

# XIAP underlies apoptosis resistance of renal cell carcinoma cells

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**Abstract.** X-linked inhibitor of apoptosis (XIAP), a key member of the inhibitors of apoptosis protein family, can inhibit apoptosis by directly binding to the initiator caspase-9, -3 and -7, thereby promoting tumor cell survival during tumor progression. In the present study, XIAP basal expression levels were investigated and its contribution to the resistance to apoptosis was evaluated, in the RCC cell lines exposed to apoptosis-inducing drugs. This was investigated by histological methods and western blot analysis. Using RNA interference, elimination of XIAP in Caki-1 cells was also studied, and its contribution to the sensitivity to apoptosis induced through the intrinsic pathway was observed. Differences in XIAP expression were detected between ClearCa-2 and ClearCa-6 cell lines. ClearCa-6 cells with lower expression of XIAP were more sensitive to apoptosis-inducing drugs, compared with ClearCa-2 cells. However, the levels of XIAP expression in both cell lines were stable during apoptosis. Furthermore, a Caki-1 cell line with no XIAP expression was used, and was demonstrated to be more sensitive to the apoptosis induced by the mitochondrial pathway. These results suggested that down-regulation of XIAP expression could enhance the sensitivity of RCC cells to apoptosis, and the basal expression of XIAP during apoptosis is stable. This may provide novel insight for targeted gene therapy against XIAP, in the clinic.

## Introduction

Renal cell carcinoma (RCC) has obvious resistance to apoptosis induced by chemical and immunological preparations and radiation, and this is why the non-surgical treatment for RCC is unlikely to be effective (1,2). However, the mechanism of RCC in apoptosis resistance is still unclear. Recently, molecular targeting therapy employing multi-kinase inhibitors (MKIs) such as tyrosine kinase inhibitors has raised hope for

patients with advanced RCC; sorafenib and sunitinib (3,4) are becoming first-line treatments for metastatic RCC. However, if the patients develop resistance to MKIs, they rapidly succumb to the disease. It is now of utmost importance to find another way to control cancer progression. Manipulation of the apoptotic mechanisms is one promising approach (5).

Different apoptotic signals such as chemicals can induce cell apoptosis in two ways, one of which is death receptor pathway (extrinsic), the other one is the mitochondrial pathway (intrinsic). Cluster of differentiation (CD)95 antibodies (CH11) are Fas ligands which can induce apoptosis through death receptor pathway (6,7). Topotecan and Etoposide (8) are anti-cancer drugs which can inhibit DNA topoisomerase I and II and induce apoptosis through the mitochondrial pathway (9,10). As an anti-cancer and anti-HIV drug, caffeic acid phenethyl ester (CAPE) is a nuclear factor (NF)- $\kappa$ B inhibitor. As NF- $\kappa$ B targets, the expression of inhibitor of apoptosis proteins (IAPs) can be promoted by NF- $\kappa$ B, so CAPE induces apoptosis through suppression of IAPs expression (11). However, regardless of which apoptosis pathway is active, the glutathione protease family (caspase) such as caspase-3, -7 and -9 must be activated, causing a chain reaction leading to apoptosis (12). More importantly, caspase-3 and -7 activation is the key to apoptosis; therefore, once they are activated, apoptosis can be carried out normally. IAPs directly bind to caspases and inhibit their activation, serving a vital role in the regulation of cell apoptosis. Caspase inhibitor XIAP is the most effective among the IAPs family. It can restrain apoptosis by suppressing the apoptosis initiation factor caspase-9 and effector caspase-3 and -7 (13). Therefore, XIAP expression levels may directly determine the sensitivity of tumor cells to apoptosis.

RNA interference (RNAi) can efficiently and specifically inhibit homologous gene expression (14-17). It interferes with the homologous sequences and gene expression at the transcriptional level, and causes the specific degradation of homologous mRNA, corresponding to silencing gene expression. RNAi technologies can effectively inhibit XIAP expression (18,19). It may reduce the experimental error, and improve the experimental accuracy and credibility.

