Isobaric tags for relative and absolute quantitation-based quantitative proteomic analysis of X-linked inhibitor of apoptosis and H2AX in etoposide-induced renal cell carcinoma apoptosis

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Abstract

Background: X-linked inhibitor of apoptosis (XIAP) is a vital factor in the anti-apoptosis mechanism of tumors and is highly expressed in renal cell carcinoma (RCC). However, the mechanism through which XIAP regulates DNA damage repair is unknown. This study investigated the regulatory mechanism of XIAP in etoposide-induced apoptosis in two Caki-1 cell lines with high or low XIAP expression.

Methods: The two cell lines were established using RNA interference technology. The differentially expressed proteins in the two cell lines were globally analyzed through an isobaric tags for relative and absolute quantitation-based quantitative proteomics approach. Proteomic analysis revealed 255, 375, 362, and 5 differentially expressed proteins after 0, 0.5, 3, and 12 h of drug stimulation, respectively, between the two cell lines. The identified differentially expressed proteins were involved in numerous biological processes. In addition, the expression of histone proteins (H1.4, H2AX, H3.1, H3.2, and H3.3) was drastically altered, and the effects of XIAP silencing were accompanied by the marked downregulation of H2AX. Protein-protein interactions were assessed and confirmed through immunofluorescence and Western blot analyses.

Results: The results suggested that XIAP may act as a vital cell signal regulator that regulates the expression of DNA repair-related proteins, such as H2AX, and influences the DNA repair process.

Conclusions: Given these functions, XIAP may be the decisive factor in determining the sensitivity of RCC cell apoptosis induction in response to chemotherapeutic agents.

Keywords: Apoptosis; H2AX; iTRAQ; Renal cell carcinoma; X-linked inhibitor of apoptosis

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant urological tumors and accounts for approximately 2% of all cancer cases worldwide.[1] The 5-year survival rate of patients with metastatic RCC is less than 10% given the chemo- and radiotherapy resistance of this malignancy. RCC is highly resistant to chemotherapy because of its high apoptosis threshold. Various factors, such as overexpression of X-linked inhibitor of apoptosis (XIAP), contribute to the progression and chemo-resistance of RCC. XIAP expression is higher in RCC than in autologous normal kidneys, and XIAP overexpression in RCC is predictive of poor prognosis.[2] Our previous research showed that RCC cells with high XIAP expression are resistant to apoptotic signals, whereas those with low XIAP expression are sensitive to apoptotic signals. XIAP expression is unaffected by apoptosis stimulation.[3]

Among all members of the inhibitor of apoptosis (IAP) family, the caspase inhibition mechanism of XIAP is the best characterized,[4] and XIAP is the most promising target for tumor therapy. XIAP contains three BIR domains and a RING domain. The Baculovirus IAP Repeats-2 (BIR-2) domain binds and potently inhibits caspase-3 and caspase-7, and the BIR3 domain and the flanking regions suppress caspase-9.[5] The RING domain of XIAP exhibits E3 ubiquitin ligase activity and labels target proteins with ubiquitin molecules, thereby promoting protein degradation through ubiquitination.[6] Recent technological advancements have furthered our current understanding of the function of XIAP. In addition to inhibiting caspase activity, XIAP inhibits apoptosis by participating in other
signal transduction pathways. Specifically, XIAP inhibits cell apoptosis by binding to the RING domain of the BMP receptor to influence the TGF-Beta Activated Kinase 1 (TAK1)-Jun Proto-Oncogene 1 (JNK1) signal transduction pathway.

