard curve for caspase-2, caspase-2S, PIDD, RAIDD, and BCL-2 and SDHA, which was used as an external standard for quantification (Fig. 1). The corresponding correlation coefficients for each standard curve were 0.98–1.0, indicating a precise log-linear relationship. The expression of caspase-2, caspase-2S, PIDD, RAIDD, and BCL-2 was normalized to SDHA expression for statistical analysis (SDHA-normalized mRNA expression levels).

2.7. *Western Blot analysis*

Tumour tissue and non-neoplastic renal tissue were lysed with ice cold Lysis Buffer (100 mM NaCl, 10 mM Tris-HCl, pH: 7.6, 1 mM EDTA, 1% NP40, protease inhibitors) by homogenization. Cell lines were lysed by exchanging cell culture medium with ice cold Lysis Buffer. Then cells were scraped off the cell culture dish and the lysate was incubated for 10 min at 4°C with occational vortexing.

The lysates were centrifuged at 4°C at 13,000 × g for 10 min. Protein concentration of the supernatant was determined by the Bradford Method (Biorad, Ger-

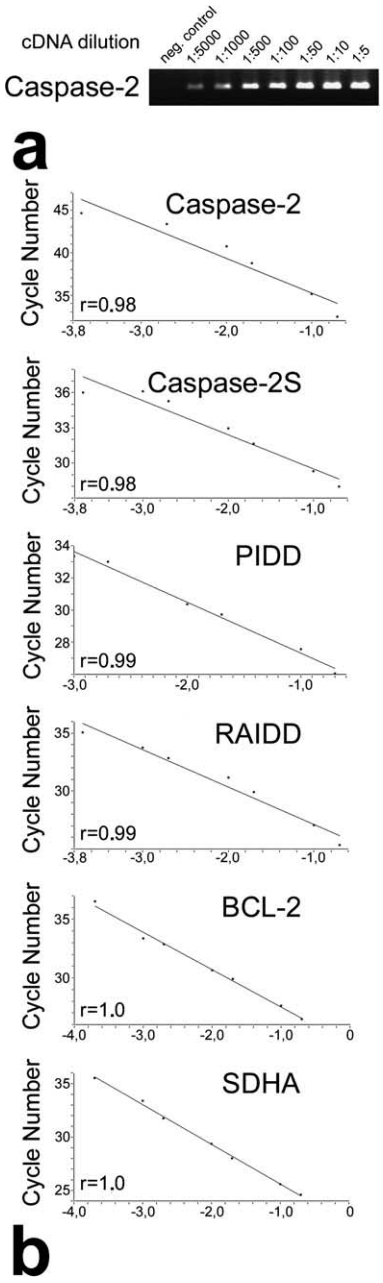

Fig. 1. Analysis of total caspase-2, caspase-2S, PIDD, RAIDD, BCL-2 and SDHA expression by real time PCR. (a) For a relative quantification, cDNA dilution series ranging from 1:1 to 1:5000 were used to create standard curves for total caspase-2, caspase-2S, PIDD, RAIDD, BCL-2 and SDHA for LightCycler PCR. Exemplarily, the agarose gel of the PCR for the total caspase-2 standard curve is shown. (b) LightCycler based standard curves for total caspase-2, caspase-2S, PIDD, RAIDD, BCL-2 and SDHA derived from PCR using the serial dilution series as template. The cycle number was plotted against the log of concentration by the second derivative maximum method. A correlation coefficient of $r > 0.97$ indicated a precise log linear relationship.

many). 250 µg of protein lysate from tumour tissue and 50 µg of protein lysate from cultured cells were separated under denaturing conditions in 7–10% polyacrylamid-gels. After blotting, the protein was transferred to a reinforced nitrocellulose membrane (Schleicher and Schuell, Germany) and equal loading of the proteins was checked by Ponceau Red staining. The membranes were then blocked with TBS containing 5% non-fat dry milk and 0.2% Tween 20 for 24 h at 4°C. Afterwards the membranes were incubated for 12 h with human specific monoclonal or polyclonal primary antibodies (mouse monoclonal caspase-2 from BD Pharmingen: concentration 1:50–1:500, PIDD rabbit polyclonal from Alexis Biochemicals: concentration 1:100–1:250, RAIDD rabbit polyclonal from Alexis Biochemicals: concentration 1:100–1:500, mouse monoclonal BCL-2 from Dako: concentration 1:1000 and mouse monoclonal β-actin from DAKO: concentration 1:10,000) and then washed with TBS for 30 min.

The blots were incubated with a fluorescence-dye conjugated anti-mouse respectively anti-rabbit secondary antibody protected from light for another 12 h at 4°C, then washed for 30 min with TBS protected from light and scanned with the Odyssey Infrared Imaging System (LI-Cor, Biosciences).

Densitometry was performed with the ImageJ 1.41 Software. For relative quantification the intensity of the bands of caspase-2L obtained by densitometry was normalized to the intensity of the band of β-actin for each sample.

2.8. Caspase-2 assay

Detection of caspase-2 activation was performed with the caspase-2 Colorimetric Assay kit according to the manufacturer's protocol (Biocat GmbH). In short, untreated cells or cells treated with Topotecan and HA14-1 in the concentrations and for the time periods indicated in the figures were harvested and lysed in 50 µl chilled Cell Lysis Buffer for 10 min. Afterwards, the lysate was centrifuged for 1 min at 10,000 $\times$ g. The supernatant cytosolic extract was transferred in a fresh tube. Protein concentration of the supernatant was determined by the Bradford Method. 250 µg cytosolic extract were diluted to 50 µl with cell lysis buffer. 50 µl of 2$\times$ Reaction Buffer were added (containing 10 mM DTT). VDVAD-pNA substrate was added to a final concentration of 200 µM and the mixture was incubated for 2 h at 37°C. Samples were read at 405 nm and extinction was compared to the untreated control.