The present study induced apoptosis in different RCC cell lines, which exhibit varying expression levels of XIAP, through death receptor, mitochondrial and NF- $\kappa$ B signaling pathways. Furthermore, RNAi was used to reduce the expression of XIAP in XIAP over-expressing RCC cells in order to study its role in apoptosis, and to investigate the mechanism

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of RCC cells in apoptosis resistance. This way, its potential application value in tumor gene therapy was investigated.

## Materials and methods

**Cell culture.** The established RCC cell lines ClearCa-2 and ClearCa-6 were obtained from Heinrich-Heine University (Dusseldorf, Germany). The Caki1 cell line was purchased from China Infrastructure of Cell Line Resources (Beijing, China), and cultured as described previously (20). CH11 (CD95-specific CH11 antibody) was purchased from Immunotech; Beckman Coulter, Inc. (Brea, CA, USA); Etoposide was from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), CAPE and Topotecan hydrochloride was from Merck KGaA.

**Western blotting.** Western blotting was performed on the cell lines, as described previously (20). A horseradish peroxidase-labelled secondary antibody (cat. no. 0101; 1:10,000; 37°C for 40 min) was used and blots were visualized using a Super Signal West Pico Substrate (Pierce; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.  $\beta$ -actin was used as a loading control. The images were analyzed using UN-SCAN-Itgel Automated Digitizing System software (version 5.1 for Windows; Silk Scientific Inc., Orem, UT, USA). The following antibodies were used: Anti-XIAP (cat. no. 2042; 1:10,000; 37°C for 40 min) from Sigma-Aldrich; Merck KGaA; polyclonal anti-inhibitor of apoptosis 1 (c-IAP1; cat. no. 4952; 1:10,000; 37°C for 40 min), anti-survivin (cat. no. 2802; 1:10,000; 37°C for 40 min) from Cell Signaling Technology Inc. (Danvers, MA, USA); anti-c-IAP2 (clone F30-2285; 1:10,000; 37°C for 40 min) from BD Biosciences (Franklin Lakes, NJ, USA) and anti- $\beta$ -actin (cat. no. 8227; 1:20,000; 37°C for 40 min) from Abcam (Cambridge, MA, USA).

**RNAi.** The XIAP-targeting short hairpin RNA vector was generated through literature review (21). The target sequence is 50-AGGTGAAGGTGATAAAGTA-30 (22) Transfection was carried out using Lipofectamine 2000 transfection reagent (Gibco; Thermo Fisher Scientific, Inc.) and a BLOCK-iT™ U6 RNAi Entry Vector kit (Kang Wei Technology, China; <http://www.cwbiotech.com>). For generation of stable transfectant clones, the transfected cells were selected with G418 for 3–4 weeks. A total of three selected clones were screened for XIAP expression (clone nos 1–3), and clone no. 2 was randomly selected for further experiments. G418-resistant mock transfectants were also isolated, produced by transfection of the plasmid without XIAP-targeting sequence.

**Measurement of cell viability and cell apoptosis.** CH11 (cat. no. 49516; 37°C for 24 h) from Abcam (Cambridge, MA, USA), Topotecan (cat. no. d1916; 37°C for 24 h) from Baomanbio (Shanghai, China), CAPE (cat. no. 211200; 37°C for 24 h) from Calbiochem; Merck KGaA (Darmstadt, Germany), and Etoposide (cat. no. 341205; 37°C for 24 h) from Calbiochem were used to induce apoptosis, and cell viability was detected by counting cells under the optical microscope. Trypan blue (cat. no. GD-jk1413; 37°C for 1 min) from Guduo (Shanghai, China) was used to detect viable cells or cell death according to the manufacturer's protocol. In additional

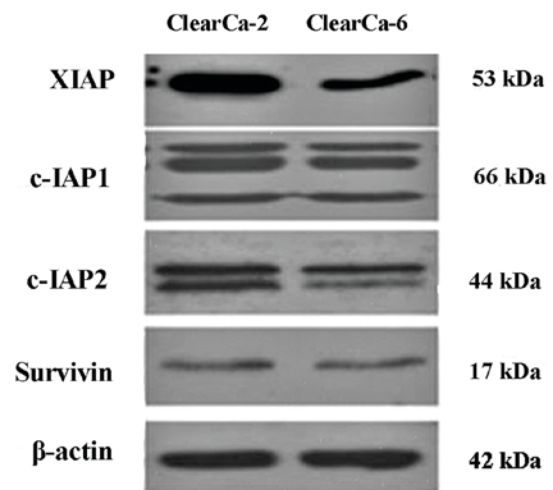


Figure 1. Protein expression of IAP1 and -2, XAP and survivin, in ClearCa-2 and ClearCa-6 cell lines, as assessed by western blot analysis. XIAP, X-linked inhibitor of apoptosis; c-IAP, inhibitor of apoptosis protein.