Whether tumor cells initiate DNA repair or apoptosis depends on the extent of DNA damage, and the histone variant H2AX plays an important role in this process. Upon DNA damage, PI3K family members ataxia telangiectasia-mutated gene (ATM), ATM and Rad-3 related (ATR), and DNA-dependent protein kinase (DNA-PK) phosphorylate H2AX on Ser139 to generate γ-H2AX. The phosphorylated form of H2AX can facilitate anchoring of signaling and repair proteins, such as Meiotic recombination 11 homolog A (MRE11), Double strand break repair protein (RAD50), MRN complex (Nijmegen Breakage Syndrome 1 [NBS1]/MRE11/RAD50), and Mediator of DNA Damage Checkpoint 1 (NFBD1)/DNA damage checkpoint 1 (MDC1), to form γ-H2AX foci at sites of DNA double-strand breaks. The phosphorylation of tyrosine 142 in H2AX on Ser139 to generate DNA-dependent protein kinase (DNA-PK) phosphorylate H2AX on Ser139 to generate γ-H2AX. The phosphorylated form of H2AX can facilitate anchoring of signaling and repair proteins, such as Meiotic recombination 11 homolog A (MRE11), Double strand break repair protein (RAD50), MRN complex (Nijmegen Breakage Syndrome 1 [NBS1]/MRE11/RAD50), and Mediator of DNA Damage Checkpoint 1 (NFBD1)/DNA damage checkpoint 1 (MDC1), to form γ-H2AX foci at sites of DNA double-strand breaks. The phosphorylation of tyrosine 142 in H2AX prevents recruitment of the repair complex and promotes binding of pre-apoptotic factors, such as JNK1, thereby directly promoting cell apoptosis. Although these findings suggest that XIAP and H2AX play important roles in apoptosis, no study has explored the interaction between these two proteins.

Methods

Cell culture and treatment

Caki-1 cells were purchased from China Infrastructure of Cell Line Resources (Beijing, China), grown in minimum essential medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Zhejiang Kang Yuan Biological Technology Co., Ltd., Zhiu, China), and maintained at 37°C in a humidified incubator with 5% CO2. At approximately 90% confluence, the cells were washed with phosphate-buffered saline and then treated with 60 μg/mL etoposide (Aldrich, Sigma, St. Louis, MO 63178, USA) or with serum-free medium as a control. The concentration of etoposide was based on our previous experimental data.

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At 70% confluence, Caki-1 cells were transfected with either XIAP small interfering RNA or control short hairpin RNA (shRNA) using Lipofectamine 2000 in accordance with the manufacturer’s instructions (Invitrogen, USA). XIAP expression after transfection was assessed through immuno-blotting. The XIAP interference target was based on that described in a study by Bilim et al. who verified the absence of homologous sequences between this fragment and other genes and showed that XIAP expression is downregulated by shRNA that contains this fragment. The target sequence was 5′-AGGTGAAGTGATAAAGTA-3′. A BLOCK-iT® U6 RNAi Entry Vector kit (Invitrogen; Thermo Fisher Scientific, Inc.) was adopted for construction. On the basis of the selected target sequence, the following shRNA oligonucleotide sequences were used: 5′-CACC-GAGTTGAAGGTGATAAAGTGATACGATTTTATCA CTTTCACC-3′ (top strand) and 5′-AAAAAGGTGAAGGT-GATAAAGTGATTCGATTTTATCACCCCTCCCTC-3′ (bottom strand). At 48 h post-transfection, the medium was replaced with fresh selection medium containing genetin (Kang Wei Technology, Beijing, China) to screen for stably transfected cells. Culture medium was replaced with fresh selection medium every 2 days until no cells were killed.

Cell lystate preparation and Western blot analysis

Total protein was extracted at different time points after etoposide induction. Protein concentration was determined using a bicinchoninic acid protein assay kit. Whole cell lysates were prepared using Radio Immunoprecipitation Assay (RIPA) reagent (Bioeasytech, China). A total of 40 μg of protein from each sample was resolved through sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked overnight with 5% non-fat milk and probed with primary antibodies against target proteins at 4°C overnight, followed by incubation with secondary anti-rabbit (1:10,000 dilution; Bioeasytech) or anti-mouse (1:20,000 dilution; Bioeasytech) antibody at 37°C for 45 min. The antibodies used in this study (targeting XIAP [1:500], β-Actin [1:2000], H2AX [1:500], and glyceraldehyde 3-phosphate dehydrogenase [1:400]) were purchased from Cell Signaling Technology. The relative intensity of each band was digitized using BandScan V4.3.
**ITRAQ analysis**

