RESEARCH ARTICLE

Suppression of Ku80 Correlates with Radiosensitivity and Telomere Shortening in the U2OS Telomerase-negative Osteosarcoma Cell Line

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Abstract

Ku70/80 heterodimer is a central element in the nonhomologous end joining (NHEJ) DNA repair pathway, Ku80 playing a key role in regulating the multiple functions of Ku proteins. It has been found that the Ku80 protein located at telomeres is a major contributor to radiosensitivity in some telomerase positive human cancer cells. However, in ALT human osteosarcoma cells, the precise function in radiosensitivity and telomere maintenance is still unknown. The aim of this study was to investigate the effects of Ku80 depletion in the U2OS ALT cell line cell line. Suppression of Ku80 expression was performed using a vector-based shRNA and stable Ku80 knockdown in cells was verified by Western blotting. U2OS cells treated with shRNA-Ku80 showed lower radiobiological parameters (D0, Dq and SF2) in clonogenic assays. Furthermore, shRNA-Ku80 vector transfected cells displayed shortening of the telomere length and showed less expression of TRF2 protein. These results demonstrated that down-regulation of Ku80 can sensitize ALT cells U2OS to radiation, and this radiosensitization is related to telomere length shortening.

Keywords: Ku80 - telomerase negative osteosarcoma - U2OS cells - radiosensitivity - telomere length - TRF2

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Introduction

Ku80 is one subunit of the Ku80/Ku70 heterodimer. The crucial role of Ku80 in DNA damage was well known, especially the NHEJ pathway. When DNA is damaged, Ku heterodimer recruits DNA-PKcs to the DSB and activates its kinase function. And then, this process stimulates DNA repair and the damage signal pathway, which may subsequently affect apoptosis and cell cycles. Besides its important role in DNA repair, many reports have suggested that Ku80 is also involved in telomere maintenance. When DNA is damaged, the Ku heterodimer protects exposed DNA ends by end-to-end bridging at sites of DNA DSBs. Unlike the DNA DSBR (double-strand DNA break repair) pathway, end-to-end joining of DNA should be avoided during repair of exposed telomeric ends (Gullo et al., 2006). During telomere repair, Ku heterodimer bind to telomeric sequences (Bianchi et al., 1999; Hsu et al., 1999) and promote the telomerase-mediated synthesis of the complimentary strand of DNA that prevents end-to-end DNA fusion (Bailey et al., 1999; Samper et al., 2000). Previous studies demonstrated that mutation in Ku80 proteins prevent repair of telomere ends (Polotnianka et al., 1998) and these results reduced telomere lengthen and enhanced the radiosensitivity in many cell lines (Yang et al., 2008). However, the cell lines selected in those studies were almost telomerase positive, the effect of Ku80 on the telomere and radiosensitivity in telomerase negative cell lines are still unclear. The aim of this study was to investigate the relationship between radiosensitivity, telomere length and Ku80 protein in telomerase negative cancer cells.

Materials and Methods

Cell lines and culture conditions.

The human osteosarcoma cell U2OS were obtained from the China center for type cultrue collection(CCTCC) in Wuhan University. The cells were cultured in RPMI 1640 medium (Sigma chemical Co., St Louis, MO, USA) at 37°C supplement with 10% fetal bovine serum and 1% penicillin/streptomycin mixture. All cultures were grown at 37°C under a humidified atmosphere of 5% carbon dioxide.

Ku80 shRNA vector constructs

Plasmid pGenesil-1 expression vector (Wuhan genesil biotechnology Co, Ltd.) was used, which contained a human

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U6 promoter, a GFP reporter gene and a neomycin resistance gene to enable antibiotic selection in mammalian cells. We designed two pairs of complementary oligonucleotide sequences according to the cDNA sequences of Ku80 (GenBank accession number: NM_021141). They analyzed BLAST research to ensure that they didn't have significant sequence homology with other genes. Target sequences of Ku80 for shRNA are showed below: 5'-CACCGCCAGGTTCTCAACAGGCTGTTCAAGA CGCAGCCTGTTGAGAACCTGG TTTTTTG-3' 5'- AG AGCTCAAAAAACCAGGTTCTCAACAGGCTGCGT CTTGAACAGCCTGTTGAGAACCTGGC-3'.

