The relationship between the down-regulation of DNA-PKcs or Ku70 and the chemosensitization in human cervical carcinoma cell line HeLa

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Abstract. The aim of this study was to clarify the function of non-homologous end-joining (NHEJ) in tumorigenesis and chemoresistance, and to explore the potential of DNA-PK as a target of reversal of chemoresistance and enhancing the sensitivity of cells to chemotherapeutic agents. Plasmid vectors pSIREN-Ku70shRNA and pSIREN-DNA-PKcsshRNA, which coded small interfering RNA of Ku70 and DNA-PKcs, were constructed and transfected into human cervical cancer cell line HeLa. The relationship between the down-regulation of Ku70 or DNA-PKcs and tumor cell proliferation and the sensitivity of cells to chemotherapeutic agents were analyzed. Down-regulation of Ku70 and DNA-PKcs expression inhibited cell proliferation, and increased cell apoptosis in DDP-treated HeLa cells. DNA-PK might play an important role in drug resistance, and inhibition of the DNA-PK expression suppressed the growth of tumor cells and enhanced the sensitivity of cells to chemotherapeutic agents.

Introduction

Non-homologous end-joining (NHEJ) is the most important mechanism of DNA repair in mammalian cells. Competent NHEJ catalyses repair DNA double-strand breaks (DSBs) by ligating two free DNA ends with little or no homology. The DNA-dependent protein kinase (DNA-PK) holoenzyme, the main executive component of mammalian NHEJ, consists of a 470-kDa catalytic subunit (DNA-PKcs), a DNA-binding regulatory component known as Ku protein, and double-stranded DNA (dsDNA) with ends (1). DNA-PKcs is a nuclear serine/threonine protein kinase that comprises of a catalytic subunit, and the Ku subunits acting as the regulatory element (2). The DNA-PK activation, an essential step in the repair process, occurs once the kinase assembles at the site of DSB. The activation of DNA-PK may affect other downstream components involved in signal transduction of the damage event (3). This active DNA-PK complex may then recruit other factors including the MRE11-RAD50-NBS1 (MRN) complex, Artemis and the DNA ligase IV/XRCC4 complex (4-6), which are potentially involved in processing of the termini and completing the repair process. It has been proposed that DNA-PK is a molecular sensor for DNA damage that enhances the signal via phosphorylation of many downstream targets. Many studies demonstrated that the expression of DNA-PK was closely related to cellular radiosensitivity. More and more investigators pay close attention to the relationship between the down-regulation of DNA-PKcs or Ku70 and the chemosensitization. In order to investigate the function of DNA-PKcs and Ku70, we constructed RNAi expression plasmid vector pSIREN-Ku70shRNA and pSIREN-DNA-PKcsshRNA which was designed against Ku70 and DNA-PKcs mRNA, and transfected the plasmids into human cervical cancer cell line HeLa to test the silencing effect of target genes on cell proliferation, cell cycle, apoptosis, and to explore the potential of DNA-PK as a target for reversal of chemo-resistance.

Materials and methods

Cell culture. Human cervical cancer cell line HeLa was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI-1640 (Gibco BRL) supplemented with 10% fetal bovine serum (Hangzhou Sijiqing Biological Engineering Materials Co. Ltd.). The cells were maintained at 37˚C in a 5% CO_2 atmosphere.

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Abbreviations: NHEJ, non-homologous end-joining; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, catalytic subunit of DNA protein kinase; Ku70, regulatory subunit of DNA protein kinase; siRNA, small interfering RNA; DSBs, DNA double-strand breaks

Key words: tumor cell, DNA-PK, shRNA, transfection, drug sensitivity

RNAi design and plasmid construction. Complementary siRNA oligonucleotides that targeted Ku70 or DNA-PKcs were designed according to 'siRNA target finder and design tool' from 'hppt//www.ambion.com' (Fig. 1A). The oligonucleotides were synthesized (Invitrogen China, Shanghai), annealed and connected with pSIREN-DNR-DsRed-Express (BD Clontech) which expressed red fluorescent protein between the *Bam*HI and *Eco*RI sites, and amplified in bacterium *E. coli*. All the recombinants were verified by DNA sequencing (Invitrogen China).