XIAP knockdown and original Caki-1 cells were treated with 60 μg/mL etoposide for 0.5, 3, and 12 h. Exactly 2 mL of lysis buffer (8 mol/L urea, 30 mmol/L N-2-hydroxyethylpiperazin-N’-2-ethanesulfonic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L ethylenediaminetetraacetic acid, and 10 mmol/L dithiothreitol) was added to each sample. Each sample was then sonicated on ice and centrifuged at 13,000 rpm for 10 min at 4°C. Proteins were precipitated with ice-cold acetone before redissolution in dissolution buffer (50% triethylammonium bicarbonate and 0.1% SDS). Proteins were quantified through a Bradford protein assay (Promega Corporation, Madison, WI, USA). Subsequently, 100 mg of protein was digested with trypsin, and the resultant peptide mixture was labeled with the appropriate ITRAQ reagents (Applied Biosystems; Thermo Fisher Scientific, Inc.). The peptide samples were subjected to nano LC-MS/MS and MS/MS data searches were performed using Proteome Discoverer 1.3 (Thermo Fisher Scientific, Inc.) to identify and fractionate using an Ultemex SCX column (Phenomenex, Torrance, CA, USA; Luna SCX 100A) via an LC-20AB HPLC pump system (Shimadzu Corporation, Kyoto, Japan) at a flow rate of 1.0 mL/min with a gradient of Buffer A (25% acetonitrile [ACN], 10 mmol/L KH₂PO₄; pH 3.0) and Buffer B (25% ACN, 2 mol/L KCl, 10 mmol/L KH₂PO₄; pH 3.0). Buffer B reached 100% within 10 min. The column flow rate was maintained at 400 nL/min, and the column temperature was maintained at room temperature under a pressure of 1000 psi. The collected fractions were desalted using a Strata C18 column (Phenomenex), vacuum centrifuged (4°C and 20,000 x g for 30 min), and reconstituted in 0.1% formic acid for subsequent LC-MS/MS analysis. Mass spectrometry (MS) analysis was performed using a Q ExactiveTM Hybrid Quadrupole-OrbitrapTM Mass Spectrometer (Thermo Fisher Scientific, Inc.). The peptide samples were subjected to nanoflow electrospray ionization MS/MS analysis on an Eksigent NanoLC-2D system (AB SCIEX, Framingham, MA, USA).

**Double-labeling immunofluorescence**

Caki-1 cells and XIAP knockdown cells were incubated with specific primary antibodies against XIAP (1:100; Cell Signaling Technology) and H2AX (1:100; Abcam Inc., Cambridge, MA, USA). As previously described, the cells were subsequently incubated for 1 h at 21°C with Alexa Fluor® 394 goat anti-rabbit or Alexa Fluor® 488 goat antimouse secondary antibody and with 4’,6-diamino-2-phenylindole (DAPI) for nuclear staining. Immunofluorescence was visualized using a confocal fluorescence microscope (Nikon A1R/A1) under 600× magnification. Fluorescence was quantified using ImageJ software, and at least 50 nuclei were analyzed.

**Statistical analysis**

SSPS 17 (SSPS Inc., IBM, Chicago, IL, USA) software was used for statistical analysis. The data were subjected to analysis of variance, and means were compared through Duncan multiple-range test. P < 0.05 was considered statistically significant.

**Results**

**Stable knockdown of XIAP by shRNA in sensitized Caki-1 cells to etoposide**

To investigate the role of XIAP in etoposide-induced apoptosis of RCC cells, we knocked down XIAP expression in Caki-1 cells lines through RNAi technology. We confirmed the interference efficiency in XIAP knockdown cells through Western blot analysis. As shown in Figure 1, XIAP expression decreased in cells transfected with XIAP shRNA relative to that in cells transfected with the control shRNA and in the original cells. To examine the effect of XIAP on apoptosis of Caki-1 cells, we used Annexin V-fluorescein (FITC)/propidium iodide (PI) Apoptosis Detection Kits to assess the apoptosis rates of XIAP knockdown cells and original Caki-1 cells. The flow cytometry results indicated that after 24 h of etoposide treatment, the total apoptosis rate of the XIAP knockdown cells was 4.2-fold higher than that of the original Caki-1 cells [Figure 2].

| Original cell | XIAP shRNA |
|--------------|------------|
| XIAP         | 53,000     |
| GAPDH        | 34,000     |

**Figure 1:** Confirmation of XIAP knockdown efficiency. Validation of XIAP expression in knockdown cells (XIAP shRNA) and original Caki-1 cells through Western blot analysis. GAPDH expression was used as a loading control. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; shRNA: Short hairpin RNA; XIAP: X-linked inhibitor of apoptosis.
Numerous differentially expressed proteins were identified through iTRAQ profiling

To investigate the global effect of XIAP on etoposide-induced apoptosis, we subjected the original Caki-1 and XIAP knockdown cells to iTRAQ-based quantitative proteomics analysis. Cells were treated with 60 μg/mL etoposide for 0, 0.5, 3, or 12 h. We used iTRAQ 117, 118, 119, and 121 tags to label the knockdown XIAP cells at these four-time points. We used iTRAQ 113, 114, 115, and 116 tags to label the original Caki-1 cells.

