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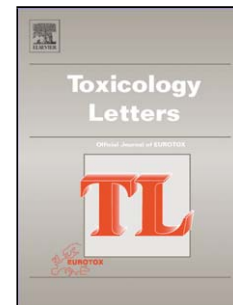
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**Increase the cisplatin cytotoxicity and cisplatin-induced DNA damage in HepG2 cells by XRCC1 abrogation related mechanisms**

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Abbreviations:

BER base excision repair;

ERCC1 excision repair cross-complementing rodent repair deficiency, complementation group 1;

HCR host cell reactivation;

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

OTM olive tail moment;

ROS Reactive oxygen species;

XRCC1 X-ray repair cross-complementing gene 1;

$\beta$ -gal  $\beta$ -galactosidase.

**Abstract**

Cisplatin is one of the most potent chemotherapeutic anticancer drugs for the treatment of various cancers. The cytotoxic action of the drug is often thought to be associated with its ability to bind DNA to form cisplatin–DNA adducts. Impaired DNA repair processes including Base Excision Repair (BER) play important roles on

its cytotoxicity. XRCC1 is a key protein known to play a central role at an early stage in the BER pathway. However, whether XRCC1 contributes to decrease the cisplatin cytotoxicity and cisplatin-induced DNA damage in HepG2 still remains unknown. Hence, the purpose of this study was to explore whether abrogation of XRCC1 gene expression by shRNA could reduce DNA repair and thus sensitize liver cancer cells to cisplatin. We abrogated the XRCC1 gene in HepG2 cell using shRNA transfection. Cell viability was measured by MTT assay and clonogenicity assay. Comet assay was used to detect the DNA damage induced by cisplatin. The host cell reactivation was employed to assess the DNA repair capacity of cisplatin-damaged luciferase reporter plasmid. Flow cytometry analysis was used to determine cisplatin induced apoptosis, cell cycle and ROS. The results showed that abrogation of XRCC1 could sensitize HepG2 cells to cisplatin. This enhanced cytotoxicity could be attributed to the increased DNA damage and reduced DNA repair capacity. Increasing cell cycle arrest and intracellular ROS production lead to more tumor cell apoptosis and then enhanced the cisplatin cytotoxicity. Our results suggested that the cisplatin cytotoxicity may increase by targeting inhibition of XRCC1.

**Keywords:** XRCC1; hepatocellular carcinoma cells; cisplatin; BER

Abbreviations: BER, base excision repair; ERCC1, excision repair cross-complementing rodent repair deficiency, complementation group 1; HCR, host cell reactivation; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OTM, olive tail moment; ROS, Reactive oxygen species; XRCC1, X-ray repair cross-complementing gene 1;  $\beta$ -gal,  $\beta$ -galactosidase.

## 1. Introduction

Cisplatin has been used widely as a chemotherapeutic drug for a variety of malignancies including hepatocellular carcinoma (Go and Adjei, 1999; Leung et al., 1999). Multiple mechanisms have been suggested to cause cisplatin induced cytotoxicity, including intracellular accumulation of cisplatin, impaired DNA-repair

processes, and decreased levels of cisplatin-inactivating factors, such as glutathione and metallothioneins (Perez, 1998; Zhang et al., 2001; Rajewsky and Müller, 2002). In tissue culture, DNA repair is clearly an important effector of resistance to platinum-based DNA-damaging agents. In vitro data showed that nucleotide excision repair (NER) is the DNA repair pathway responsible for DNA damage induced by cisplatin and other platin compounds (Dijt et al., 1998; Zamble et al., 1995; Jordan and Carmo-Fonseca, 2000; Kartalou and Essigmann, 2001; Reed, 1998). However, the involvement of other DNA repair pathways in cisplatin-induced DNA damage repair has not been completely clarified.

Although the NER pathway seemed related with the repair mechanism for cisplatin-DNA adducts, some reports support the involvement of overlapping repair pathways in alternative repair of cisplatin adducts, such as the DNA base excision repair (BER) pathway (Weaver et al., 2005). The XRCC1 (X-ray repair cross-complementing gene 1), is a platform protein and a key factor in the BER and single strand break repair (SSBR) pathways (Thompson and West, 2000).

Biochemical data has indicated that XRCC1 has no enzymatic activity, but instead interacts with a number of proteins involved in BER/SSBR, suggesting that XRCC1 operates as a scaffold protein to facilitate efficient DNA damage processing (Fan and Wilson, 2005). Studies also indicated that XRCC1 played a role in replication initiation and replication-coupled DNA repair in S-phase cells (Taylor et al., 2002; Kubota and Horiuchi, 2003; Fan et al., 2004). XRCC1 may also be involved in the repair of other types of DNA damage caused by cisplatin including DNA double-strand break (DSB) repair (Weaver et al., 2005; Levy et al., 2006; Wong and Wilson, 2005). Recently, there has been report that XRCC1 transcript abundance level decreases the cytotoxicity of cisplatin in non-small cell lung cancer (Weaver et al., 2005). Overexpression of XRCC1 resists the cytotoxicity of anticancer drug camptothecin in CHO cell (Park et al., 2002; Barrows et al., 1998). Furthermore, association studies have suggested a possible link of human XRCC1 polymorphisms with DNA repair defects, increased chromosome instability, and cancer susceptibility (Fan et al., 2007).

In this work, we had established an XRCC1 deficient cell line in HepG2 cell using shRNA. We evaluated the cytotoxicity and DNA damage induced by cisplatin in XRCC1 deficient HepG2 cells. Our results showed that the XRCC1 deficient could increase the cytotoxicity of cisplatin in HepG2 cells. The present study would provide a strategy for a more efficacious sensitization of cisplatin-resistant tumor cells.

## 2. Materials and Methods

### 2.1 Cell Culture

The HepG2 cell line was derived from China Center for Type Culture Collection (CCTCC). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Gibco, Grand Island, NY) with 10% heat inactivated fetal calf serum (FCS; Invitrogen, Gibco, Grand Island, NY), penicillin-streptomycin (100 µg/ml, respectively; Life Technologies, UK) and L-glutamine (2 mmol/l; Life Technologies, UK). Cultures were incubated in an atmosphere of 5% CO<sub>2</sub> at a temperature of 37 °C.