Transfection and drug interfering. The cells were seeded at a density of $5x10^5$/ml on a 6-well plate, according to the manufacturer's instructions, when the cell confluence reached ~70-90%, the mock vector pSIREN, and the recombinant plasmids pSIREN-Ku70shRNA1, 2 and pSIREN-DNA-PKcsshRNA1, 2 were transfected into HeLa cells by Lipofectamine 2000 (Invitrogen). The transfection efficiency was determined by a fluorescence microscope.

For drug interfering, HeLa cells were exposed to the cisplatin (DDP), etoposide (VP-16) and topotecan (TPT) at different concentrations (DDP: 5, 10, 20, 40, 80, 160 μmol/l; VP-16: 8, 16, 32, 64, 128, 256 μmol/l; TPT: 10, 20, 40, 80, 160, 320 μmol/l). The cells were divided into four groups: the control, RNAi, chemotherapy (DDP, VP-16 or TPT) and RNAi + chemotherapy groups. Each group had 3 parallel wells.

RT-PCR. Cellular total RNA was extracted using Trizol (Invitrogen) and cDNA was synthesized with M-MLV reverse transcriptase (Promega) as recommended by the supplier. PCR reactions were performed using the primer pairs as follows: P1 5'-AAA GGA GGA CTC TGG AAA-3' and P2 5'-TGA TGT GGA TTT ATT G-3' to detect the 510-bp fragment of the Ku70; P1 5'-TTA TGC AGA AGC CCA GCT-3' and P2 5'-ATT ATG GAG TTT ACC ACG AC-3' for a 377-bp DNA-PKcs fragment, and P1 5'-ACG GAT TTG GTC GTA TTG GG-3' and P2 5'-TGA TTT TGG AGG GAT CTC GC-3' for a 230-bp fragment-coded internal control glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reaction cycle number was 30 cycles for Ku70 and DNA-PKcs, 28 cycles for GAPDH. PCR was performed as follows: 95˚C for 1 min; 95˚C for 30 sec, 50˚C for 45 sec (Ku70), or 53˚C for 45 sec (DNA-PKcs), or 56˚C for 30 sec (GAPDH), and 72˚C for 10 min for above mentioned cycles; the reactions were ended with 72˚C for 10 min.

Western blot analysis. Cells were lysed in RIPA buffer [50 mmol/l Tris (pH 8.0), 150 mmol/l NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate]. The protein concentration of suspension was measured by the Coomassie Blue method. Equivalent amounts of whole-cell extracts (80 μg) were separated in 10% polyacrylamide gel and transferred to Hybond-ECL nitrocellulose filter paper (Amersham). Filters were blocked in 25 mmol/l Tris (pH 8.0) containing 125 mmol/l NaCl, 0.1% Tween-20, and 5% skim milk. Protein bands were probed with antibodies against Ku70 and DNA-PKcs at 1:300, and ß-actin (1:1000), stayed overnight at 40˚C, and then with alkaline phosphatase-labeled secondary antibodies (1:500) at 37˚C for 1 h, the blots were developed by BCIP/NBT.