shRNA vector stable transfection

One hundred thousand U2OS cells in a total volume of 2 ml RPMI 1640 medium were seeded in 6-well plates and incubated for 24 h. The cells were rinsed with PBS and then were treated with shRNA and scrambled control according to the manufacturer's instructions. shRNA plasmids and Oligofectamine 2000 (Invitrogen, USA) were mixed separately with opti-MEM and incubated for 5 min at room temperature. These reagents were combined and incubated for another 20 min before adding to the cells. The effectiveness of transfection was visualized by fluorescence microscope 24 h after transfection. After 48 h incubation, 500 μg/ml of hygromycin (G418) was added to the medium for selection individual colonies which were isolated after addition 2 weeks culture. The colonies which contained GFP were selected by fluorescence microscope and continue to culture for at least 10 passages. As a control, a subset of U2OS cells was transfected with the control plasmid carrying scrambled sequence.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacture's instructions. The first strand of cDNA was obtained using Revert aidTM first strand cDNA synthesis kit (Fermentas). For quantitative analysis of Ku80 mRNA, human GAPDH gene was used as an internal control. DNA primer sequences were designed as follows: F: 5'-GGGGTACCATGGTGCGGTCGGGGAAT-3', R: 5'-GCTCTAGATCCCCATACATCCACGAC-3'for Ku80, F: 5'-ATCACTGCCACCCAGAAGAC-3', R: 5'-AGCGTCAAAGGTGGAGGAGT-3'for GAPDH. Amplification cycles were 94°C for 3 min; the 30 cycles at 94°C for 30 sec, 57°C for 30 s, 72°C for 1 min, 72°C for 5 min. PCR products were identified using electrophoresis on 1.5% agarose gels containing 1% ethidium bromide (EB). Gel images were obtained using Bio-ID gel analysis software (Vilber lourmat, France). All experiments were repeated at least three times.

Western blot analysis

Cultured cells were rinsed twice with phosphate buffered saline (PBS) and mixed with 200 μl of lysis buffer (Beyotime biotechnology). The cells in lysis buffer in the dish were removed using a scraper and transferred to an Eppendorf tube. The cells were homogenized and centrifuged 1,2000 rpm for 5 min , and the supernatant were stored at -20°C. The protein concentration of the whole cell was determined using a BCA protein assay kit (Beyotime biotechnology). The protein extracts (50μg) were incubated in loading buffer (60 mmol/L Tris-Hcl, 25% Glycerol, 2% SDS, 14.4 mmol/L Mercaptoethanol, 0.1% Bromophenol blue), and boiled for 5 min. These samples were electrophoresed on a 10% SDS-PAGE. After electrophoresis, proteins were transferred to a PVDF membrane and processed for immunoblotting. For the detection of Ku80 and β-actin, blots were incubated with 1:300 dilution mouse monoclonal antibodies (Santa cruze biotechnology) and further incubated with Horseradish peroxidase-conjugated secondary antibody diluted at 1:5000 and specific bands were visualized by ECL (Beyotime biotechnology), Autoradiographs were recorded onto X-Omat AR film (Eastman kodak Co.) The density of bands in the resulting film was quantified using the Image J analysis program.