### 2.2 Plasmid construction.

The cDNA sequence of XRCC1 was obtained from Genbank (NM\_006297) and targeting sequence was designed using Invitrogen's RNAi algorithm available on line (<https://rnaidesigner.invitrogen.com/rnaiexpress/index.jsp>). Sequence was verified using BLAST to avoid off-target gene silencing. The short hairpin RNAs (shRNA) duplexes were synthesized by Sangon (Shanghai). The sequences were as follows, sense: 5'-gat cca ttg cca gct cca act cgt ttc aag aga acg agt tgg agc tgg caa ttt ttt tgg aaa-3', Antisense: 5'-agc ttt tcc aaa aaa att gcc agc tcc aac teg ttc tct tga aac gag ttg gag ctg gca atg-3' to target the XRCC1 transcript and Sense: 5'-gat ccg act tca taa ggc gca tgc ttc aag acg gca tgc gcc tta tga agt ctt ttt tgt cga ca -3', Antisense: 5'-agc ttg teg aca aaa aag act tca taa ggc gca tgc cgt ctt gaa gca tgc gcc tta tga agt cg -3'(scrambled control, containing the nucleotides of a region of the XRCC1 transcript in a random

order). The oligonucleotides were phosphorylated by T4 Polynucleotide Kinase (Taraka) at 37 °C for 5 min and then denatured at 95 °C for 5 min. The following hairpin RNAs (shRNA) duplexes were inserted into the pSilencer-H1 (Ambion) vector using the BamHI and Hind III (NEB) restriction sites. The restructured vectors with shRNA XRCC1 was named pSilencer-H1-XRCC1 and with random order shRNA was named pSilencer-H1-control. The identity of the insert was confirmed by DNA sequencing by Invitrogen Company.

### 2.3 Cell transient transfection

HepG2 cells, grown to 95% confluence, were transfected with purified recombinant vectors using lipofectamine 2000 reagent (Invitrogen, Life Technologies, UK) according to the manufacturer's instructions, with a final recombinant vectors concentration of 50 nM. Cells were split 48 h after the start of transfection and treated with indicated concentrations of cisplatin 72 h after the start of transfection. The pSilencer-H1-XRCC1 transfected cell was called XRCC1(-) cell and pSilencer-H1-control transfected cell was called vector control cell. The knockdown levels were tested by Quantitative RT-PCR and western blot analysis.

### 2.4 Quantitative RT-PCR

In order to monitor the modulation of XRCC1 mRNA levels, a quantitative RT-PCR approach was used. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol and cDNA was prepared with 3µg total RNA using the reverse transcription system (Promega). Equal amounts of cDNA were subjected to PCR, in the presence of SYBR green dye with the Platinum SYBR Green qPCR SupperMix-UDG kit (Invitrogen) and the ABI Fast quantitative PCR 7900HT system (Applied Biosystems). PCR without template was used as a negative control. The GAPDH mRNA was used as an internal control. XRCC1, GAPDH and negative control were amplified on the same plate. The XRCC1 and GAPDH specific primers

were designed using the Primer Premier 5 software and were as follows, XRCC1 sense: 5'-CTG TCG CCA TCT GTT CCC -3', antisense: 5'-CCA CTC AGC ACC ACT ACC A -3'. GAPDH sense: 5'-CCA TGT TCG TCA TGG GTG TGA ACC A -3', antisense: 5'-GCC AGT AGA GGC AGG GAT GAT GTT C-3'. PCR was performed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C after a 2 min initial denaturation at 95 °C. Each sample was normalized by using the difference in critical thresholds (CT) between XRCC1 and GAPDH. The following equation was used to describe the result:

$$\Delta\Delta CT_{XRCC1} = \Delta CT_{XRCC1} - \Delta CT_{GAPDH}$$

$\Delta CT_{XRCC1}$  was the difference in CT between XRCC1 and negative control, and  $\Delta CT_{GAPDH}$  was the difference between GAPDH and negative control. The mRNA levels of each sample were then compared using the expression  $\Delta\Delta CT_{XRCC1}$ . All experiments were independently performed for three times and the average was used for comparison.

## 2.5 Immunoblotting assay

In order to monitor the modulation of XRCC1 protein levels, an immunoblotting approach was used. After transfection for 48h, cells were washed twice with cold PBS and harvested on ice in lysis buffer containing 150mM NaCl, 50mM Tris/HCl (pH 7.6), 1% Triton, 1µg/ml aprotinin, and 100 µg/ml phenylmethylsulfonyl fluoride. The suspension was incubated at 4 °C for 30 min. Cellular debris was then pelleted by centrifugation for 10 min at 13,000 rpm at 4 °C, supernatants were collected, and protein concentration was estimated using BCA kit (Pierce). The equivalent volume of loading buffer (100mM Tris/HCl (pH 6.8), 4% SDS, 20% glycerin, 10% β-mercaptoethanol and 0.2% bromophenol blue) was added and mixed again. The samples were then denatured at 95 °C for 10 min. Proteins (About 70µg of XRCC1 and 50µg of GAPDH) mixture was loaded in each well and separated in 10% SDS-polyacrylamide electrophoresis gels. After running about 60min, the proteins were transferred onto nitrocellulose membranes (Bio-Rad). The membranes were saturated and blocked with 5% fat-free milk at 37 °C for 1 h, and were incubated with

rabbit polyclonal anti-XRCC1 (Santa Cruz; 1:300 dilution) or mouse monoclonal anti-GAPDH (Kangchen; 1:2000 dilution) for 2 h at 37 °C. After extensive washing, the second antibody (goat anti-rabbit HRP and goat anti-mouse HRP (Pierce) were added, respectively, and the membranes incubated for 45 min followed by extensive washes (1-2 h). Specific antibody-antigen complexes were detected by using the ECL Western blot detection kit (Pierce). Graphs of blots were obtained in the linear range of detection and were quantified for the level of specific induction by scanning laser densitometry. All experiments were independently performed for three times and the average was used for comparison. Protein expression was quantified by densitometry with Gel pro3.0 image software (Media Cybernetics, Silver Spring, MD)

## 2.6 Tetrazolium test

The cell survival ratio was assessed by the ability of cells to reduce the tetrazolium salt 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT). Briefly, the cells were seeded into 96 well plates at a density of  $1 \times 10^4$  cells/ml (200  $\mu$ l per well) in DMEM (10% FCS) and incubated for 24 hours. Then the cells were treated with varying doses of cisplatin (Sigma) for 24 h (the concentration of cisplatin was 5, 10, 20, 40 and 80  $\mu$ M, respectively). In time-course experiments, the 5  $\mu$ M cisplatin treated cells were washed with PBS and were further incubated for varying posttreatment periods (i.e., 24 h, 48 h, and 72 h). Aliquots of 10  $\mu$ l of MTT (5 mg/ml) were added to each well and incubated at 37 °C for 4 hours. Thereafter, the color formed was quantitated by a spectrophotometric plate reader (BIO-TEK Instruments, Inc.) at 490 nm wavelength after solubilization in 150  $\mu$ l of DMSO. Results were presented as percentage of the control values. IC50 was defined as the concentration of drug that inhibited cell growth by 50%. The experiments were independently performed for three times and the average was used for comparison.