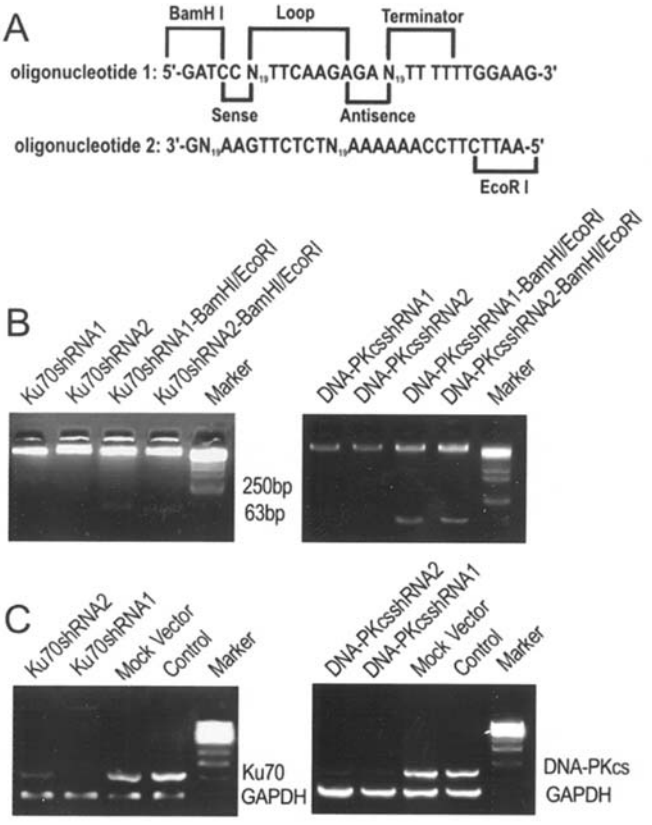

Figure 1. (A) DNA sequences that targeted the genes under study were inserted into the N_{19} both in forward and reverse. The complementary oligonucleotides were annealed to form a double-strand DNA which contained *Bam*HI and *Eco*RI sites at both ends. Then the annealed DNA was ligated with *Bam*HI/*Eco*RI-treated pSIREN-DNR-DsRed-Express. (B) *Bam*HI/*Eco*RI digested recombinant plasmids. Each successful recombinant plasmid showed a 63-bp fragment in the agarose gel. All the plasmids were then sequenced to confirm no errors. (C) DNA-PKcs and Ku70 mRNA expression in transfected HeLa cells was detected by RT-PCR 48 h after transfection. DNA-PKcs and Ku70 mRNA were down-regulated dramatically after transfection with recombinant plasmids compared with mock plasmid-transfected and untransfected cells.

Cell immunohistochemistry. Sterilized coverslips were put into a 24-well plate, cells were seeded at 10^5 cells/well. When the confluence reached ~50-70%, the recombinant plasmids were transfected into the cells with Lipofectamine 2000 according to the manufacturer's instructions, and the transfected cells with no plasmids were set as the control. The coverslips were put into acetone-alcohol solution for 30 min, and then fixed on the slides with glue. Cells were incubated with mouse anti-human monoclonal Ku70 and DNA-PKcs antibodies (NeoMarkers Corporation), immunohistochemistry staining was performed according to the SP kit manual (Beijing Tianwei Time Biotechnology Co. Ltd.). For negative control, primary antibodies were displaced by PBS solutions.

MTT assay. MTT assays were performed to assess the effect of siRNA treatment on cell proliferation. Briefly, $5x10^3$ cells/well were plated on a 96-well plate for 24 h, then Ku70 and DNA-PKcs siRNA plasmids were transfected into the cells by Lipofectamine 2000. After 24, 48, 72 or 96 h of culture at 37˚C, 10 μl of MTT (5 mg/ml) were added to each well. The