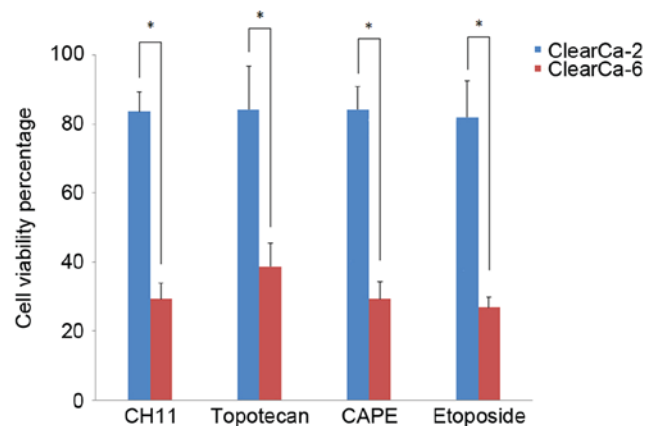


Figure 2. Cell viability percentage of ClearCa-2 and ClearCa-6 cell lines following CH11, Topotecan, CAPE and Etoposide treatment. Data are expressed as the mean  $\pm$  standard deviation. \* $P$ <0.05 ClearCa-2 vs. ClearCa-6. CAPE, caffeic acid phenethyl ester; CH11, CD95-specific antibody.

experiments, in order to improve the experiment efficiency, an MTT kit was used (Gibco; Thermo Fisher Scientific, Inc.) and flow cytometry (BD Biosciences) were used to detect early apoptosis according to the manufacturer's protocol.

**Statistical analysis.** SPSS software version 13.0 was used (SPSS, Inc., Chicago, IL, USA). All data are expressed as the mean  $\pm$  standard deviation of three independent experimental replicates. Statistical analysis was performed using a one-way analysis of variance followed by the Least Significant difference post hoc test or Student's t-test.  $P$ <0.05 was considered to indicate a statistically significant difference.

## Results

**Basal expression of XIAP is significantly different between ClearCa-2 and ClearCa-6 cell lines.** The basal protein expression of XIAP, cIAP-1, cIAP-2 and survivin, which are the main members of the IAPs family, were analyzed by western blot analysis. It was demonstrated that there were no