## 2.7 Clonogenicity assays

Clonogenicity assay was performed according to the method of Janes (2004) with a



small modification. Briefly, after treatment with cisplatin for 24 h, cells were trypsinized and 300 cells were plated in 6-well plate. 14 days later the cultures were fixed in 2ml methanol for 15 minutes and stained with Giemsa for 10 minutes. All colonies (that is, consisting of 2 cells or more) were scored. Colony forming efficiency was calculated as the percentage of plated cells that formed colonies. Abortive colonies were defined as colonies that contained fewer than 50 cells. All experiments were performed in triplicate and more than 100 colonies were scored per experimental condition.

Cloning efficiency (%) = the counts of colonies with more than 50 cells/the counts of all scored colonies)  $\times 100\%$ .

## 2.8 Cell cycle and apoptosis analyses by Flow cytometric

Cell cycle and apoptosis analyses were performed by flow cytometry. Cells were grown in DMEM medium supplemented with 10% FBS and penicillin/streptomycin, and plated into 24-well plates at a density of  $0.5 \times 10^5$  cells per well. 24 h after treatment with cisplatin in different concentration, the cells were harvested. The cell cycle and percentages of apoptotic were measured according to the manufacturer's instruction. Briefly, the cells were washed twice with cold PBS, resuspended in 2 mL of 70% ethanol, and kept at 4 °C overnight. They were subsequently rinsed twice with PBS and incubated with 100  $\mu$ L RNase (10 mg/mL). Finally, the cells were stained with propidium iodide (PI; 0.5  $\mu$ g/ml final concentration, Sigma) for 15 min in the dark at room temperature. Distribution of the cell cycle and the rate of apoptosis were determined using a FACS420 flow cytometer (Becton Dickinson, USA). Each assay was performed in triplicate.

## 2.9 Measurement of intracellular reactive oxygen species (ROS)

The intracellular reactive oxygen species (ROS) was detected using 2',7'-dichloro-fluorescein diacetate (DCFH-DA) as a probe (Wang and Joseph, 1999).

After 24 h exposure on cisplatin, ROS was detected with Reactive Oxygen Species Assay Kit (Beyotime) according to the manufacturer's instructions. After treated with cisplatin (The concentration was 5, 10, 20, 40 and 80 $\mu$ M, respectively) for 24 h, cells were washed twice and loaded with 10  $\mu$ M DCFH-DA for 30 min at 37 °C in the dark. The formation of the fluorescent-oxidised derivative of DCF-DA was monitored using a FACS420 flow cytometer (Becton Dickinson, USA) at emission wavelength of 530 nm and excitation wavelength of 485 nm. Finally, ROS generation was quantified by the median fluorescence intensity of 10,000 cells.

#### 2.10 Single cell gel electrophoreses assay (comet assay)

After 24 h exposed to cisplatin,  $1 \times 10^5$  cells suspended in 0.5% low melting agarose were spread on the normal melting agarose-coated (1%) slides. The slides were covered with a coverslip and put at 4 °C to solidify the low melting agarose. Thereafter, the coverslips were removed and the slides were transferred to a lysis buffer (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris base, 1% Triton-X 100 and 10% DMSO) over 1 h at 4 °C. Then slides were transferred to an electrophoretic box containing 300 mM NaOH and 1 mM Na<sub>2</sub>EDTA (pH > 13) for 20 min at 4 °C, and electrophoresis for 25 min at 25 V (300 mA) at 4 °C (Singh et al., 1988). Subsequently, all slides were washed with 0.4 M Tris-HCl (pH 7.5) for 5 min for three times, and stained with 20 mL ethidium bromide (10 mg/mL). 100 randomly chosen cells (comets) were scored visually using fluorescence microscope (Olympus, Japan) equipped with an excitation filter of 515-560 nm and a barrier filter of 590 nm. The "olive tail moment" of each comet was calculated using CASP analyzed software (Olive Tail Moment = percent of DNA in the tail  $\times$  distance between the center of gravity of DNA in the tail and the center of gravity of DNA in the head). The Olive tail moment was scored by CASP image-analysis program (Konca et al., 2003).

#### 2.11 Host cell reactivation assay

HCR of luciferase activity was determined as described previously with modification

(Yen et al., 1997; Wu et al., 2003). Briefly, the reporter plasmid pGL3 (Promega Biotech, Madison, WI) containing the luciferase gene driven by the SV40 promoter was treated for 12 h at 37°C with increasing concentrations of cisplatin (0.5, 1, 2 and 4µM) in TE buffer [10 mM Tris (pH 8.0)-1 mM EDTA] at a DNA concentration of 200 µg/ml. The damaged plasmid was recovered by precipitation with ethanol, and after it was dissolved in TE buffer, the concentration of plasmid was determined. The HepG2 cells and XRCC1<sup>-</sup> cells were seeded in 24-well plates and were grown to 50–80% confluence. The cells were transiently transfected with 1 µg of cisplatin-damaged reporter plasmid and 1 µg of control plasmid (pSV-β-gal; Promega) by Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were collected 48 h after transfection, and cell extracts were used to determine luciferase activity (Luciferase Assay System with Reporter Lysis Buffer; Promega) and β-gal activity according to the protocol described previously and the manufacturer's instructions. Luciferase activity values were quantified with a luminometer (Lumat, LB9507; EG&G) and were normalized for β-gal activity.

## 2.12 Statistical analysis

Data were expressed as means±SD(standard deviation). Statistical analysis was performed by ANOVA and Pearson correlation coefficient using SPSS (version 13.0). Each data point was an average of three independent experiments. Differences were considered significant when  $P < 0.05$ .

## 3. Results

### 3.1 Down-regulation of XRCC1 mRNA and protein levels by shRNA target to XRCC1.

We adopt an RNAi approach to modulate XRCC1 expression in human hepatocellular carcinoma cells line HepG2. HepG2 cells were successfully transfected with the recombinant plasmid containing shRNA target to human XRCC1 cDNA.

Seventy-two hours after the start of transfection, quantitative RT-PCR revealed a

reduction of XRCC1 mRNA levels of 80% in XRCC1 shRNA-transfected HepG2 cells compared with levels in control cells (Table 2). Western blotting confirmed these results and showed that mRNA and protein levels were closely correlated (Fig. 1).