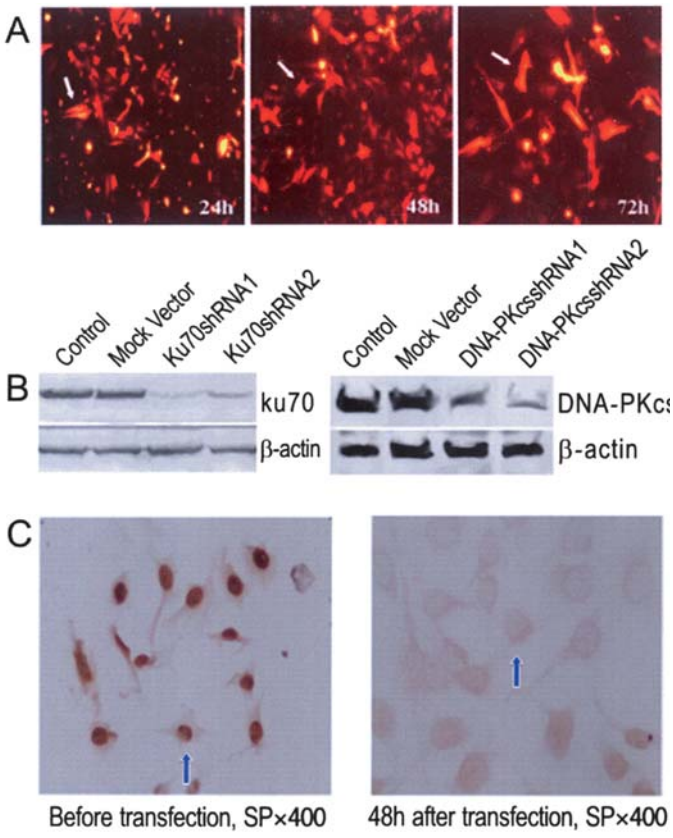

Figure 2. (A) Cells were observed under a fluorescent microscope 24, 48 and 72 h after transfected with recombinant PSIREN-Ku70shRNA1. The highest transfection efficiency was obtained 48 h after transfection. Similar results were also obtained in the cells transfected with other recombinant plasmids as well as the mock plasmid. (B) In Western blot assay, down-regulation of DNA-PKcs and Ku70 protein were observed 48 h after transfection with recombinant plasmids. (C) In pSIREN-DNA-PKcsshRNA2-transfected HeLa cells, DNA-PKcs expression was clearly reduced 48 h after transfection.

reaction was stopped after 4 h of incubation by adding 150 μl of dimethyl sulfoxide (DMSO). The optical density (OD) value was obtained by measuring absorbance at the wavelength of 570 nm, and the proliferation index calculated by the ratio of OD570 of experimental group to OD570 of control group. The MTT assay was done in triplicate, and repeated 3 times.

FCM. Cells were harvested and fixed in cold 70% ethanol for 24 h at -20˚C, washed with PBS, treated with RNase A for 30 min at room temperature, and then stained with propidium iodide (PI) for 30 min, PI stained cells were analyzed using a flow cytometer (Becton Dickinson).

Statistical analysis. Statistical analysis was performed using statistical package SPSS12.0. To determine the significant difference (95% probability) of parameters between sample groups, ANOVA and q-test were utilized.

Results

Restriction endonuclease analysis of pSIREN-Ku70shRNA and pSIREN-DNA-PKcsshRNA recombinant plasmids. pSIREN-Ku70shRNA and pSIREN-DNA-PKcsshRNA recombinant plasmids were digested by *Bam*HI and *Eco*RI respectively, the acquired bands were consistent with theoretical anticipated 63-bp bands on the agarose gel electrophoresis (Fig. 1B). The sequences were confirmed with no errors by DNA sequencing.

mRNA silence effect of DNA-PKcs and Ku70 detected by RT-PCR. DNA-PKcs and Ku70 mRNA expression in transfected HeLa cells was detected by RT-PCR at 48 h after transfection. Compared with the control and mock vector transfected cells, the mRNA expression level of Ku70 and DNA-PKcs in HeLa cells was reduced dramatically after transfection with pSIREN-Ku70shRNA1, 2 or pSIREN-DNA-PKcsshRNA1, 2, indicating the silence effect of the plasmids, and the inhibition effect of pSIREN-Ku70shRNA1 or pSIREN-DNA-PKcsshRNA2 was more obvious (Fig. 1C).