Real-time quantitative PCR

Genomic DNA was extracted from cells using TIANamp genomic DNA kit (TIANGEN biotech Co,. Ltd.) and stored at -70°C. Relative telomere length was detected by using the approach described by Cawthon previously (Cawthon., 2002). This method measures the factor by which the ratio of telomere repeat copy number to single-gene copy number differs between the sample and the reference sample. PCR amplification was achieved using telomere (T) and single copy gene(S), 36B4 (encodes acidic ribosomal phosphoprotein) primers which serves as a quantative control. The mean telomere repeat gene sequence to a reference single copy gene is represented as T/S ratio which calculated to determine the relative telomere length. Standard curves were generated for telomere lengths and the single gene copy amplification reactions from a reference DNA sample serially diluted with Milli-Q water by 2-fold per dilution to produce four concentrations of DNA ranging from 50 to 3.125 ng/μl. Duplicate PCR reactions using the TaKaRa realtime PCR kit (TaKaRa biotechnology Co, Ltd) according to the manufacture's instructions. The primers for telomeres and the single copy gene 36B4 were added to final concentrations of 0.2 μM. The telomere and single copy gene specific primers used for the experiment were given as below: Tel 1: 5'- GGTTTTTGAGGGTGAGGGTGA GGGTGAGGGTGAGGGT -3'; Tel 2: 5'-TCCCGACTA TCCCTA TCCCTATCCCTATCCCTATCCCTA -3'; 36B4u: 5'-CAGCAAGTGGGAAGGTGTAATCC-3', 36B4d: 5'-CCCATTCTATCATCAACGGGTACAA-3'. The cycling conditions consisted of a preincubation for 5 sec at 95°C and followed by 35 cycles of 15 sec at 95°C, 2 min at 54°C. All experiments were repeated at least three times. Thermal amplification was carried out on Mx3000P (Stratagene) and the result was analyzed using the MXP 3000 analysis program.

Grouping and Irradiation

The cells were divided into 3 groups: untreated cells (negative control), Cells stable transfected with shRNA-Ku80, shRNA-scramble. Cells were seeded in 6 wells and incubated at 37°C under humidified 5% CO_2, 95% air in culture medium. Cells were exposed to γ –rays from a

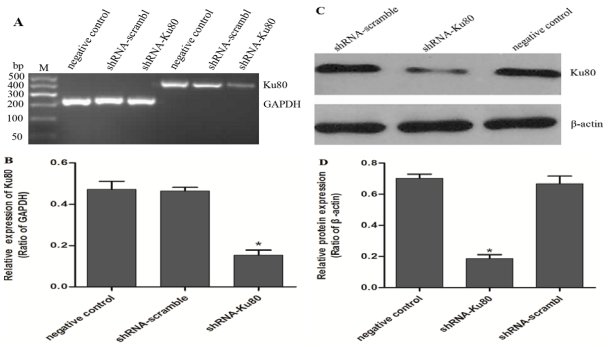

Figure 1. Ku80 Gene Down-regulation in U2OS Cells. RT-PCR detected Ku80 expression in U2OS cells treated with shRNA-Ku80, shRNA-scrambl and negative control. (A) RT-PCR was used to detect the Ku80 gene expression in the different groups. (B) The PCR products were semiquantified for relative levels of mRNA using image analysis by comparing Ku80 with GAPDH. (C) Ku80 protein expression was detected by Western blotting in the different groups. (D) The bar chart shows the semiquantitative analysis of Ku80 protein expression. Data represent means ±SD. $P^*<0.05$ is considered significant

^{60}Co-ray source (Atomic energy of Canada Ltd, Canada and located in Institute, Seoul, Korea) at a dose rate of 61.3 cGy/min.

Colony formation assay

An appropriate number of cells which stable transfected with shRNA-Ku80, shRNA-scramble and untreated cells were plated into 6-well plates. Each group of cells were irradiated at the dose point of 0, 1, 2, 4, 6, 8, 10 Gy respectively. After 14 days of incubation, the colonies were fixed and stained with crystal violet (1% in absolute alcohol). Those colonies containing >50 cells were scored as viable colonies. The data were fit into the single-hit multi-target model, and survival curve of each group were demonstrated by Graphpad prism 5.0 software. Radiobiological parameters, such as D 0, Dq and SF 2were calculated according to the survival curves.

Statistic analysis

All of the experiments were replicated three times. Data were obtained from triplicate samples and expressed as the mean ±standard deviation. Statistical analysis were performed by two-factor ANOVA. Statistical analysis was performed using software SPSS 13.0 and Graphpad prism 5.0 software. $P<0.05$ was considered to be statistically significant.