### 3.2 Increasing cisplatin toxicity in XRCC1 deficient HepG2 cell.

To determine whether decreased expression of XRCC1 enhanced cisplatin sensitivity, the cells were exposed to increasing doses of cisplatin for 24 h, and cell viability was measured by the MTT assay. As shown in Fig. 2A, XRCC1(-) cells exhibited increased sensitivity to cisplatin. The statistical analysis revealed that the cell toxicity in XRCC1(-) cell was correlated significantly with an increasing concentration of cisplatin ( $r=0.9131$ ,  $P < 0.01$ ). The cytotoxicity in XRCC1(-) was increased about 2.2-fold in regard to IC<sub>50</sub> (Fig. 2A). In time-course experiments, the cells were treated with 5  $\mu$ M cisplatin for 24 h, and were further incubated for 24-72 h. The HepG2 cells with XRCC1 deficient showed a greater reduction in cell viability over time, compared with the HepG2 cells ( $P < 0.05$ , Fig.2B).

In addition, the effect of deficient XRCC1 on clonal growth of human hepatocellular carcinoma cells was also investigated. After treatment with cisplatin, the colony formation rate was calculated (Fig. 2C). As shown in Fig. 2C, the clonogenicity in XRCC1(-) cell was decreased significantly after treatment with cisplatin and also correlated significantly with increasing concentrations of cisplatin. When XRCC1(-) cell was treated with 80 $\mu$ M cisplatin for 24h, the conloning efficiency(%) was decreased about 3.8-fold compared with HepG2 cell.

Emerging evidence has suggested that a reduced apoptotic response result in the decrease cytotoxicity of cisplatin. (Gonzalez et al., 2001). In this case, increased levels of DNA damage would be required to induce the signal that initiates apoptosis. After 24 h of cisplatin treatment, both the detached and the attached cells were collected for the flow cytometry assay. Both after treatment with 5 and 80 $\mu$ M cisplatin, the number of apoptosis cells was increased about 2.5-fold in XRCC1 (-) cell compared with HepG2 cells. The results from this study demonstrated that the inefficient repair of the DNA damage induced by cisplatin in the XRCC1 (-) cells

could lead to enhanced apoptosis (Fig. 2D).

### 3.3 XRCC1-deficient cells display delayed cell-cycle progression following treated with cisplatin

As XRCC1-deficiency has been linked to the perturbation of DNA replication, we next assessed whether reduced XRCC1 levels impacted cell-cycle progression with cisplatin treatment. Cells were exposed to cisplatin and their cell cycle distribution was analyzed (Fig. 3). Treatment for 24 h with 40 or 80 $\mu$ M cisplatin caused a partial arrest in S phase, increasing the percentage of cells in S phase by 1.39-fold and 1.54-fold in HepG2 cells with unmodulated XRCC1 levels, respectively, which is in agreement with previous reports (Yen et al., 1997). At the same time the fraction of cells in G0/G1 or G2/M phases decreases, which indicated cisplatin could cause perturbations in cell cycle distribution (Nguyen et al., 1993).

### 3.4 Decreased the repair capacity of XRCC1 deficient HepG2 cells following cisplatin treatment

To investigate whether the enhanced cytotoxicity to cisplatin in cells expressing decreased levels of XRCC1 was due to reduced DNA repair capacity, a host-cell reactivation assay (HCR) was conducted in which cisplatin damaged reporter plasmid was transiently transfected into cells. As shown in Fig. 4, the inhibition of XRCC1 expression did lead to a significant decrease in reactivation of the reporter plasmid damaged by various doses of cisplatin. The greater the relative reporter gene activity, the more proficient the DNA repair of host cell. These results indicated that decreasing of DNA repair capacity in XRCC1(-) contributed to, at least in part, the enhanced cytotoxicity to cisplatin in these cells.

### 3.5 Increased DNA damage of XRCC1-deficient HepG2 after cisplatin treatment

Comet assay is sensitive to detect primary DNA lesions including single-strand breaks, double-strand breaks, incomplete excision repair sites and alkali labile sites that can be converted to DNA single-strand breaks during the test procedure. Using comet

assay, we detected the DNA fragmentation induced by cisplatin in human hepatoma cells. (Fig.5) Comet assay results showed that cisplatin induced more severe DNA fragmentation in XRCC(-) cell compared with HepG2 cell at 20, 40 and 80 $\mu$ M concentration ( $P < 0.01$ ).

3.6 Oxidative stress in XRCC1(-) cell maybe closely associated with cisplatin sensitivity.

As shown in Fig.6, XRCC1(-) cell showed an increased intensity of DCF fluorescence following 20, 40 and 80 $\mu$ M cisplatin treatment, respectively, suggesting that intracellular ROS was increased and then induced the intensive cytotoxicity. The increase in ROS was dose-dependent and the level of ROS was significantly increased compared with HepG2 cell ( $P < 0.01$ ).

#### 4. Discussion

DNA repair appeared to be the most important contributor to prevent the cytotoxicity of cisplatin. XRCC1, as a base excision repair and single strand break repair protein, may play an important role in resistance to a variety of DNA damaging agents. In vitro, Chinese hamster ovary and breast cancer cells lacking functional XRCC1 protein were hypersensitive to a broad range of DNA damaging agents and XRCC1 transcript levels correlate negatively with cisplatin cytotoxicity in cancer cell lines(Weaver et al., 2005). In this report, we demonstrated that down-regulation of XRCC1 protein by shRNA strategy inhibited DNA repair capacity and induced the more serious DNA damage (Fig.4 and 5), and, thus, enhanced the cytotoxicity to cisplatin in a human liver cancer cell line(Figs. 2A, B,C). Moreover, we found XRCC1 deficiency could significantly increase cisplatin cytotoxicity and the IC50 was decreased about 2.2-fold in XRCC1 (-) cell compared with HepG2 cells.

Although some reports consistently report that ERCC1 in NER or Glutathione-related mechanism are essential to resist cisplatin toxicity (Dijt et al., 1998; Thompson and West, 2000), recent study indicated that XRCC1 transcript levels correlated negatively with cisplatin cytotoxicity in cancer cell lines(Weaver et al., 2005). Our observations

further suggested that the increasing of cisplatin cytotoxicity could be attributed to the reduced BER capacity as a result of a reduced level of XRCC1 mRNA and protein expression.

One important mechanism of translation of cisplatin-DNA damage into cell death is apoptosis. In this case, increased levels of DNA damage would be required to induce the signal that initiates apoptosis. Our results showed that the DNA damage was increased in XRCC1 deficient cells, while DNA repair capacity induced by cisplatin was decreased significantly (Fig.4 and 5). Hence, this study demonstrated that the inefficient repair of the increasing DNA damage induced by cisplatin in XRCC1 deficient cells could lead to enhanced apoptosis (Fig. 2 D), which maybe associated with increased cisplatin cytotoxicity.