Transfection efficiency. pSIREN-DNR-DsRed-Express expressed red fluorescent protein, the transfected cells were observed under a fluorescent microscope. PSIREN-Ku70shRNA1, 2 and pSIREN-DNA-PKcsshRNA1, 2 transfected into HeLa cells with high efficiency. Comparing the transfection efficiency of different time points, we found that of 48 h was the highest (Fig. 2A).

Protein blocking effect of DNA-PKcs and Ku70 detected by Western blotting. The reduction of mRNA was reflected at protein level, as shown by Western blot analysis (Fig. 2B). Recombinant plasmid-transfected HeLa cells, particularly pSIREN-Ku70shRNA1 or pSIREN-DNA-PKcsshRNA2 transfected cells, demonstrated a high inhibition effect of the target genes 48 h after transfection. The results were matched with those we acquired from RT-PCR.

Cell immunohistochemistry. We verified the down-regulation effect of pSIREN-DNA-PKcsshRNA2 in living cells by immunohistochemistry. We observed that DNA-PKcs expression was concentrated on the cellular nucleus, and was weakened at 48 h after pSIREN-DNA-PKcsshRNA2 was transfected into HeLa cells (Fig. 2C).

The effect of the recombinant plasmid transfection on cell proliferation. Cell proliferation was clearly inhibited in recombinant plasmid-transfected HeLa cells when compared with untransfected and mock plasmid-transfected cells ($F=5.022$, $P=0.018$) (Fig. 3A).

HeLa cell proliferation was inhibited when DNA-PKcs and Ku70 expression was knocked down by RNAi, the cell growth inhibition rate reached as high as 30 and 37% after pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 treatment for 72 h (Fig. 3A). The dose response curve was drawn according to cell inhibition rate and drug concentration logarithm (DDP, VP-16, TPT) at 24 h after the drugs were added to the cells, which had been transfected with pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 for 48 h, and obtained 50% inhibiting concentration (IC_{50}) of DDP, VP-16 and TPT (Fig. 3B), the result showed that the sensitivity of HeLa cells to DDP ($P=0.001$), VP-16 ($P=0.001$) and TPT ($P=0.001$) increased after transfection, and transfection with pSIREN-Ku70shRNA1 was similar with pSIREN-DNA-PKcsshRNA2.