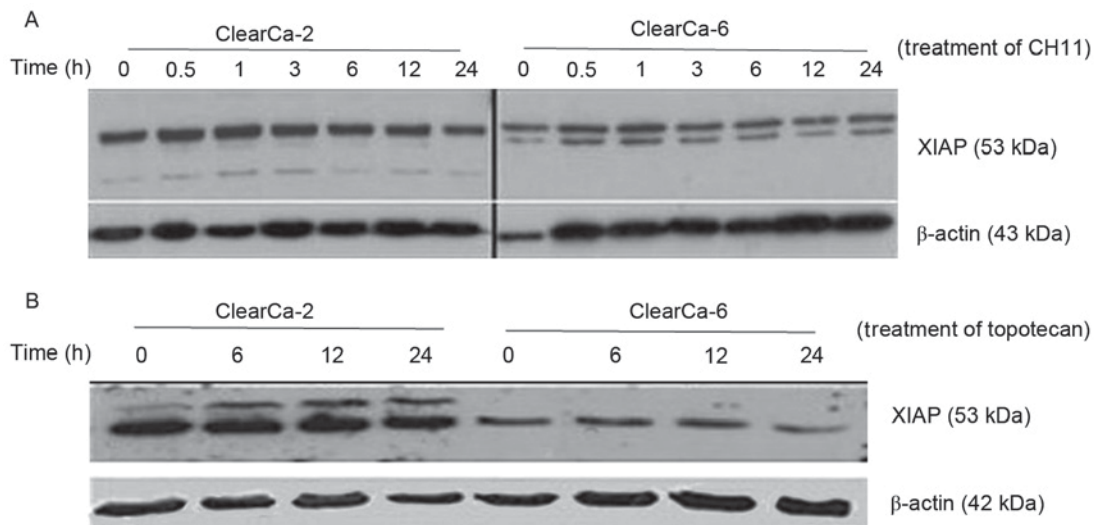


Figure 3. XIAP protein expression following Topotecan and CH11 treatment, as assessed by western blot analysis. Representative western blot images of Clear-Ca-2 and -6 cells following (A) CH11 and (B) Topotecan treatment. XIAP, X-linked inhibitor of apoptosis; CH11, CD95-specific antibody.

differences between cIAP-1 and survivin expression levels in both cell lines, and cIAP-2 had a slight difference, but the expression XIAP between the two cell lines was very different. The expression of XIAP in ClearCa-2 was much higher than ClearCa-6 (Fig. 1).

*ClearCa-2 cells with high expression XIAP are resistant to apoptosis; however, ClearCa-6 cells with low-expression XIAP are sensitive to apoptosis.* Because of the complicated mechanism of apoptosis, four different drugs which could induce cell apoptosis through different pathways were used. The results demonstrated that ClearCa-2 cells with higher expression of XIAP were resistant to apoptosis induced by all four drugs (Fig. 2). Following drug treatment for 24 h, the cell viabilities (compared with the control group) were 83.54% for CH11, 84.07% for Topotecan, 84.04% for CAPE, and 81.85% for Etoposide. However, ClearCa-6 cells with lower expression of XIAP were sensitive to apoptosis induced by all four drugs (Fig. 2). This difference in the expression levels between the two cell lines was statistically significant ( $P < 0.05$ ).

*Different apoptotic signals did not impact on the expression of XIAP during apoptosis.* ClearCa-2 and -6 cell lines demonstrated a different sensitivity to apoptosis, because of different basal protein expression levels of XIAP. However, whether these levels would change during apoptosis induced by different drug sat different time points or not was investigated. Following 24 h treatment with CH11 (Fig. 3A) and Topotecan (Fig. 3B), both cell lines had no change in XIAP expression but XIAP expression levels were higher in ClearCa-2 cells compared with the ClearCa-6 cells.

*RNAi technology inhibits the expression of XIAP in the Caki-1 cell line.* To further determine the role XIAP serves in RCC cell apoptosis, another XIAP-high-expression RCC cell line was studied (Caki-1), by inhibiting XIAP expression through RNAi. A total of three stable transfection cell lines were used (clone nos. 1-3), and western blot analysis was performed to detect XIAP expression in them. Expression

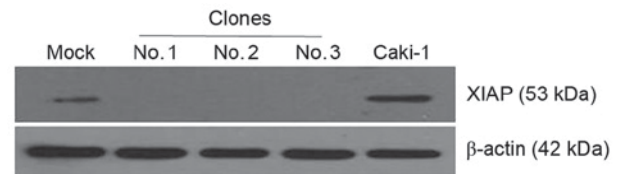


Figure 4. XIAP expression in RNA interference clone cell lines, as assessed by western blot analysis. XIAP, X-linked inhibitor of apoptosis.

levels of XIAP were effectively silenced in all three clone cell lines, but in the parental and the mock group, the expression of XIAP was normal (Fig. 4). Therefore, clone no. 2 was selected in further experiments, and was termed as XIAP no-expression Caki-1 cells.