Our data clearly indicate that abrogation of XRCC1 increased DNA damage in HepG2 cell responding to cisplatin. It is well known that cisplatin regulates DNA damage either directly or indirectly through other mechanisms like regulating cell cycle checkpoints. Cell cycle checkpoint regulatory mechanisms involve various cyclins and cyclin-dependent kinases (CDKs)(Hartwell and Kastan, 1994). The most studied checkpoint mechanism involves cell cycle arrest at the G1/S, following DNA damage. G1/S arrest, which is believed to be required for DNA repair, occurs through the interaction between p53 and the CDK inhibitor, p21WAF1, or other cell related genes such as PCNA, RPA and GADD45(Smith et al., 1994). Our results demonstrated that the cisplatin induces cell cycle arrest at S stage (Fig. 3). Furthermore, abrogation of XRCC1 enhanced cell cycle arrest and this may underlie the observation that gadd45 and p53 mRNA are overexpressed in these cells (data not shown).

Our results indicated that inhibition of DNA repair can enhance cytotoxicity and induce apoptosis by cisplatin, but the mechanisms still require further study. Cisplatin induced cytotoxicity is believed to be caused by the formation of DNA adducts, which has been suggested to accumulate preferentially in mtDNA (Forastiere, 1994; Yang et al., 2006). Cisplatin has also been associated with the increased production of ROS, and biochemical manipulations aimed at reducing ROS production or detoxifying ROS had resulted in decreased toxicity of cisplatin (Spitz et al., 1993; Jing et al.,

2007). Meurette et al reported the production of superoxide anion during apoptosis induced by combined treatment with TRAIL and cisplatin in HepG2 hepatocarcinoma cell (Meurette et al., 2005). And other reports suggested that exposure of cancer cells to agents that induce mitochondrial dysfunction, such as azidothymidine (AZT), causes significant sensitization to cisplatin-induced toxicity via disruptions in thiol metabolism and oxidative stress (Mattson et al., 2009). Whereas a role for GSH in resistance to platinum compounds has been well described, In fact, the detoxification of cisplatin by GSH via nonenzymatic or enzymatic reaction with GSH S-transferases had been shown (Godwin et al., 1992). These findings provide a biochemical mechanism to evaluate the cytotoxicity of cisplatin by oxidative stress. In this study, cisplatin could induce more intracellular ROS production in XRCC1 deficient cell which maybe related with the increasing cisplatin cytotoxicity in HepG2 cells (Fig.6).

In summary, our results suggest that shRNA transfected HepG2 could silence the XRCC1 mRNA and protein expression. The repair process of cisplatin-induced DNA damage is now recognized as an important mechanism of its cytotoxicity. XRCC1 plays a key role in the repair of cisplatin-induced DNA damage. However, a detailed mechanistic study about this repair process requires additional investigation.

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### Figure legends

Fig.1 shRNA targeting the XRCC1 transcript efficiently reduces XRCC1 protein levels in HepG2 cells.

Cells were transfected with shRNA against the XRCC1 transcript, a scrambled siRNA or untransfected. Protein levels were analyzed 72 h after the start of transfection by quantitative western blotting. Protein levels were calculated by dividing the levels of transfected cells by the levels of untransfected cells. The reduction of XRCC1 protein levels of about 69% in XRCC1 shRNA-transfected HepG2 cells compared with levels in HepG2 cells. There was no significantly difference between vector control transfected cells and HepG2 cells.

Fig.2 The effect of XRCC1 deficiency on the cellular cisplatin cytotoxicity.

A, the cells were treated with various doses of cisplatin for 24 h, and the cell viability was measured by a MTT assay. XRCC1 deficient cells exhibited significantly increased cytotoxicity to cisplatin compared with the HepG2 cells (IC<sub>50</sub> was decreased about 2.2-fold in XRCC1 (-) cell compared with HepG2 cell), whereas this increased cytotoxicity was not observed in colonies of vector control transfected cells.

B, the cells were treated with 5 $\mu$ M cisplatin for 24 h, washed with PBS, and further incubated for 24-72 h; then the cell viability was determined by MTT assay. XRCC1 (-) cells exhibited significantly increased sensitivity to cisplatin compared with the HepG2 cells in different post treatment period ( $P < 0.01$ , post treated cisplatin for 24, 48, 72 h, respectively).

C, the cells were treated with cisplatin for 24h and then evaluated for colony-forming ability after further incubated for 14 day. The cloning efficiency (%) was significantly decreased in XRCC1(-) cell compared with HepG2 cell treated by different concentration of cisplatin (5, 10, 20, 40 and 80 $\mu$ M, respectively,  $P < 0.01$ ).

D, the apoptosis rate(%) of cells induced by cisplatin were significantly increased in XRCC1(-) cell compared with HepG2 cell at different concentration of cisplatin (5, 10, 20, 40 and 80 $\mu$ M, respectively,  $P < 0.01$ ).

Data were expressed as means $\pm$ SD(standard deviation). Data significant in relation to

HepG2 cell at  $^{**}P < 0.01$ /one-way ANOVA Dunnett's multiple comparison test.

Each data point was an average of three independent experiments.

Fig.3 Cell-cycle progression of cells with differential expression of XRCC1 following exposure to cisplatin.

Cells were treated with different concentration cisplatin for 24h and then cell-cycle distribution was assessed by flow cytometry. Cell-cycle distribution was different between HepG2 cells and XRCC1(-) cells after treated with cisplatin.

Compared with HepG2 cell, the fraction of S phase cells were increased significantly(posttreated with 40 and 80 $\mu$ M cisplatin).

Fig.4 DNA repair capacity of XRCC1 deficient cells significantly decreased following exposure to cisplatin.

XRCC1 deficient in HepG2 cell reduced HCR of cisplatin-damaged luciferase reporter plasmid. The reporter plasmid pGL3 was treated with 0.5, 1, 2, and 4 $\mu$ M cisplatin for 12 h, respectively, and was recovered by precipitation with ethanol. The cells were transiently transfected with 1 $\mu$ g of cisplatin-damaged reporter plasmid and 1 $\mu$ g of control plasmid (pSV- $\beta$ -gal). At 48 h after transfection, cells were harvested; then luciferase and  $\beta$ -gal activity were analyzed. The repair capacity in XRCC1(-) cell was significant decreased responding to cisplatin-damage. Data were expressed as means $\pm$ SD(standard deviation). Data significant in relation to HepG2 cell at  $^{**}P < 0.01$ /one-way ANOVA Dunnett's multiple comparison test. Each data point was an average of three independent experiments.