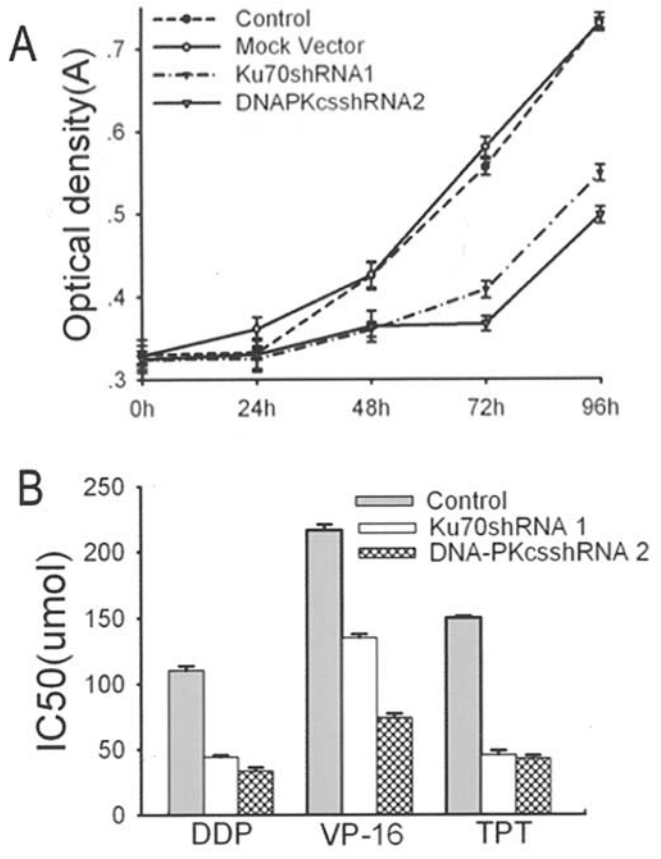

Figure 3. (A) Cell proliferation curves were drawn at different time points after transfection. After transfected with pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 72 h or later, cell proliferation was inhibited when compared with mock plasmid-transfected cells and untransfected cells. (B) After transfection of 48 h with pSIREN-Ku70shRNA1, pSIREN-DNA-PKcsshRNA2 and mock plasmids, HeLa cells were incubated with different concentrations of DDP, VP-16 and TPT. After 24 h, cell proliferation curves were drawn. pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 transfected cells showed increased sensitivity to drugs compard to mock plasmid-transfected and untransfected cells.

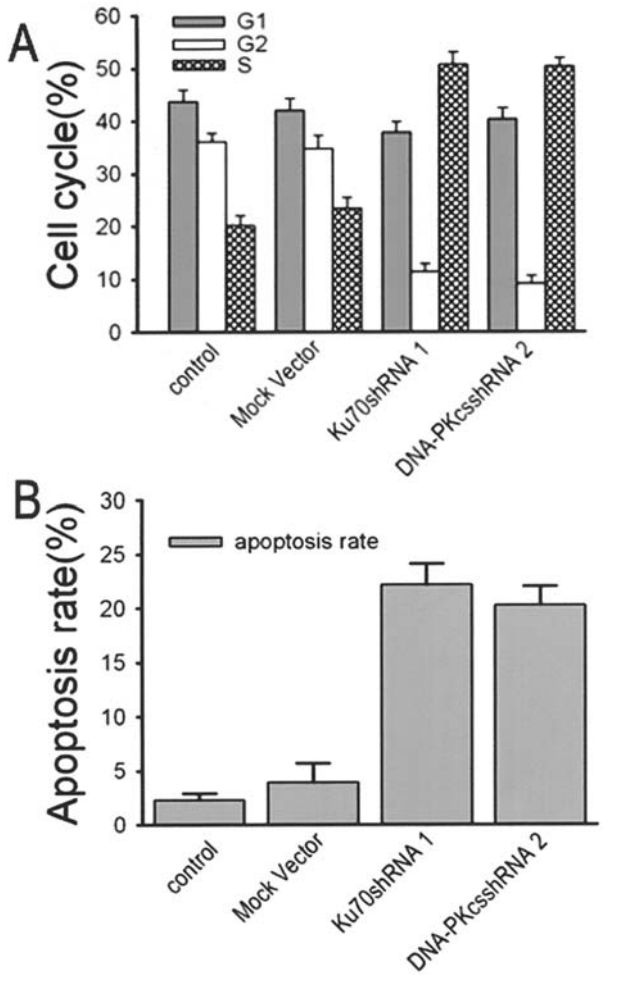

Figure 4. (A) After 48 h transfection with pSIREN-Ku70shRNA1, pSIREN-DNA-PKcsshRNA2 and mock plasmids, HeLa cells were treated for cell cycle analysis by flow cytometry. Most pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 transfected cells were arrested in S phase. (B) After 48 h transfection with pSIREN-Ku70shRNA1, pSIREN-DNA-PKcsshRNA2 and mock plasmids, HeLa cells were incubated with DDP (40 μmol/l). after 24 h, cell apoptosis rates were assayed by a flow cytometer. More apoptotic cells were detected in pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 transfected cells than in the mock plasmid-transfected cells.

The effects of transfection of the recombinant plasmids on cell cycle and apoptosis. Cell cycle and apoptosis were observed by flow cytometry after 48 h of pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 treatment, the results indicated that the HeLa cell growth was arrested in S phase of the cell cycle following Ku70 and DNA-PKcs knockdown (P=0.001, P=0.001; Fig. 4A), apoptotic cells increased (P=0.001, P=0.001; Fig. 4B). Cell cycle and apoptosis were observed by flow cytometry at 24 h after 40 μmol/l of DDP was added to the cells which had been transfected with pSIREN-Ku70shRNA1 and pSIREN-DNA-PKcsshRNA2 for 48 h, flow cytometric analysis indicated that apoptosis rates were clearly increased in these cells compared with in that of mock plasmid-transfected cells (Fig. 4B).