Results

Efficient knockdown of Ku80 in U2OS cells

RT-PCR and western blot were used to analyze the Ku80 expression. Compared with negative control group and shRNA-scramble group, the expression of Ku80 transcripts (Figure 1A and Figure 1B) and Ku80 proteins (Figure 1C and Figure 1D) were reduced in shRNA-Ku80 group. However, negative control cells and shRNA-scramble cells showed no apparent change of Ku80 expression. The results indicated that the shRNA-Ku80 could downregulate the expression of Ku80 efficiently in gene and protein level.

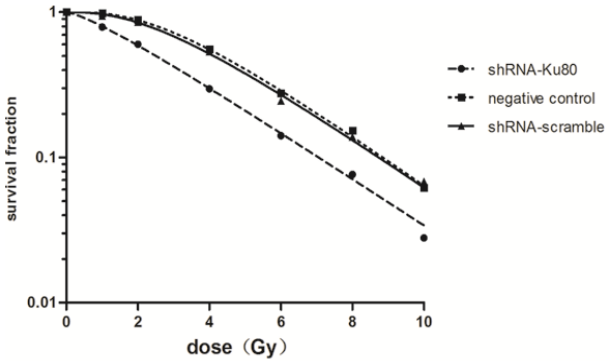

Figure 2. Clonogenic Assays. Survival curves in U2OS cells treated with shRNA-Ku80, shRNA-scramble and negative control. An appropriate number cells in different groups were plated into 6-well plates. Each group of cells were irradiated at the dose point of 0, 1, 2, 4, 6, 8, 10 Gy respectively. After 14days of incubation, the colonies were fixed and stained with crystal violet (1% in absolute alcohol). Those colonies containing >50 cells were scored as viable colonies. The data were fit into the single-hit multi-target model, and survival curve of each group were demonstrated by Graphpad prism 5.0 software.

Table 1. Radiobiological Parameters in Different Groups

Grouping	D0	Dq	SF2
shRNA-Ku80	2.106±0.033*	1.028±0.012*	0.602±0.009*
shRNA-scarmble	2.384±0.040	2.967±0.015	shRNA-KU80
Negative control	2.421±0.042	2.692±0.198	0.883±0.007

Data from triplicate clonogenic assay were fit into the single-hit multi-target model, and survival curves of both groups were drawn by software Graphpad Prism, the SF2, D0, Dq values were calculated (PD0, Dq, SF2<0.05). Data were expressed as the mean±SD. $P^*<0.05$ is considered significant. SF, surviving fraction; D0, mean lethal dose, is known as final slope; Dq, quasi-threshold dose

Radiobiological parameters of clonogenic assay.

The survival curves describe the radiobiological parameters of each group (Figure 2). Compared to the negative control and shRNA-scramble group, the survival fractions of shRNA-Ku80 group were much lower at each point. The radiobiological parameters calculated are showed in Table 1. D0, Dq and SF 2 values in shRNA-Ku80 group are significantly lower than the control groups (P<0.05). Negative control and shRNA-scramble groups showed no significantly changed. The result suggested that downregulating Ku80 protein expression could enhance the radiosensitivity of U2OS cells.

Relative telomere length

Telomere length was determined from U2OS cell stable transfected shRNA-Ku80 plasmid, shRNA-scramble plasmid and negative control by using real-time PCR. The dissociation curve showing a unique peak from the PCR amplification of telomere and single copy gene (36B4) attested to the specificity of the amplification. The standard curve derived from the amplification of various concentrations indicated that there was linear relationship between Ct values and DNA copy number, suggesting that our realtime PCR approach would be useful for quantifying (data not shown). The relative telomere length among the cells stable transfected with shRNA-Ku80,