Fig.5 XRCC1 deficient increased DNA damage of HepG2 cell exposure to cisplatin

Cells were treated with various concentration of cisplatin for 24 h, and the DNA fragmentation was measured by a comet assay.

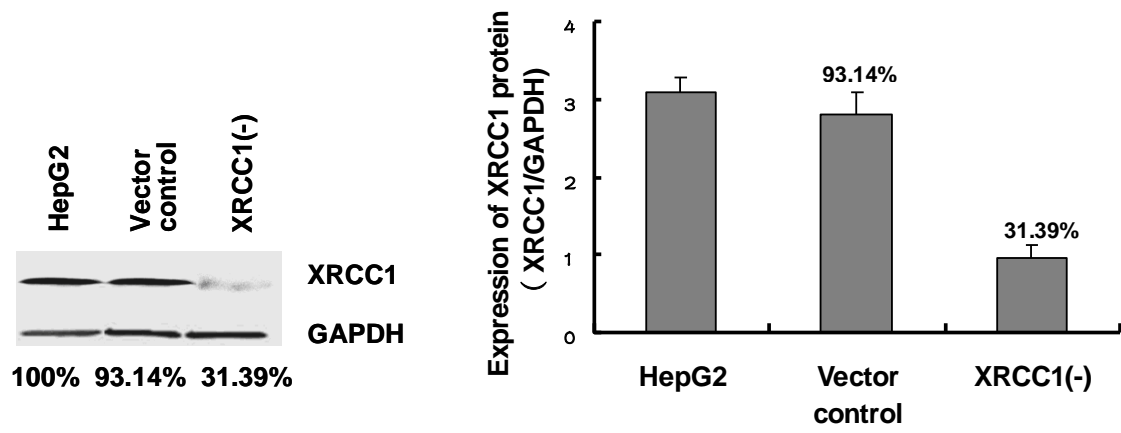
A: Image of DNA damage detected by comet assay in HepG2 cell and XRCC1(-) cell with different treated with concentration of ciplatin (0, 5, 10, 20, 40, 80 $\mu$ M, respectively.).( $\times 20$ )

B: Statistical analysis showed that XRCC1 deficient cells exhibited significantly increased DNA damage in response to cisplatin compared with the HepG2 cells, whereas this increased cytotoxicity was not observed in colonies of vector control transfected cells. Data represent the mean values $\pm$ standard error of three-independent experiments. Compared with the HepG2 cell,  $**P < 0.01$ .

Fig. 6. ROS in XRCC1 deficient cell was closely correlated with cisplatin cytotoxicity.

Cells ( $1 \times 10^6$ ) were incubated with cisplatin (0, 5, 10, 20, 40 and 80  $\mu\text{M}$ ) for 24 h. Then the cells were harvested and incubated with the free serum medium added with 10  $\mu\text{M}$  of DCFH-DA for 15 min at 37°C in the dark. DCF fluorescence was measured using a flow cytometer with FL-1 filter. Fluorescence results were expressed as mean fluorescence intensity obtained from the histogram statistics. Data were expressed as means $\pm$ SD(standard deviation). Data significant in relation to HepG2 cell at  $**P < 0.01$ /one-way ANOVA Dunnett's multiple comparison test. Each data point was an average of three independent experiments.

Fig.1



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Fig.2

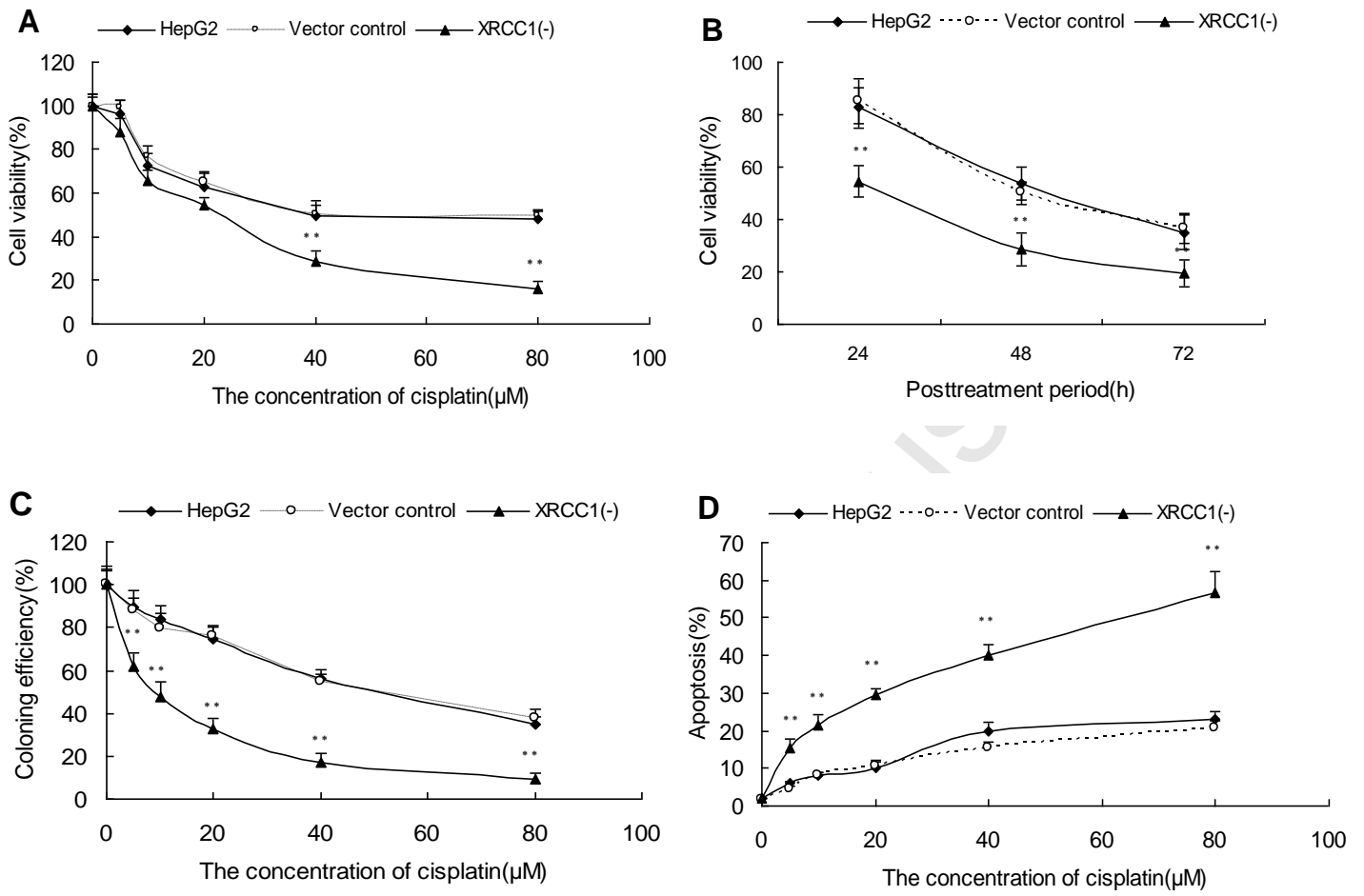
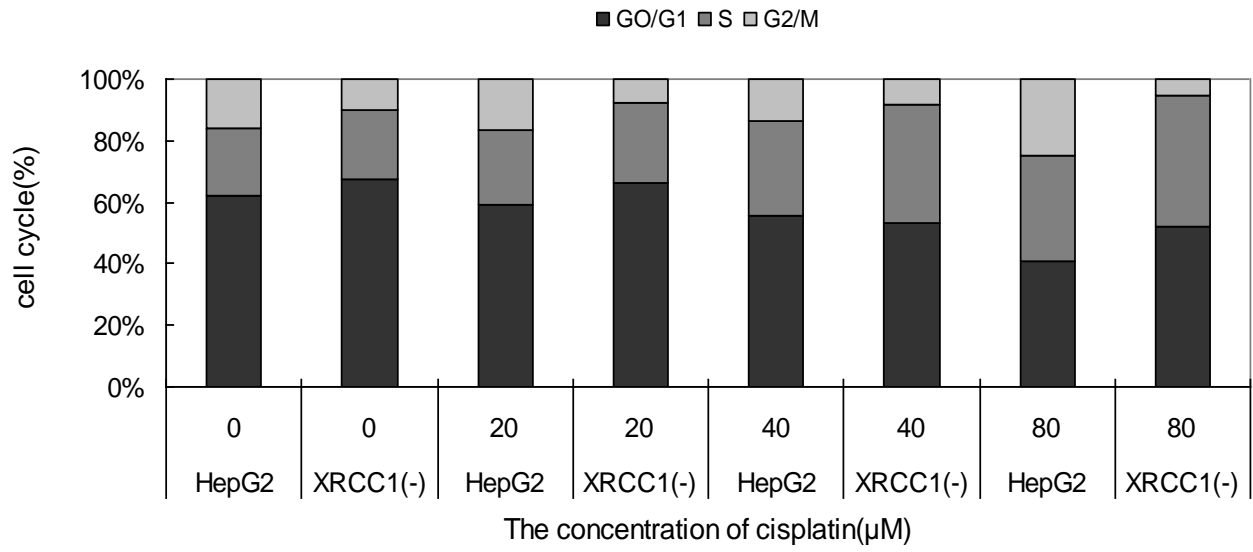