We identified 1783 non-redundant differentially expressed proteins in the two cell lines. For the subsequent relative quantification analysis, although statistical analysis is part of the ProteinPilot software, we applied an additional ≥1.3 or ≤0.77 (1/1.3)-fold cut-off to all iTRAQ ratios to minimize false positives when proteins were identified as over- or underexpressed. We adopted this cut-off value because the overall technical variation in the data from duplicate experiments was estimated to be less than 30% and because this value has been widely used in other investigations that used the iTRAQ approach.[20] Proteins with iTRAQ ratios below 0.77 were considered underexpressed, whereas those with ratios greater than 1.3 were considered overexpressed.

By applying this filter, we identified 255, 375, 362, and 5 significantly altered proteins in Caki-1 and XIAP knockdown cells at 0, 0.5, 3, and 12 h, respectively. Then, we conducted gene ontology (GO) enrichment analysis to better understand the molecular and functional characteristics of these differentially expressed proteins. Figure 3A-C shows the distribution of differentially expressed proteins in cellular component, biological processes, and molecular functions at 0.5 h of etoposide treatment. A total of 3521 biological processes, 577 protein classes, and 710 molecular functions were classified. At the same time,
many classifications related to the nucleus and DNA were found, such as DNA repair complex, damaged DNA binding, and DNA damage checkpoint. All these classifications included numerous differentially expressed proteins. Functional annotations were obtained from the GO database (http://www.geneontology.org/).

**XIAP knockdown changed the expression of many histone variants during etoposide-induced apoptosis**

Etoposide can cause DNA double-strand breaks. Histone modifications can serve as damage markers and play a crucial role in DNA repair.[21] GO analysis revealed numerous differentially expressed proteins that participate in various nuclear-related cellular components, including the nucleus, DNA damage, and DNA repair. Thus, we studied changes in histones in the two cell lines. We identified 14 histone protein variants. By using the above criteria (\( P < 0.05, \text{iTRAQ ratio} \geq 1.3 \) or \( \leq 0.77 \)), we found that at 12 h after stimulation of apoptosis, the number of differentially expressed proteins between the two cell lines drastically decreased. Table 1 shows that the expression levels of five proteins (H1.4, H2AX, H3.1, H3.2, and H3.3) at three-time points (0, 0.5, and 3 h) were significantly altered by etoposide treatment and that only H2AX was downregulated at 0 h. The ratios of 113:117, 114:118, 115:119, and 116:121 indicate the relative abundance levels of H2AX protein [Figure 4] in Caki-1 cells compared with those in the XIAP knockdown group at four different time points after etoposide treatment.

**The H2AX expression trend detected through Western blot analysis was consistent with that detected through iTRAQ analysis**

The phosphorylation status of the histone variant H2AX S139 changes after DNA damage. Thus, H2AX was selected for further validation on the basis of its highly...
significant ratios and its relevance to apoptosis. Western blot analysis results for H2AX expression in the two cell lines are shown in Figure 5. H2AX was downregulated in Caki-1 cells but not in XIAP knockdown cells. Although this change in expression detected through immunoblot analysis was consistent with that detected through iTRAQ, some differences were detected between the two techniques. These differences could be attributed to various factors, such as antibody potency, differences inherent to each technical approach, and other uncertainties. Nevertheless, both techniques consistently showed that H2AX expression was significantly downregulated in Caki-1 cells compared with XIAP knockdown cells.