*Sensitivity of Caki-1 cells not expressing XIAP to Etoposide-induced apoptosis is greatly enhanced.* It was demonstrated that RCC cells were more sensitive to Etoposide-induced apoptosis than with the other drugs; therefore, XIAP no-expression Caki-1 cells, Caki-1 cells and Mock cells (transfected by plasmid without XIAP interference gene) were treated with Etoposide for 24 h, and the cell death percentage was measured by MTT. It was demonstrated that the cell death percentage of XIAP no-expression Caki-1 cells was much higher than the Caki-1 and Mock cells, ( $P < 0.05$ ; Fig. 5). Between Caki-1 and mock cells, there was no statistical significance in the cell death percentage ( $P = 0.519$ ; Fig. 5A). It was also observed that following the treatment of Etoposide, the early apoptosis rate in XIAP no-expression Caki-1 cells was much higher than in the Caki-1 cells. At 0, 0.5, 1, 3, 6, 12 and 24 h, the early apoptosis rates of XIAP no-expression Caki-1 cells were 1.23, 11.7, 13.87, 22.07, 29.14, 31.81 and 43.21%, respectively. However, the early apoptosis rates of Caki-1 cells were 1.16, 1.48, 2.62, 4.61, 4.61, 3.26, and 5.20%, respectively (Fig. 5B).

*Expression of XIAP is stable during apoptosis induced by Etoposide.* In order to observe the level of expression of XIAP

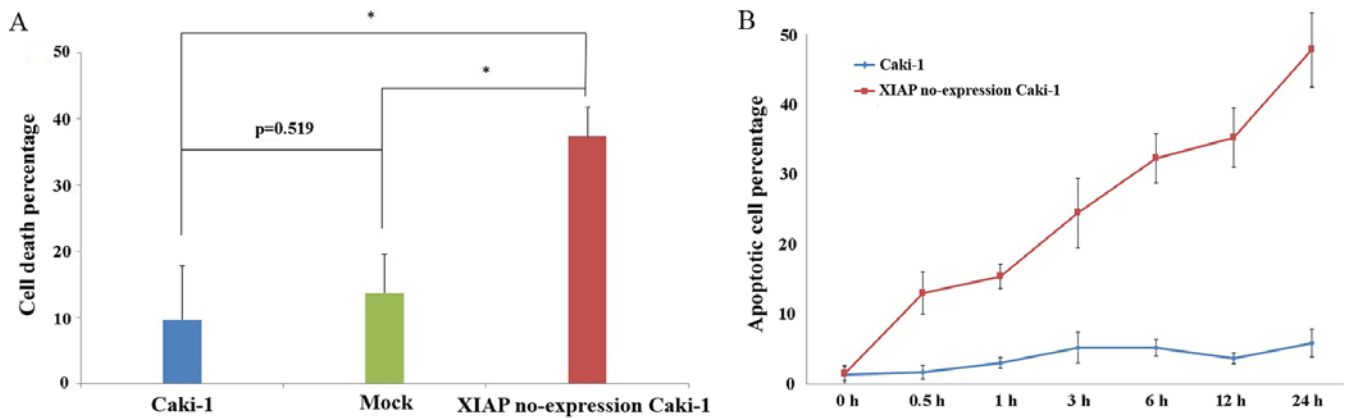


Figure 5. (A) Cell death percentage of Caki-1, mock and RNA interference clone no.2 cells detected by MTT. (B) Time course measurement of the apoptotic cell percentage of Caki-1 and XIAP no-expression Caki-1 cells following Etoposide treatment. As assessed by flow cytometry. Data are expressed as the mean  $\pm$  standard deviation. \* $P < 0.05$  vs. XIAP no expression Caki cells. XIAP, X-linked inhibitor of apoptosis.

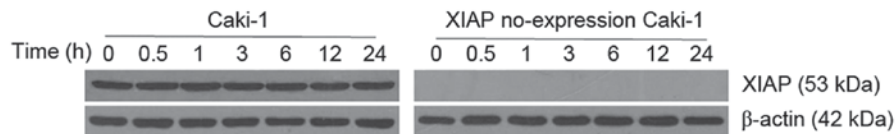


Figure 6. XIAP protein expression levels following Etoposide treatment, as assessed by western blot analysis. XIAP, X-linked inhibitor of apoptosis.

in the progress of apoptosis, the expression of XIAP in Caki-1 and XIAP no-expression Caki-1 cells was detected by western blot analysis, at different time points during apoptosis induced by Etoposide. It was observed that there was no change in the XIAP protein expression levels, as was also observed in the ClearCa-2 and ClearCa-6 cell lines (Fig. 6).