Fig.3



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Fig.4

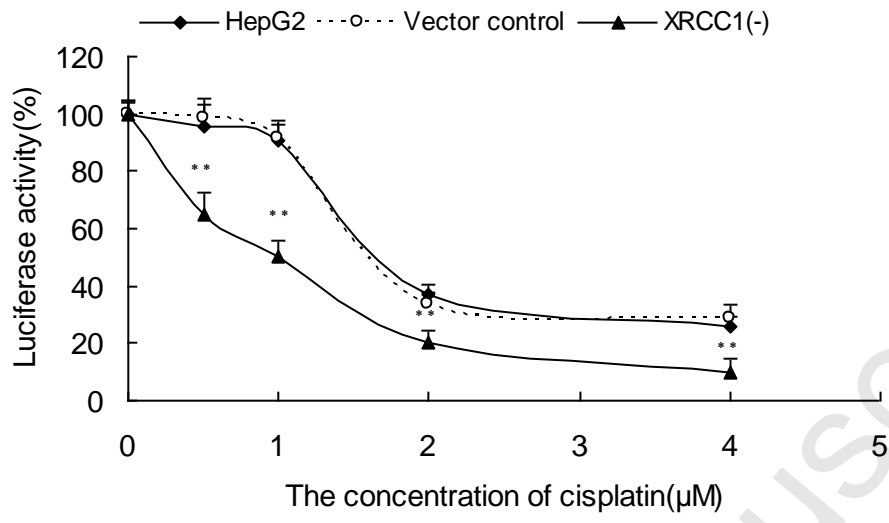
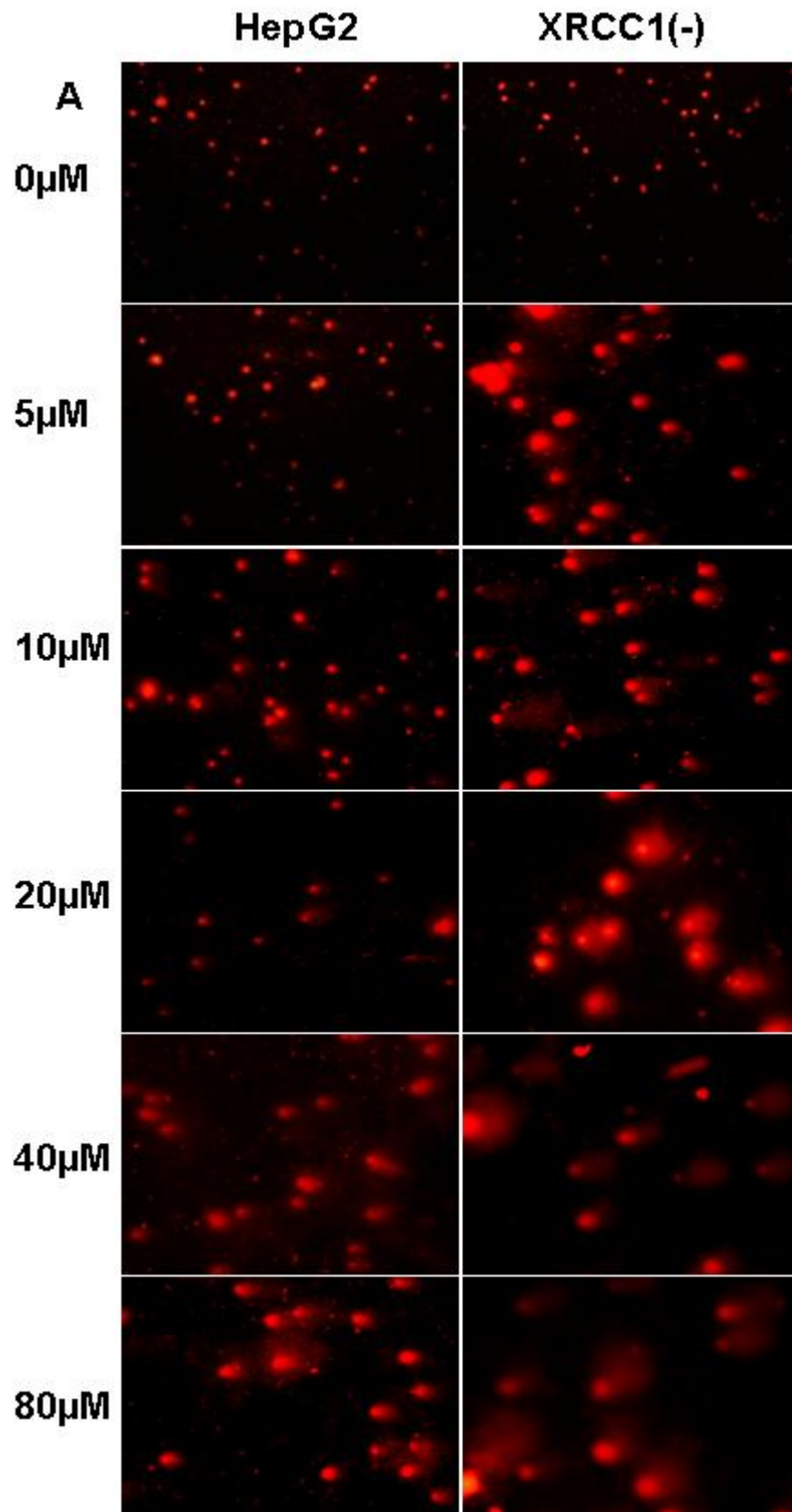