3. Results

3.1. Total caspase-2 and caspase-2S are upregulated during tumour progression in RCCs whereas caspase-2L is downregulated

mRNA expression of total caspase-2 and caspase-2S was found in all RCCs and in all corresponding non-neoplastic renal tissues.

Thus, the relative caspase-2 mRNA expression (i.e. SDHA-normalized caspase-2 expression) (Fig. 2a) showed no differences between non-neoplastic renal tissue and pT1 carcinomas, whereas caspase-2 expression was highly significantly increased in pT2 and pT3 tumours compared to non-neoplastic renal tissue and pT1 RCCs. However, no significant differences were found between pT2 and pT3 RCCs.

In contrast, relative mRNA expression of antiapoptotic caspase-2S (i.e. SDHA-normalized caspase-2S mRNA expression) was not only highly significantly increased during tumour progression from pT1 to pT2 RCCs but also from non-neoplastic renal tissue to pT1 RCCs as well as from pT2 to pT3 RCCs (Fig. 2b). Thus, the relative caspase-2S mRNA expression levels revealed a continuous highly significant increase from non-neoplastic renal tissue to pT3 RCCs.

We additionally performed Western Blot experiments on 4 arbitrarily selected non-neoplastic renal tissues as well as 4 pT1, pT2 and pT3 RCCs each (Fig. 2c) to determine, which influence the observed changes in relative mRNA expression of total caspase-2 and caspase-2S had on the protein expression levels of proapoptotic caspase-2L. Here, caspase-2L revealed only a very weak protein expression in all tissue samples. It could just be detected after application of as much as 250 µg total cell lysate per lane in the Western Blot and was expressed in non-neoplastic renal tissue and pT1 carcinomas with no detectable differences by densitometric analysis (Fig. 2c, d). However, this overall weak caspase-2L protein expression was even decreased in pT2 carcinomas compared to non-neoplastic renal tissue and pT1 RCCs and further declined and nearly vanished in pT3 RCCs.

Interestingly, no caspase-2S protein expression could be detected, though using an antibody capable of detecting both isoforms, confirming that caspase-2S mRNA is obviously very unstable and subjected to rapid degradation without generation of considerable amounts of protein in RCCs [26].