**Cellular distribution of XIAP and H2AX**

We used double immunofluorescence labeling to investigate the sub-cellular distribution of H2AX and its potential interaction with XIAP. We subjected Caki-1 cells and XIAP knockdown cells to XIAP-specific immunofluores-
XIAP, one of the most important IAPs, is characterized by the presence of a RING finger that confers E3 ubiquitin ligase activity. In addition to its function in apoptosis regulation, XIAP also performs other non-apoptotic biological functions. For example, XIAP participates in signal transduction and ubiquitination. The current understanding of XIAP, particularly the function of its E3 ubiquitin ligase, remains limited. Therefore, we conducted an in-depth study on the potential mechanisms of XIAP in the apoptosis of Caki-1 cells, which are derived from a typical RCC cell line. We combined RNAi of XIAP with an iTRAQ-based quantitative proteomics approach to globally profile the function of XIAP in etoposide-induced apoptosis. Our iTRAQ results revealed that under apoptosis stimulation, changes in the XIAP protein level induce differential expression of proteins between the original Caki-1 cells and XIAP knockdown cells. The findings suggest that XIAP may be involved in regulating the expression of the identified differentially expressed proteins. We identified 1784 differentially expressed proteins between the two cell lines. We characterized proteins showing a ratio fold-change ≥1.3 or ≤-0.77 as significantly altered proteins. GO enrichment analysis showed that the identified differentially expressed proteins are widely distributed in the cell and are involved in many aspects of cell biology. Collectively, these results indicate that XIAP is involved in numerous novel biological processes. Notably, we observed the highest number of differentially expressed proteins at 0.5 h after etoposide treatment. The number of differentially expressed proteins then drastically decreased at 12 h after etoposide treatment. Thus, we inferred that the functions of XIAP are mainly confined to a 3 h window after apoptosis induction and that initiation of the apoptotic signaling pathway is completed at 12 h after apoptosis induction.

Etoposide-induced DNA strand breaks are major triggers of DNA repair activation through histone proteins. DNA damage and repair are critical in a cell-free apoptosis system. Thus, we examined whether XIAP affects histones. XIAP silencing altered the expression of five histone variants (H1.4, H3.1, H3.2, H3.3, and H2AX) in etoposide-induced apoptosis. We found that except for H2AX, the remaining four proteins were highly expressed before the induction of apoptosis and that their ratios decreased after etoposide treatment. However, in Caki-1 cells, H2AX was downregulated at each time point. H1 histone variants directly affect the tightness of nucleosome packaging and the advanced structure of chromosomes by interacting with DNA. The H3 family is composed of H3.1, H3.2, and H3.3 variants, which are highly conserved. H3.3 is a variant of histone proteins involved in transcriptional activation. The caspase-3/CAD...
(DFF40) pathway cannot induce DNA fragmentation in the absence of H2AX. Therefore, H1 and H3 may have minor roles in apoptosis induction, and their participation deserves further investigation.

Our Western blot analysis results revealed for the first time that XIAP knockdown induces upregulation of H2AX. Our Western blot analysis results validated our iTRAQ results. Therefore, our results collectively suggest that etoposide-induced DNA damage and DNA repair mechanisms likely depend on XIAP. Considering the E3 ubiquitin ligase activity of XIAP, we speculated that XIAP may decrease H2AX protein expression by directly ubiquitinating the H2AX protein via the ubiquitin proteasome pathway. However, under ordinary conditions, XIAP is distributed in the cytoplasm, and H2AX is distributed in the nucleus. Thus, to verify whether XIAP directly interacts with H2AX, we used confocal microscopy to detect the localization of XIAP-IF and H2AX-IF in the two cell lines. As shown in Figure 6, there was no colocalization of XIAP and H2AX. Therefore, XIAP may not directly regulate the protein level of H2AX through ubiquitination but instead affect H2AX through other signaling pathways in cells or regulate the transcriptional level of H2AX. It has been reported that the mitogen-activated protein kinase (MAPK) pathway regulates the protein and transcription level of histone H2AX protein. Therefore, in follow-up studies, we will continue to explore whether XIAP can affect H2AX through the MAPK pathway in RCC. In addition, the effect of XIAP on apoptosis-related events in the nucleus can be clarified.

Conclusions
Our results indicate that XIAP can mediate apoptosis through multiple pathways, including DNA damage and DNA repair mechanisms, through downregulation of H2AX. The apoptotic pathway is activated if repair fails.
Although the regulation of H2AX expression in human RCC is not completely understood and demands further investigation, our data suggest that RCC cells with high XIAP expression are resistant to apoptosis and to reductions in H2AX. These findings provide a new direction for studying the radiation resistance of RCC and the potential function of XIAP.

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Conflicts of interest
None.

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