Fig.5



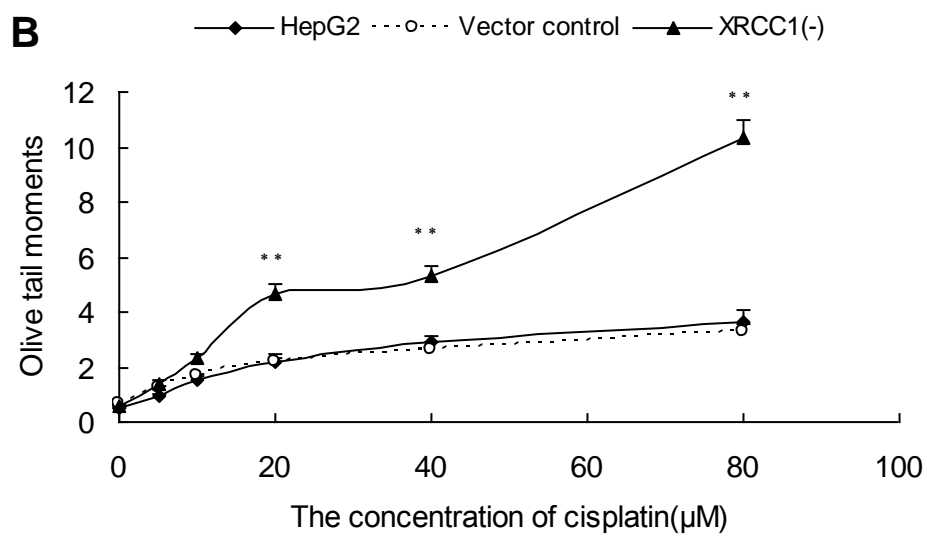
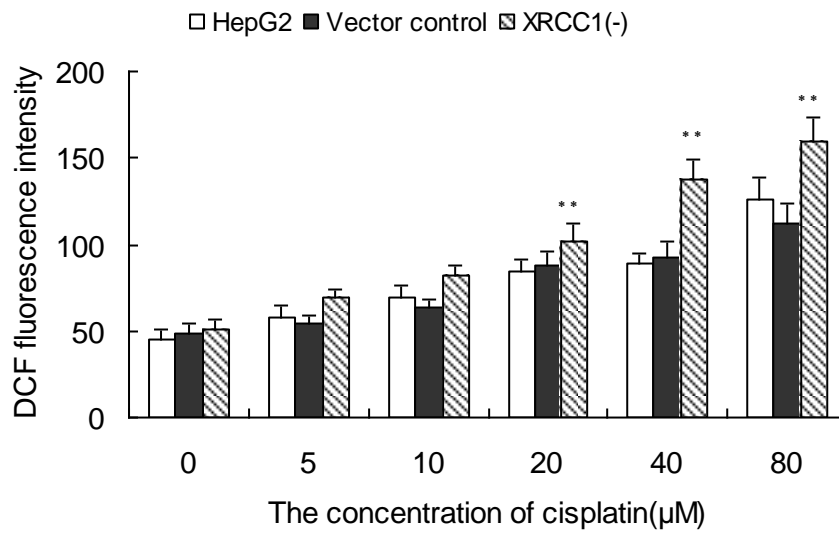


Fig.6



**Table 1 The sequence of shRNA target to XRCC1 mRNA in HepG2 cell**

Group		Sequence(5'→3')	Site
XRCC1 target	Sence	gat cca ttg cca gct cca act cgt ttc aag aga acg agt tgg agc tgg caa ttt ttt tgg aaa	925bp-955bp
	Antisense	agc ttt tcc aaa aaa att gcc agc tcc aac tgc ttc tct tga aac gag ttg gag ctg gca atg	
Scrambled control	Sence	gat ccg act tca taa ggc gca tgc ttc aag acg gca tgc gcc tta tga agt ctt ttt tgt cga ca	a random order
	Antisense	agc ttg tgc aca aaa aag act tca taa ggc gca tgc cgt ctt gaa gca tgc gcc tta tga agt cg	

**Table 2 Expression of XRCC1 mRNA after shRNA transfection in HepG2 cell**

Group	C <sub>T</sub> (XRCC1)	C <sub>T</sub> (GAPDH)	$\Delta C_T$ XRCC1-GAPDH	$\Delta\Delta C_T$	Relative contents ( $2^{-\Delta\Delta C_T}$ )
HepG2	17.50±1.30	23.19±0.42	5.69±0.89	0	1 (0.72-1.38)
Vector control	16.90±0.95	22.68±1.02	5.78±0.26	0.09	0.9395 (0.74-1.18)
XRCC1(-)	16.34±0.18	24.64±0.47	8.30±0.04	2.61	0.2088** (0.12-0.22)

Data were expressed as means±SD(standard deviation). Data significant in relation to HepG2 cell at \*\*P < 0.01/one-way ANOVA test. Each data point was an average of three independent experiments.