Discussion

DNA-PK is the most important component of mammalian NHEJ, competent DNA-PK participates in the course of transcription and apoptosis via phosphorylation of many protein substrates, and has significance in maintaining the genetic stability (7). Many studies have described that the start of mammalian DNA duplication is Ku-dependent. Ku with activation of ATP enzyme and joining enzyme connected with some duplicate initiation factors, and the absence of Ku induced a decrease of DNA duplication to an elementary level (8,9). Ku interacts with some members of the RecQ family, which belongs to DNA untwisting enzyme and relates to DNA recombination, to participate in liver nucleic acid metabolism (10,11). Interaction between Ku and Msx2, Runx2, Tbdn100 regulated the expression of bone Gla protein gene; interaction between Ku70, which replaces heat shock element (HSE), and heat shock transcription factor 1 (HSF1) inhibited gene promoter of heat shock protein 70 (HSP70) (12). By interactions with human telomerase transcriptase hTERT, Ku regulated telomere longitude and maintained the stability of telomeres (13). DNA-PKcs is

recognized to function in the pathways of NHEJ of DSBs and V(D)J recombination. In addition, DNA-PKcs is essential for maintaining telomere length and stability, and it can phosphorylate *in vitro* a number of important proteins including some oncogene products and transduction factors such as c-fos, c-myc, and c-jun. Recent data demonstrated that suppression of the DNA-PK expression enhanced the sensitivity of radio- and chemotherapy in tumor cells, which suggested the potential role of DNA-PK in radio- and chemosensitivity (14-17).

Cisplatin (DDP), etoposide (VP-16) and topotecan (TPT) are widely used clinically for the treatment of tumors. Cisplatin produces a variety of platinum-DNA adducts, including intra- and inter-strand crosslinks, and it is generally accepted that these lesions underlie most of the cytotoxic effects of the drug (18,19). The topoisomerase II is a nuclear enzyme that functions during both DNA replication and transcription, topoisomerase II inhibitors, such as VP-16, represent a major class of anticancer agents with documented activities against a broad spectrum of human malignancies (20), can stabilize the complex formed by topoisomerase II and the 5'-cleaved ends of the DNA, thus forming stable (non-repairable) protein-linked DNA double-strand breaks. Cells are apparently able to recognize such DNA damage and, in turn, eliminate the injured cells by apoptosis (21). Topotecan selectively poisons topoisomerase I by trapping topoisomerase I cleavage complexes, which correspond to enzyme-linked DNA breaks. It induces replication-dependent DNA lesions and arrests cells in the S and G2 phase of cell cycle. DNA damage induced by topotecan probably consists of replication-mediated DNA double-strand ends and formation of abnormal replication intermediates, consequent to encounters of replication forks with topotecan-stabilized topoisomerase I-DNA complexes (22).

However, the emergence of tumor cell drug resistance remains a major clinical problem and the frequent cause of failure in the long-term efficacy of cancer therapy (18,19,23). Extensive experimental research has shown that cellular resistance mechanisms are generally multifactorial (24-27). Possible mechanisms of acquired resistance include: a) pretarget events, including drug uptake, metabolism, and intracellular distribution; b) drug-target interactions; and c) post-target events, including macromolecular syntheses, DNA repair, cell cycle progression, and regulation of cell death (26,28).

Because of the reactivity of DDP and DNA topoisomerase inhibitors and the complexity of the cellular response to DNA damage, the molecular mechanisms that underlie chemoresistance are largely unknown. Therefore, the goal of our experiment is to explore the underlying mechanisms responsible for this phenomenon, with the hope that more effective therapies can be devised.