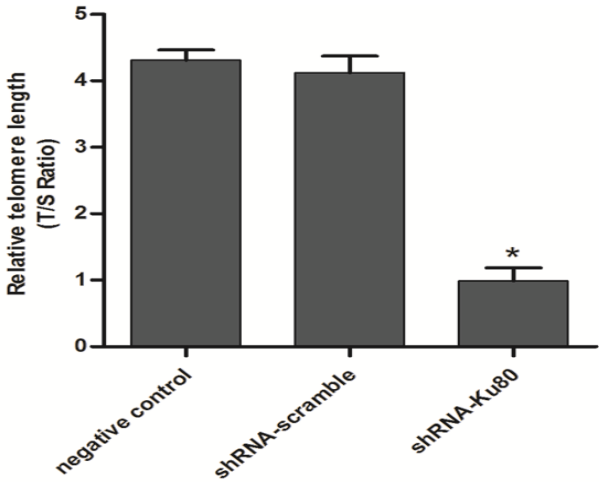

Figure 3. Effect of Suppression of Ku80 on Telomere Length in U2OS Cells. Realtime PCR detected the relative telomere length in U2OS cells treated with shRNA-Ku80, shRNA-scrambl and negative control. The Bar graph shows the mean±SD value of relative telomere length. *P**<0.05 is considered significant

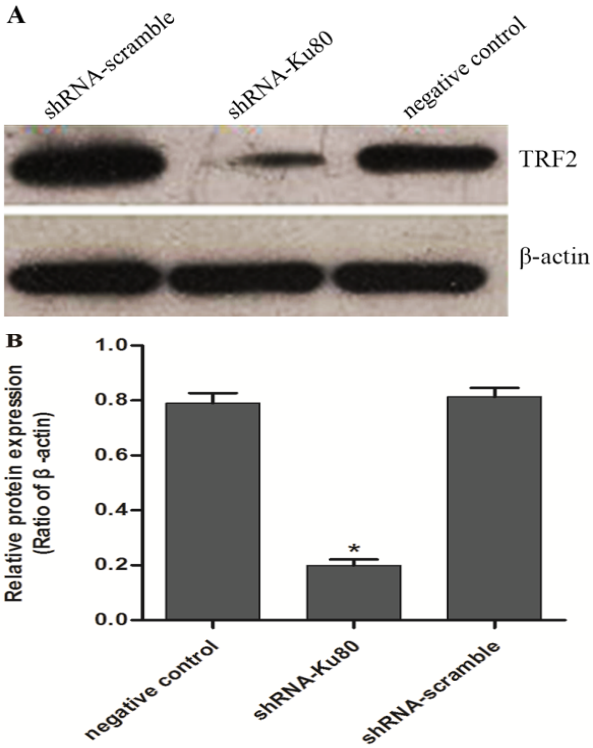

Figure 4. Effect of Ku80 Down-regulation on The Expression of TRF2 Protein in U2OS Cells. (A)Western blotting analysis shows the protein expression of TRF2 in U2OS cells treated with shRNA-Ku80, shRNA-scramble and negative control. (B) The bar chart shows the semiquantitative analysis of Ku80 protein expression. Data represent means±SD. P*<0.05 is considered significant

shRNA-scramble and negative control was determined to be 1.07±0.07, 4.42±1.30, 4.11±0.84, respectively. (Figure 3) The result suggested that downregulation of Ku80 protein expression could significantly shorten the telomere length.

The protein expression of TRF2 in different groups

Western blot were used to analyze the TRF2 expression. Compared with negative control group and shRNA-scramble group, the expression of TRF2 protein (Figure 4) was reduced in shRNA-Ku80 group. However, negative control cells and shRNA-scramble cells showed no change. The results suggested that downregulating the Ku80 expression could depress the TRF2 expression.

Discussion

Ku80 is a versatile regulatory protein that has been shown to play a crucial role in multiple nuclear processes such as DNA repair, telomere maintenance and apoptosis. Recent reports suggested that there is a positive relationship between Ku80 and telomere length in various cancer cells (Nimura et al., 2007). However, significant discoveries on the role of Ku80 in telomerase negative cancer cells have been uncovered. To study the role of Ku80 in telomerase negative cancer cells, we have successfully constructed shRNA-Ku80 vector and selected the positive colonies cells to suppress the endogenous Ku80 expression efficiently. In this study, we used the vector-based shRNA expression systems in our research, overcoming the limitations of transient transfection of small interference RNA.