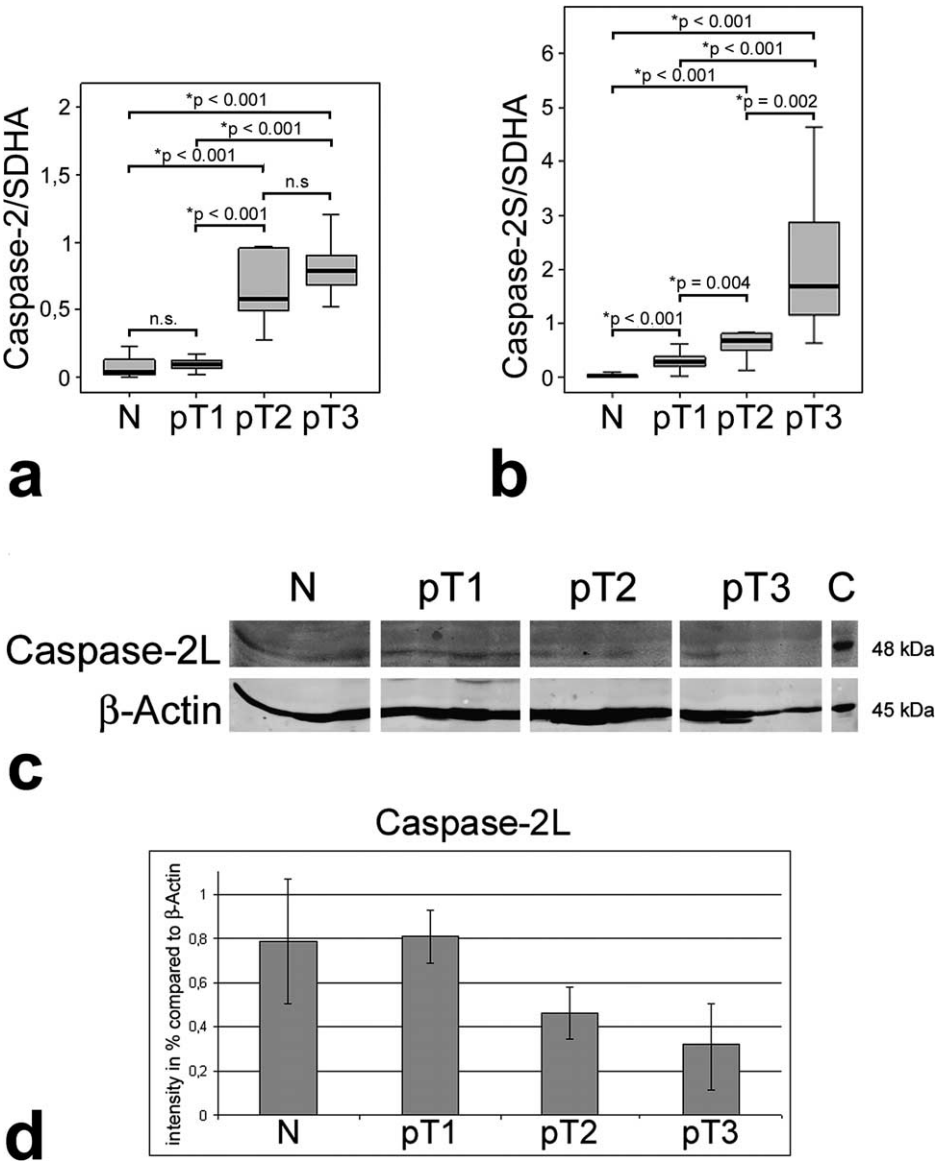

Fig. 2. SDHA-normalized total caspase-2 and caspase-2S mRNA expression as well as caspase-2L protein expression in non-neoplastic renal tissue and in RCCs of different tumour stages. (a) Significantly increased relative total caspase-2 mRNA expression (i.e. SDHA-normalized total caspase-2 expression) in pT2 and pT3 RCCs when compared to non-neoplastic renal tissue (N) and pT1 RCCs. No difference between relative total caspase-2 mRNA expression in RCCs infiltrating beyond the kidney borders (pT3) and pT2 RCCs. (Significant results are indicated $^*p$.) (b) Significantly increased relative caspase-2S mRNA expression (i.e. SDHA-normalized caspase-2S expression) in pT1 RCCs when compared to non-neoplastic renal tissue. Further increase in relative caspase-2S mRNA expression in pT2 RCCs compared to pT1 RCCs and in pT3 RCCs compared to pT2 RCCs. (Significant results are indicated $^*p$.) (c) Four arbitrarily selected non-neoplastic tumour tissues and four arbitrarily selected tumour tissues of each stage were analysed by Western Blot. No difference in caspase-2L protein expression between non-neoplastic renal tissue and pT1 RCCs. Decreased caspase-2S expression in pT2 RCCs compared to non-neoplastic renal tissue and pT1 RCCs with further decrease in pT3 RCCs. (d) For relative quantification the intensity of the Western Blot band of caspase-2L obtained by densitometry was normalized to the intensity of the band of β-actin for each sample (given is the relative intensity in % of the β-actin band). Data represent the means and the standard deviations of the four non-neoplastic renal tissues respectively the four RCC samples of each tumour stage investigated by Western Blot.

3.2. The PIDDosome members PIDD and RAIDD are upregulated during tumour progression in RCCs

Transcription of the proapoptotic PIDDosome members PIDD and RAIDD was found in all RCCs and in all corresponding non-neoplastic renal tissues. Interestingly, they revealed a similar change in relative mRNA expression during tumour progression.

Relative PIDD mRNA expression (i.e. SDHA-normalized PIDD expression) (Fig. 3a) did not significantly differ between non-neoplastic renal tissues and pT1 RCCs but highly significantly increased in pT2 and pT3 RCCs compared to non-neoplastic renal tissue and pT1 RCCs without significant differences between pT2 and pT3 carcinomas.

In contrast, a significant increase in relative RAIDD mRNA expression (i.e. SDHA-normalized RAIDD expression) could already be observed comparing non-neoplastic renal tissue with pT1 carcinomas (Fig. 3b). Again, a highly significant increase in relative RAIDD expression was observed when comparing pT2 and pT3 carcinomas with non-neoplastic renal tissue as well as pT1 RCCs, whereas no significant difference in relative RAIDD expression between pT2 and pT3 carcinomas occurred.

On the protein level, neither PIDD nor RAIDD could be detected in the Western Blot of total protein lysates obtained from non-neoplastic renal tissues and tumor tissues by standard procedures (data not shown).

3.3. Expression of antiapoptotic BCL-2 is significantly increased in pT1 and pT2 RCCs but significantly decreased in pT3 RCCs

mRNA Expression of antiapoptotic BCL-2 could be observed in all RCCs and in all corresponding non-neoplastic renal tissues. However, the course of relative mRNA expression differed from that of the other genes examined in this study. Thus, relative BCL-2 mRNA expression (i.e. SDHA-normalized BCL-2 mRNA expression) revealed a highly significant increase comparing pT1 RCCs with non-neoplastic renal tissues, with a further highly significant increase in pT2 RCCs compared to non-neoplastic renal tissues as well as pT1 RCCs. Interestingly, relative BCL-2 mRNA expression in pT3 carcinomas significantly decreased compared to pT1 and pT2 carcinomas and even non-neoplastic renal tissues (Fig. 4c).

3.4. Caspase-2 cleavage correlates with sensitivity to Topotecan-induced apoptosis in RCC cell lines

Sensitivity of multiple RCC cell lines towards Topotecan induced apoptosis is well known from previous experiments [36]. However, the involvement of caspase-2 in this Topotecan-induced apoptosis has not been investigated so far. We, therefore, compared Topotecan mediated caspase-2 activation in 3 RCC