## Discussion

In recent years, many apoptosis controlling genes have been reported (23-25). XIAP, an important member of the anti-apoptotic IAPs family, has a strong inhibitory effect on caspase-3, -7, and -9 and it can also inhibit pro-caspase-3. It can also affect signaling pathways, such as c-Jun N-terminal kinase where, it is thought to serve a key role in the regulation of apoptosis (26-28). Many malignant tumors, such as breast, ovarian, lung, pancreatic and prostate cancer, express high levels of XIAP (29-34). Some reports have mentioned that increasing expression levels of XIAP through transfection can enhance their resistance to apoptosis induced by the death receptors or gamma-rays. Conversely, reducing the expression of XIAP increases the sensitivity of cancer cells to apoptosis (35,36). A previous study illustrated that XIAP is expressed in different histologic types of RCC, and further confirmed the universality of XIAP expression in human cancer (37). Notably, the expression of XIAP in RCC is increased, from early to late cancer stages, at both mRNA and protein levels (37). This indicated that tumor progression coincided with higher expression of XIAP. Similar reports have been presented regarding non-small cell lung cancer and acute myeloid leukemia (38). High expression levels of XIAP may reduce the sensitivity of RCC cells to apoptosis, and provide favorable conditions for tumor cell survival and development.

The present study demonstrated that the expression levels of XIAP have important effects in RCC cells apoptosis. The ClearCa-2 and ClearCa-6 RCC cell lines have different basal protein expression levels of XIAP. Apoptosis may be induced through extrinsic or intrinsic signaling pathways, and ultimately activate caspases leading to apoptosis. A total of four drugs were chosen that function through the extrinsic death receptor pathway (CH11), the intrinsic mitochondrial pathway (Etoposide and Topotecan) and the nuclear factor (NF)- $\kappa$ B inhibiting IAPs (CAPE) induced apoptosis. It was demonstrated that the ClearCa-6 cell line was sensitive to apoptosis, whereas ClearCa-2 cells were not, and the intrinsic mitochondrial pathway was the strongest in inducing apoptosis. Furthermore, another RCC cell line was employed, termed Caki-1, which has also high expression of XIAP, to verify our conclusion that XIAP may have important effects in RCC cell apoptosis (22). The protein expression of XIAP in the Caki-1 cells was eliminated through RNAi, and it was confirmed that the sensitivity of RCC cells to apoptosis was increased with decreased expression of XIAP.

Previous studies have reported that the expression of XIAP increases following exposure of tumor cells to tumor necrosis factor-related apoptosis-inducing ligand or gamma-rays (39); however, other reports demonstrated the opposite result (40,41). In the present study, CH11 and Topotecan induced apoptosis, but had no effect in the expression of XIAP in either ClearCa-2 or ClearCa-6 cell lines. The expression of XIAP in both cell lines was stable during apoptosis, although ClearCa-2 cells expressed much higher XIAP than the ClearCa-6 cells. To verify this result, the expression of XIAP was investigated in Caki-1 cells and no-expression XIAP Caki-1 cells during apoptosis induced by Etoposide, and the same phenomenon was observed. Therefore, it is evident that the role of XIAP in apoptosis is very important.

RNAi can effectively suppress protein expression, with a range in the inhibition rate (14-17). Regarding XIAP, Wang *et al* (18) has reported that RNAi could reduce the expression of XIAP by 56.2% in laryngeal carcinoma cells. Cao *et al* (19) has reported that RNAi could reduce its expression by 79.86% in pancreatic carcinoma cells. Regarding the RCC cells, Bilim *et al* (22) used RNAi to decrease XIAP by 85.3%, in Caki-1 cells. In the present study, the interference plasmid transfected Caki-1 cells and it completely inhibited XIAP expression by 100%. To the best of our knowledge, no previous study has ever reported such an effect. This study provides novel insights on XIAP and gene therapy in clinical practice.

In conclusion, there are so many factors inducing apoptosis, and the present study only studied the effect of XIAP on apoptosis. There was a difference in the basal expression of XIAP in two RCC cell lines, and those with higher expression of XIAP resisted apoptosis. At the same time, reducing the expression of XIAP enhanced the sensitivity to apoptosis. However, the underlying mechanism(s) of this phenomenon need to be elucidated further.

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