Overexpression of DNA-PK has been reported in a wide variety of human tumors, and many studies suggest that high expression of DNA-PK correlates with radiosensitivity (17,29). Since many antitumor drugs produce a marked effect through damaged DNA in tumor cells, close attention is paid to the relationship of DNA-PK and chemosensitivity. The recombinant plasmids were successfully transfected into HeLa cells and verified by fluorescence microscope (Fig. 2A), the mRNA and protein expression levels of DNA-PKcs and Ku70 decreased significantly after transfection (Fig. 2), and then showed that DNA-PKcs and Ku70 was knocked down successfully. HeLa cell proliferation was inhibited and the cell cycle arrested in S phase after that Ku70 and DNA-PKcs had been knockdown (Figs. 3A and 4A), while, apoptotic cells increased obviously (Fig. 4B). These findings indicated that DNA-PKcs and Ku70 are related to cell proliferation and apoptosis. Then, we used DDP, VP-16 and TPT to treat the cells 48 h after transfection. The IC_{50} of cells was distinctly decreased compared with the control cells which were treated with drugs alone (Fig. 3B). The cell cycle arrested in S phase in transfected cells (Fig. 4C). The results indicated that the activation of DNA-PK is a critical factor in determining chemosensitivity of tumor cells.

Deriano *et al* (29) found that at 15 min after irradiation, the levels of NHEJ (as measured by an *in vitro* DSB end-ligation assay) and DNA-PKcs activity were, respectively, 2-fold and 4-fold higher in radio-resistant than in radio-sensitive B-CLL cells; Ku70/Ku80 heterodimer DNA end-binding activity was also 2- to 3-fold higher in the resistant B-CLL cell subset compared with the sensitive B-CLL cell subset. Durant *et al* (30) inhibited the activity of DNA-PK by Vanillins, a DNA-PK inhibitor, and found it significantly potentiated the cytotoxicity of cisplatin. Eriksson *et al* (31) inhibited the activity of DNA-PK by trifluoperazine and drew a similar conclusion. We have shown in this study that the inhibition of DNA-PK by mRNA silence of Ku70 or DNA-PKcs decreased the activity of DNA-PK and thus induced cell apoptosis. Most importantly, this inhibition correlated with the chemosensitivity of certain drugs, and then DNA-PK possibly participated in the process of drug resistance.

Belenkov *et al* (28) investigated down-regulation of the Ku86 gene by transfection of human glioma cell line (M059K) with 200 nmol/l Ku86 antisense ASOs which markedly increased cell death after treatment with ionizing radiation, bleomycin, and etoposide; however, no sensitization occurred to the DNA cross-linking agent cisplatin. Ayene *et al* (32) reported that the expression of Ku70 in HCT116 (colon cancer cell line) had negative correlation with the sensitivity of γ-ray and VP-16. Frit *et al* (33) studied a murine CDDP-resistant L1210 cell line (L1210/3R) that exhibits cross-resistance to IR, and found an increased DNA-end binding activity compared with parental cells (L1210/P). Many studies have described that Ku is related to radiosensitivity and sensitivity of VP-16, but the relationship between Ku and DDP is not clear. Our findings indicated that inhibition of expression of Ku70 enhanced the sensitivity of tumor cells to DDP and VP-16. These results suggest that Ku activity may be an important molecular target in cancer therapy.

Although the studies on the relation between TPT and DNA-PK are few, the studies between TPT and capacity of DNA damage repair have attaracted wide interest (34,35). Our results showed that inhibition activity of DNA-PKcs and Ku70 enhanced the cytotoxicity of TPT.

In conclusion, inhibition of the DNA-PK expression suppressed the growth of tumor cells and enhanced the sensitivity of cells to chemotherapeutic agents DDP, VP-16 and TPT. We provided evidence that DNA-PK may contribute to tumor resistance in chemotherapy. The study strongly

supported the potential of DNA-PK as a target for reversal of drug resistance.

Acknowledgements

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