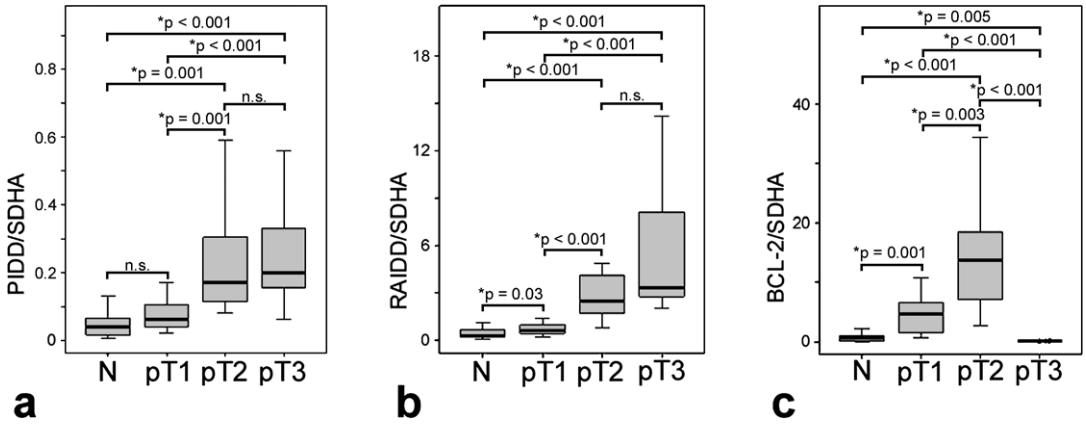

Fig. 3. SDHA-normalized PIDD, RAIDD and BCL-2 mRNA expression in non-neoplastic renal tissue and in RCCs of different tumour stages. (a) Significantly increased relative total PIDD mRNA expression (i.e. SDHA-normalized total PIDD expression) in pT2 and pT3 RCCs when compared to non-neoplastic renal tissue (N) and pT1 RCCs. No difference between relative total PIDD mRNA expression in RCCs infiltrating beyond the kidney borders (pT3) and pT2 RCCs. (Significant results are indicated $^{*}p$.) (b) Significantly increased relative RAIDD expression (i.e. SDHA-normalized RAIDD expression) in pT1 RCCs when compared to non-neoplastic renal tissue. Further increase in relative RAIDD mRNA expression in pT2 RCCs compared to pT1 RCCs. No difference between relative RAIDD mRNA expression in pT3 and pT2 RCCs. (c) Significantly increased relative BCL-2 expression (i.e. SDHA-normalized BCL-2 expression) in pT1 RCCs when compared to non-neoplastic renal tissue. Further increase in relative BCL-2 mRNA expression in pT2 RCCs compared to pT1 RCCs. Significantly decreased relative BCL-2 mRNA expression in pT3 RCCs compared to non-neoplastic renal tissues, pT1 as well as pT2 RCCs. (Significant results are indicated $^{*}p$.)