Ku80 is an important DNA repair protein in the NHEJ pathway with the other proteins such as DNA–PKcs, XRCC4 and DNA Ligase IV. Ku80 is a critical nuclear protein that not only involved in DNA repair, but also it is essential for the maintenance of telomere length. During telomere repair, Ku80 bind to telomere sequences (Bianchi et al., 1999; Hsu et al., 1999) and associate with human telomerase reverse transcriptase, which is essential for the addition of telomeric repeat sequences (Chai et al., 2002). Moreover, the direct relationship between Ku80 and telomerase has been shown in recent reports (Hsu et al., 2000; Bailey et al., 2004). Suppression of Ku80 expression resulted in losing of telomerase activity, shortening the chromosome ends gradually, and apoptosis (Lucero et al., 2003; Kim., 2008). The human telomerase complex is a ribonucleoprotein containing an integral RNA (hTR), a reverse transcriptase protein subunit (hTERT), and other associated proteins. It is reported that Ku protein associated with hTERT physically to protect telomere ends and regulate the access of telomerase to telomere DNA ends (Chai et al., 2002). However, in telomerase negative cancer cells, how does the Ku80 protein works without the association of hTERT? Here, we have demonstrated that in the telomerase negative cancer cells U2OS, decreased Ku80 expression exhibit significant telomere shortening. Yet, the control group remained unchanged. It means that in the cancer cells lacking of telomerase, Ku80 is probably a positive factor in telomere maintenance. It is reported that following targeted Ku80 knockdown, the steady state levels of TRF2 protein which bind to chromatin are reduced. It implicates that Ku80 protein enhances TRF2 chromatin association. Our results also manifested that the expression of TRF2 protein were depressed by down-regulating the expression of Ku80 protein in U2OS cells. Therefore, it is supposed that Ku80 protein will maintain telomere length with TRF2 protein together. Nevertheless, the underlying molecular mechanisms of

telomere maintenance of Ku80 in telomerase negative cancer cells remain unknown and require further research.

In our study, the effect of silencing Ku80 gene on radiosensitivity was also investigated. D0 and SF2 values in U2OS cells transfected shRNA-Ku80 plasmid are significantly lower than the control group ($P<0.01$). This result suggested that Ku80 depletion enhanced the U2OS cells sensitivity to radiation. Ku80 contributes to genomic stability by binding to DNA ends. This binding represents a key, initiating step in the non-homologous end joining (NHEJ) pathway for DNA repair (Fink et al., 2010). The binding results in recruitment of the catalytic subunit of DNA-dependent protein kinase DNA-PKcs to form the holoenzyme DNA-PK, followed by the exonuclease Artemis and the XRCC4-like factor XLF (Silver et al., 2012). In addition to its role in DNA repair, multiple lines of evidence indicate that the Ku heterodimer functions at the telomere length (Boulton et al., 1998). A great number of studies manifested that cellular radiosensitivity is related to the cellar reproductive ability, so there should be some kind of intrinsic correlation between the telomere length and radiosensitivity (Barwell et al., 2007; Zongaro et al., 2008). Our previous research also revealed that radiosensitivity negatively correlated with telomere length, the longer telomere length, the lower radiosensitivity. (Zhong et al., 2008). It is believed that telomere is a key structure to determine the radiosensitivity (Zhou et al., 2010). So, Ku80 depletion may be radiation sensitizer which would become a radiotherapeutic strategy.

In conclusion, our result indicated that Ku80 depletion enhanced telomerase negative cancer cells U2OS radiation sensitivity by shortening the telomere length. So Ku80 depletion may be radiation sensitizer, which would become a potential therapeutic strategy. However, the underlying molecular mechanisms of this action of Ku80 remain unclear, we believe that this mechanism is worthy of further research

Acknowledgements

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