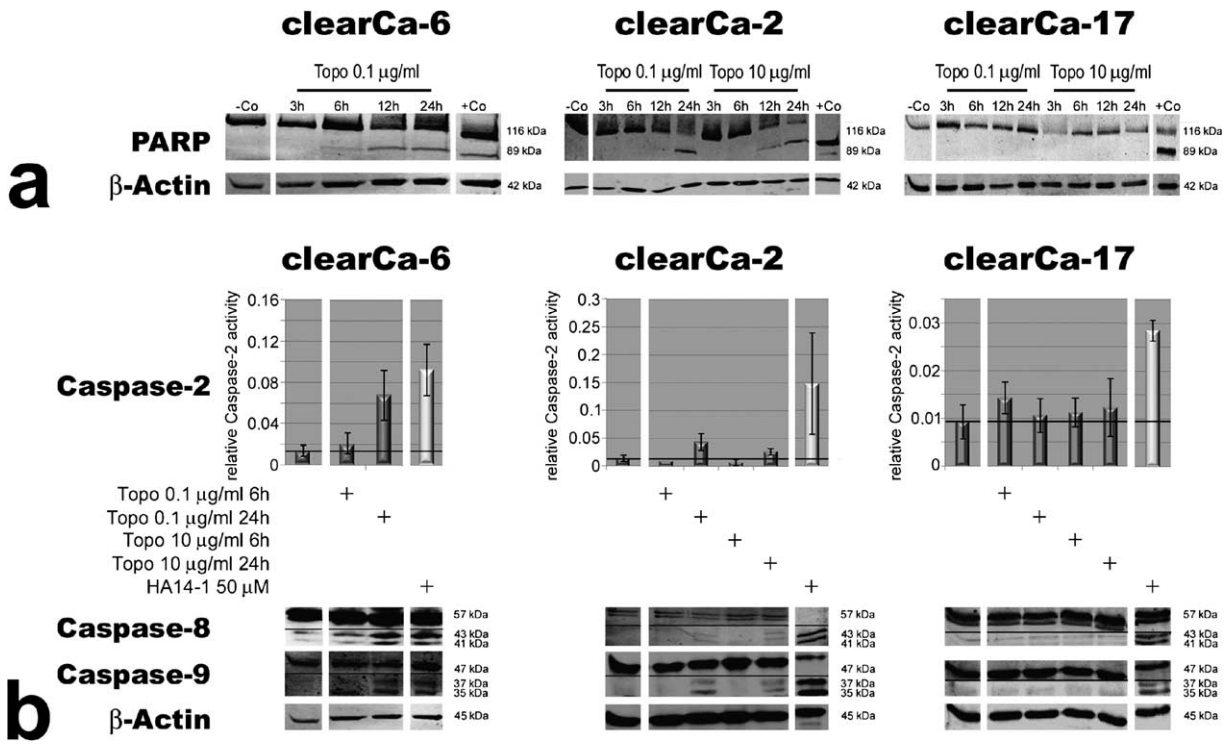

Fig. 4. PARP-cleavage (a) and activation of the Initiator caspases-2, -8 and -9 (b) in clearCa-6, clearCa-2 and clearCa-17. In the very sensitive cell line clearCa-6 Topotecan treatment (0.1 µg/ml) induced an early and weak PARP-cleavage already after 6 h and which was even increased after 12 and 24 h. PARP-cleavage was accompanied by a barely detectable activation of caspases-2, -8 and -9 at 6 respectively moderate activation after 24 h. In the moderately sensitive cell line clearCa-2 Topotecan treatment induced a very slight PARP cleavage after 12 h (0.1 µg/ml) whereas a stronger activation could be detected after 24 h (0.1 µg/ml) respectively 12 h (10 µg/ml). PARP-cleavage was accompanied by weak activation of caspases-2, -8 and -9 at 24 hours. In the resistant cell line clearCa-17 Topotecan treatment induced no PARP cleavage even after 24 h (0.1 and 10 µg/ml). No caspase-2, -8 or -9 activation could be detected.

cell lines exhibiting a high (clearCa-6), a moderate (clearCa-2) and no (clearCa-17) sensitivity towards Topotecan-mediated apoptosis to the activation of the initiator caspases-8 and -9. Caspase-2 activation coincidenced with PARP-cleavage in all cell lines after treatment with Topotecan (Fig. 4). However, coincidental activation could also be shown for the initiator caspases-8 and -9 (Fig. 4).

Conventional Western Blot analysis revealed only a very weak and barely detectable caspase-2L protein expression in the highly and moderately sensitive RCC cell lines clearCa-6 and clearCa-2, whereas the resistant cell line clearCa-17 showed a stronger caspase-2L expression (Fig. 5a). Interestingly, relative caspase-2S mRNA expression as determined by light Cycler PCR was significantly stronger in clearCa-6 and clearCa-2 than in clearCa-17 (Fig. 5b). This was in concordance with our *in vivo* results of caspase-2L protein downregulation upon upregulation of caspase-2S mRNA expression. BCL-2 protein expression was weakest in clearCa-2 and did not differ between clearCa-6 and clearCa-17. Thus, sensitivity towards Topotecan-induced apoptosis could not be concluded from caspase-2L, caspase-2S or BCL-2 expression in the RCC cell lines.

To further clarify the role of caspase-2 activation in Topotecan-induced apoptosis we performed experiments with caspase-inhibitors: Cotreatment of the cells with Topotecan and a pan-caspase-inhibitor (50 µM) (Fig. 6) prevented PARP-cleavage as a marker of Topotecan-induced, caspase-mediated apoptosis, whereas cotreatment with a specific inhibitor of caspase-2 at the same concentration had no effect on Topotecan mediated PARP-cleavage (Fig. 6).

3.5. Topotecan induced apoptosis and caspase-2 cleavage can be enhanced by HA14-1

It is well known, that caspase-2 cleavage can be inhibited by overexpression of BCL-2 [29]. We, there-

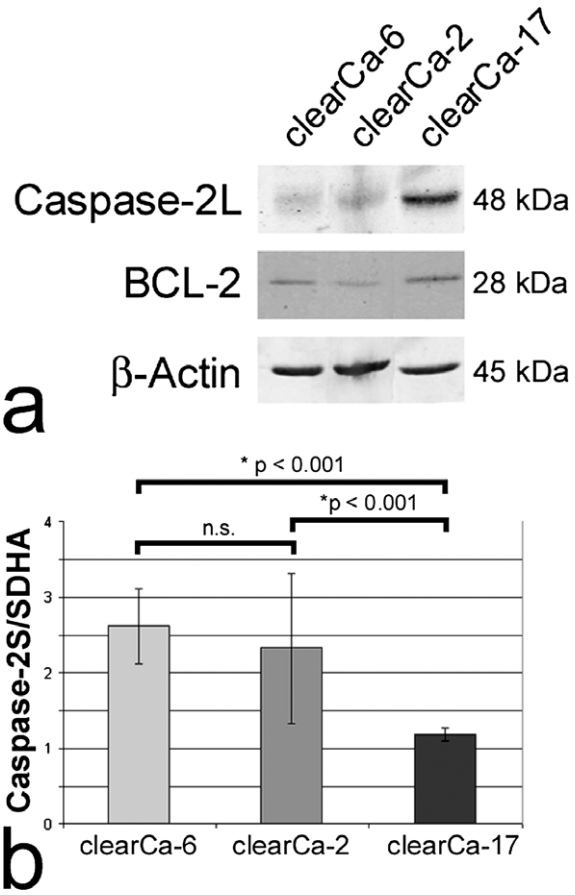

Fig. 5. Expression of caspase-2L, caspase-2S and BCL-2 does not correlate with sensitivity towards Topotecan-induced apoptosis in RCC cell lines. (a) Caspase-2L protein expression was strongest in clearCa-17 and only weak and barely detectable in clearCa-6 as well as clearCa-2. BCL-2 protein expression was weakest in clearCa-2 and did not differ between clearCa-6 and clearCa-17. (b) Caspase-2S mRNA expression was significantly higher in clearCa-6 and clearCa-2 than in clearCa-17 with no significant differences between clearCa-6 and clearCa-2. (Shown are the mean and the standard deviations of 3 independent light cycler runs for each sample. Significant results are indicated $^{*}p$.)

fore, tested whether HA14-1, a putative BCL-2 inhibitor, could enhance Topotecan-induced caspase-2 cleavage and thereby apoptosis in RCC cell lines. Pretreatment of all cell lines with HA14-1 in a dose just barely inducing apoptosis (25 μM) for 2 hours and consecutive cotreatment with HA14-1 (25 μM) and Topotecan (clearCa-6: 0.1 μM, clearCa-2 and clearCa-17: 10 μM) for 12 h (clearCa-6) respectively 24 h (clearCa-2 and clearCa-17) resulted in a synergistic enhancement of caspase-2 cleavage and apoptosis as determined by PARP-cleavage (Fig. 7). However, not only caspase-2 but also the initiator caspases-8

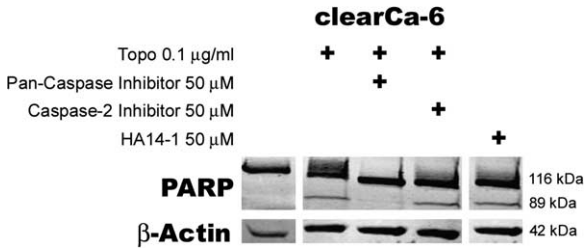

Fig. 6. Inhibition of caspase-2 does not prevent Topotecan-induced PARP cleavage. In the sensitive cell line clearCa-6 Topotecan treatment (0.1 μg/ml) for 12 h resulted in PARP-cleavage. Pretreatment with a Pan-caspase-inhibitor (50 μM) for 2 h followed by a cotreatment of the cells with Topotecan (0.1 μg/ml) and the Pan-caspase-inhibitor (50 μM) prevented PARP cleavage. Pretreatment with a specific caspase-2-inhibitor (50 μM) for 2 h followed by a cotreatment of the cells with Topotecan (0.1 μg/ml) and the caspase-2-inhibitor (50 μM) could not prevent PARP cleavage as a marker of apoptosis.

and -9, were synergistically activated after this cotreatment (Fig. 7). It remains to be said, that after single and combined treatment of clearCa-2 with HA14-1 the standard deviation in the caspase-2 assay was very high: Obviously, sensitivity towards an HA14-1 induced respectively enhanced apoptosis differed between different passages of this cell line.

4. Discussion

The present study investigated for the first time expression of caspase-2 – which can be alternatively spliced into caspase-2S and caspase-2L – and the PIDDosome members RAIDD and PIDD during tumour progression of clear cell RCCs *in vivo*. It reveals an increase in total caspase-2 mRNA expression during tumour progression paralleled by an increase in antiapoptotic caspase-2S mRNA expression. This resulted in an overall decrease of proapoptotic caspase-2L expression on the protein level. Furthermore, an increase in the mRNA expression of the other PIDDosome members, PIDD and RAIDD, during tumour progression could be observed. Finally, the importance of caspase-2 activation for Topotecan-induced apoptosis in RCCs is analysed *in vitro* and HA14-1 (a putative BCL-2 inhibitor) is recognized as a sensitizing agent towards Topotecan-induced apoptosis in RCCs.

Apoptosis is under tight control of a multitude of pro- and antiapoptotic genes, with caspase-2 being one important of these proapoptotic genes involved not only in the intrinsic but also in the extrinsic pathway of apoptosis. For its proapoptotic functions formation

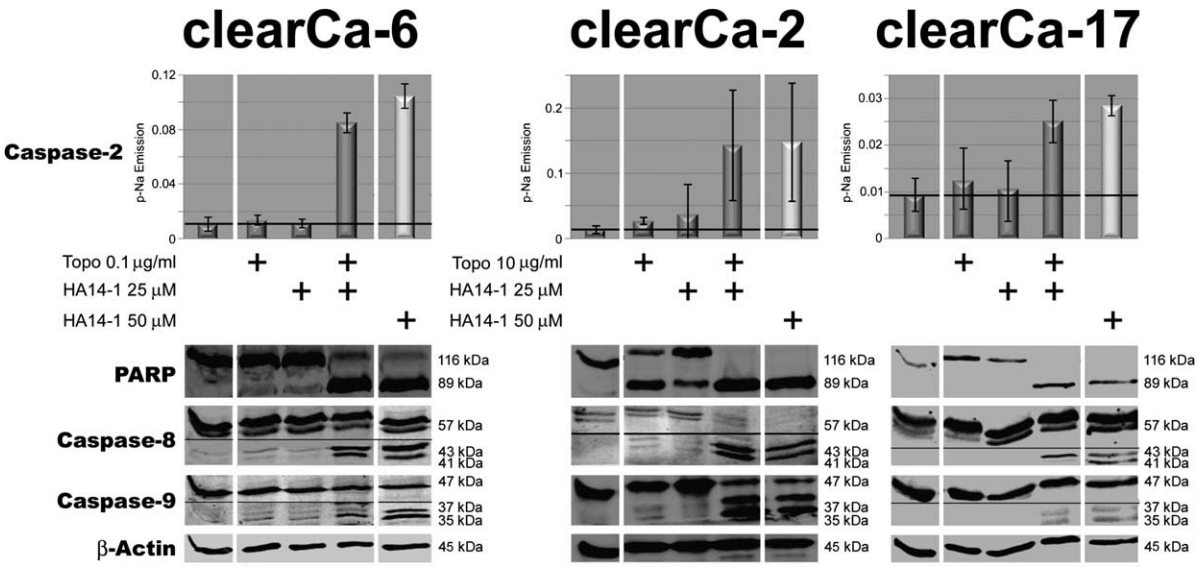

Fig. 7. HA14-1 is able to synergistically enhance activation of the initiator caspases-2, -8 and -9 as well as PARP-cleavage in all RCC cell lines. Cotreatment of the sensitive cell line clearCa-6 with Topotecan (0.1 µg/ml) and HA14-1 in a dose inducing apoptosis just weakly (25 µM) for 12 h after pretreatment with HA14-1 (25 µM) for 2 h induced a synergistically enhanced PARP-cleavage as well as caspase-2, caspase-8 and caspase-9 activation compared to separate treatment with Topotecan (0.1 µg/ml for 12 h) and HA14-1 (25 µM for 14 h) alone. In the moderately sensitive cell line clearCa-2 cotreatment with Topotecan (10 µg/ml) and HA14-1 in a dose (25 µM) barely inducing apoptosis for 24 h after pretreatment with HA14-1 (25 µM) for 2 h induced a synergistically enhanced caspase-2, caspase-8 and caspase-9 activation compared to separate treatment with Topotecan (10 µg/ml for 24 h) and HA14-1 (25 µM for 26 h) alone. Since Topotecan treatment (10 µg/ml) alone already induced a subtotal PARP-cleavage after 24 h a synergistic cleavage of PARP could not be seen after cotreatment with HA14-1. In the resistant cell line clearCa-17 cotreatment with Topotecan (10 µg/ml) and HA14-1 in a sublethal dose (25 µM) for 24 h after pretreatment with HA14-1 (25 µM) for 2 h induced PARP-cleavage as well as caspase-2, caspase-8 and caspase-9 activation whereas separate treatment with Topotecan (10 µg/ml for 24 h) and HA14-1 (25 µM for 26 h) alone could not induce PARP-cleavage or caspase-2, caspase-8 and caspase-9 activation.

of a multi-protein complex consisting of caspase-2L, RAIDD and PIDD, the so-called PIDDosome, is necessary [7].

Here, we found expression of total caspase-2, caspase-2S and the PIDDosome members PIDD and RAIDD in all non-neoplastic renal tissues and all clear cell RCCs, thereby confirming the ubiquity of their expression in renal tissue *in vivo*. Relative mRNA expression of total caspase-2 mRNA – comprising caspase-2L and caspase-2S expression – revealed no significant differences between non-neoplastic renal tissue and pT1 carcinomas whereas it was significantly increased in pT2 and pT3 RCCs with no differences between pT2 and pT3 RCCs. At the same time, antiapoptotic caspase-2S mRNA expression highly significantly increased already from non-neoplastic renal tissue to pT1 carcinomas as well as during further tumour progression from pT1 to pT2 and again from pT2 to pT3 RCCs. Interestingly, a continuous decrease of proapoptotic caspase-2L expression during tumour progression from pT1 to pT3 RCCs could be observed on the protein level. This decrease in caspase-2L expression (as the member of the PIDDosome that transfers the apoptotic signal to more downstream signalling pathways) might contribute to the well known apoptosis resistance of RCCs and it is reasonable to assume that the observed increase in caspase-2S mRNA expression might participate in this downregulation. However, caspase-2L protein expression was weak in all tissue samples and could only be detected after application of large amounts of protein lysate to the Western Blot. Caspase-2S protein could not be detected by Western Blot, confirming the hypothesis of rapid degradation of its mRNA without significant translation of a functional protein [26].

Of the other PIDDosome members, PIDD expression revealed no significant differences between non-neoplastic renal tissues and pT1 RCCs, whereas expression was highly significantly increased in pT2 and pT3 RCCs, without differences between pT2 and pT3 tumours. Interestingly, at first glance this proposed a shift towards a more proapoptotic cellular context in advanced tumour stages. However, PIDD is subjected to post-translational procession [19] and thus partici-

pates not only in the proapoptotic PIDDosome formation but also in prosurvival NFκB-pathway activation. Therefore, the observed increase in PIDD expression could also mean a growth advantage for tumour cells. Furthermore, mRNA isoforms of PIDD have been described [18] exhibiting pro- and antiapoptotic properties, which we did not address in this study but which could also contribute to apoptosis resistance in RCCs. Be it as it may, neither PIDD nor its cleaved forms could be shown on the protein level in the Western Blot under standard conditions, demonstrating that PIDD protein is only very weakly expressed in all renal tissues and thus proposing only a minor role for tumour development and progression in RCCs.

Relative RAIDD mRNA expression was highly significantly increased already in pT1 carcinomas compared to the level in non-neoplastic renal tissue and continuously increased highly significantly during further tumour progression from pT1 to pT2 with no significant difference between pT2 and pT3 RCCs. However, taking into account that caspase-2L expression decreased during tumour progression and RAIDD as well as PIDD were only very weakly expressed in RCCs on the protein level, it is of doubt, that this increase in RAIDD expression alone is able to compensate the downregulation respectively weak expression of the other PIDDosome members.

Besides the transcriptional regulation of caspase-2L by caspase-2S the proapoptotic activity of caspase-2L is on the protein level under the control of antiapoptotic genes like BCL-2 [29]. Therefore, we also analysed the relative mRNA expression of BCL-2 in our RCCs.

BCL-2 mRNA expression could be shown in all non-neoplastic renal tissues and all RCCs *in vivo*. Hereby, expression of BCL-2 was increased in pT1 RCCs compared to non-neoplastic renal tissue and again in pT2 compared to pT1 RCCs. However, its relative mRNA expression significantly declined from pT2 to pT3 RCCs. Regrettably, we had no follow up data of the patients. However, taking into account, that tumour stage is a major prognostic factor in RCCs, our results are in accordance with the findings of other groups [30,31], who revealed a reverse correlation between BCL-2 expression and prognosis of RCCs. Furthermore, this increase of BCL-2 in early tumour stages proposes an important role especially in tumour transformation of non-neoplastic tissue and during early tumour progression. In late stage RCCs (pT3) the low BCL-2 expression is paralleled by low caspase-2L expression.

Taken together, an overall decrease in proapoptotic caspase-2L expression during tumour progression of RCCs was paralleled by an increasing expression of PIDD and an increase in expression of RAIDD. In contrast, antiapoptotic BCL-2 expression was significantly increased only during early tumour stages and decreased in pT3 RCCs. However, the very weak and even decreasing expression of proapoptotic caspase-2L in advanced RCCs – as the executioning and thus the apoptotic signal transferring PIDDosome member – in concert with the overall very weak PIDD expression, with unclear posttranslational processing reflects a more antiapoptotic context during tumour progression of RCCs, probably contributing to the well known apoptosis resistance of RCCs. Furthermore, we could previously show an overexpression of ARC in RCCs of all tumour stages [41], another very important caspase-2 inhibiting protein, which adds to this more antiapoptotic transcriptional context of RCCs.

However, the role of caspase-2 mediated apoptosis in RCCs was not clear. Thus, to further establish the importance of caspase-2 activation for apoptosis in RCCs, we analysed caspase-2 activation in 3 RCC cell lines exhibiting a high (clearCa-6), a moderate (clearCa-2) and no (clearCa-17) sensitivity towards Topotecan-mediated apoptosis. Here, caspase-2 activation coincidenced with sensitivity towards Topotecan-induced apoptosis. However, a similar activation could also be demonstrated for the initiator caspases-8 and -9. This sensitivity towards Topotecan-induced apoptosis could neither be concluded from the protein expression levels of caspase-2L and BCL-2 nor from the mRNA expression levels of caspase-2S. Obviously other cellular mechanisms than the expression levels of caspase-2L, caspase-2S or BCL-2 are responsible for Topotecan sensitivity in RCC cell lines. Nevertheless, caspase-2L protein expression was inversely correlated to caspase-2S expression in the cell lines *in vitro*. This was in concordance with our *in vivo* results of decreasing caspase-2L protein levels upon increase of caspase-2S mRNA in the primary tumour tissues. Importantly, inhibition of caspase-2 with a specific caspase-2 inhibitor in the Topotecan-sensitive cell line clearCa-6 could not prevent apoptosis, whereas a pan-caspase-inhibitor in the same concentration clearly inhibited apoptosis as determined by PARP-cleavage.

In conclusion these results showed that the caspase-cascade was involved in Topotecan mediated apoptosis in RCCs, whereas caspase-2 was not essential for this caspase-mediated apoptosis, confirming the results of Davidson and coworkers [42]. Obviously the proapoptotic function of caspase-2 could be re-

placed or bypassed by other proapoptotic caspase-dependent signalling pathways in RCCs. All cell lines expressed BCL-2 protein to a similar extend providing a rationale for treatment with the putative BCL-2 inhibitor HA14-1. Interestingly, Topotecan mediated-apoptosis – determined by caspase-2 activation as well as caspase-8, caspase-9 and PARP-cleavage – could be synergistically enhanced in the Topotecan sensitive cell lines by HA14-1. Importantly, even the apoptosis resistant cell line clearCa-17 could be sensitized towards Topotecan-induced apoptosis by HA14-1. These results go in concert with our previous observation that HA14-1 could sensitize RCC cell lines towards TRAIL-induced apoptosis [37]. Although the role of HA14-1 as BCL-2 inhibitor has recently been questioned and it seems to have multiple mechanism of action by which it induces apoptosis [43], our findings could provide a way of overcoming the well known chemotherapy resistance of RCCs, especially taking into account that BCL-2 expression in RCC metastases is higher than in high-risk primary tumours [44].

Taken together, we observed changes in the expression levels of caspase-2, the other PIDDosome members – PIDD and RAIDD – and caspase-2 inhibiting BCL-2 during carcinogenesis and tumour progression as well as dedifferentiation in RCCs *in vivo*, showing expression of caspase-2, caspase-2S, RAIDD and PIDD in RCCs *in vivo* for the first time. Our experiments revealed a decrease in proapoptotic caspase-2L expression that might be due to an increase in antiapoptotic caspase-2S mRNA expression, an only weak but nevertheless increasing PIDD expression and a weak but increasing proapoptotic RAIDD expression during tumour progression of RCCs, whereas antiapoptotic BCL-2 was increased only in early tumour stages and decreased during tumour spread beyond the kidney border. Taking into account the dual role of PIDD as apoptosis inhibitor and enhancer together with the well known overexpression of ARC in RCCs, these results on the expression of caspase-2 and associated genes confirms the concept of a shift towards an antiapoptotic transcriptional context during tumour progression in RCCs.

Furthermore, our *in vitro* results propose that caspase-2 participates in chemotherapy-induced apoptosis in RCCs, although it is not mandatory for it. Much more, its proapoptotic functions can obviously be bypassed and replaced by other initiator caspases.

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