HELQ reverses the malignant phenotype of osteosarcoma cells via CHK1-RAD51 signaling pathway

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Abstract. HELQ is a DNA helicase important for repair of DNA lesions and has been linked to several types of cancer. However, little is known about its relationship with osteosarcoma (OS) and its mechanism. In the present study, the expression of HELQ and its downstream mediators in OS cells was assayed by quantitative PCR and western blot analysis. The function of HELQ in OS cells was investigated by Transwell invasion, wound healing, CCK8 assays and Comet assay. The results demonstrated that HELQ gene and protein were expressed in OS cells. OS cell invasion, migration, proliferation and DNA damage repair were enhanced by HELQ knock-down with shRNA-lentivirus and inhibited by HELQ overexpression with lentivirus transfection. Furthermore, the antitumor activities of HELQ may be associated with upregulated expression of the DNA damage-related proteins CHK1 and RAD51. Our findings indicated that HELQ confers an anti-invasive phenotype on OS cells by activating the CHK1-RAD51 signaling pathway and suggested that HELQ could be recognized as a promising therapeutic target for OS and other types of malignant tumors.

Introduction

Osteosarcoma (OS) represents a type of highly aggressive bone tumor prevalent in adolescents and is characterized by composite genetic defects (1-4). The failure in osteosarcoma therapy is mainly due to tumor recurrence or lung metastasis (5-7). Therefore, identification of new molecular targets and relative mechanism of metastasis is urgently needed for the effective management of OS.

In clinical specimens from different stages of human tumors, the early precursor lesions commonly express markers of an activated DNA damage response (8). DNA helicases have crucial roles in maintaining genome stability and stable DNA replication in all organisms (9). Mammalian HELQ is a 3'-5' DNA helicase with strand displacement activity (10). It opens up the parental strands at a blocked DNA replication fork and remodels nascent lagging strand intermediates to facilitate the loading of subsequent factors required for DNA damage processing or restart of DNA replication (11). Adelman *et al* uncovered a critical role for HELQ in germ cell maintenance and tumor suppression in mammals, which attributed to its role in replication-coupled DNA repair by interacting with RAD51 paralogue (12). Recent studies have identified single nucleotide polymorphisms at loci within or near HELQ that are associated with increased risks for several different cancers including upper aerodigestive tract cancers, ovarian cancers, and head and neck cancers (13). However, whether HELQ is involved in OS tumorigenesis and the molecular mechanisms of its tumorigenesis have yet to be defined.

Chk1 is the principal direct effector of the DNA damage and replication checkpoints (14). Inhibition of Chk1 promoted genomic DNA damage and decreased homologous recombination repair. Studies showed that defects in CHK1-RAD51 signaling result in defective homologous recombination and chromosome instabilities, and Rad51 inactivation induced aberrant replication dynamics, consequently leading to tumorigenesis (15,16). HELQ has a role in promoting CHK1 activation and HELQ colocalizes with Rad51 involved in the repair of damaged forks by homologous recombination (11). It was also reported that the *Caenorhabditis elegans ortholog helq-1* plays a role in meiotic DSB repair by promoting post-synaptic RAD-51 filament disassembly (17). All these findings suggested the association between HELQ, CHK1-RAD51 pathway in DNA repairing which may affect OS phenotype.

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In the present study, we tested this hypothesis and found that HELQ may be involved in OS cell malignant phenotype via activating the CHK1-RAD51 signaling pathway.

Materials and methods

Cell lines. The human osteosarcoma cell lines U2-OS and 143B were purchased from the cell bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The U2-OS cells were cultured in 1640 medium (Gibco) and 143B maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco), both supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 100 U/ml streptomycin. Cells were cultured at 37˚C in 5% CO_2.

RNA isolation and qPCR. Total RNA from U2-OS and 143B cells was extracted using TRIzol (Invitrogen) method. HELQ expression level was evaluated by quantificational real time-PCR, and GAPDH was used as the endogenous reference genes. All amplifications were performed in the final reaction mixture (20 μl). Primer sequences used were: HELQ forward primer 5'-GAAGGTGTCACTATTGAACCTGG-3', HELQ reverse primer 5'-GAGGATGACTTCCAATCCCTTTC-3'; GAPDH forward primer 5'-GGAGCGAGATCCCTCCAAAAT-3', GAPDH reverse primer 5'-GGCTGTTGTCATACTTCTCATGG-3'. The amplification reaction was performed using StepOne Real-Time PCR System for 40 cycles. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Lentivirus-Vector construction and cell transfection. The Lentivirus-Vectors were prepared by Yingqi Biotechnology Company (Wuhan, Hubei, China). The cells were transfected with Lentivirus-Vectors of upregulating HELQ (Lv-HELQ), Lentivirus-Vectors of downregulating HELQ (Lv-shHELQ), and negative Lentivirus-Vectors (NC), respectively.

Migration assays. In brief, cells were grown to confluence in 6-well tissue culture plastic dishes to a density of approximately 5x10^6 cells/well. The cells were wounded by drawing a line with a rubber policeman (Fisher Scientific, Hampton, NH, USA) through the center of the plate. Cultures were rinsed with PBS and replaced with fresh quiescent medium alone or containing 10% FBS, following which the cells were incubated at 37˚C for 24 h. Photographs were taken at 0 and 24 h, and the migrated distance was measured by ImageJ (NCBI). The cell migration rate was obtained by counting three fields per area and represented as the average of six independent experiments done over multiple days.

Transwell invasion assays. Invasion of U2-OS and 143B cells was measured using the BD BioCoat™ BD Matrigel™ Invasion Chamber (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. The medium in the lower chamber contained 5% fetal calf serum which acts as a source of chemoattractants (in the absence of FCS in the upper chamber). Cells were suspended in serum-free medium and added to the upper chambers at the same time. Cells that passed through the Matrigel-coated membrane were stained with Diff-Quik (Sysmex, Kobe, Japan) and photographed (x400). Photographs were taken at 24 h, and cell counting was measured by ImageJ (NCBI). The values for invasion were obtained by counting three fields per membrane and represented as the average of six independent experiments done over multiple days.

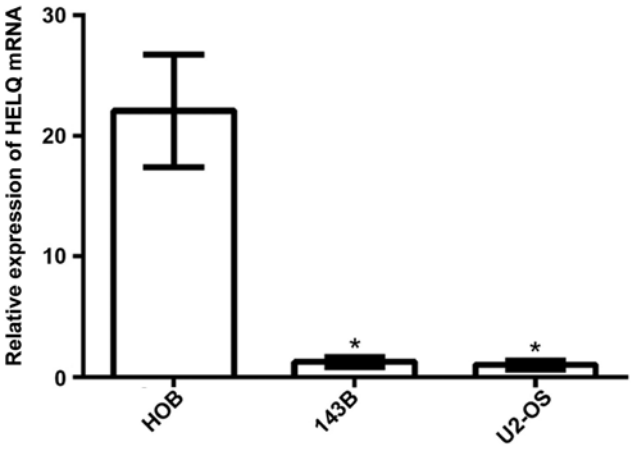

Figure 1. Quantitative PCR analysis showing the level of HELQ gene in U2-OS, 143B cells and HOB cells (n=6). *p<0.05 vs. HOB groups.

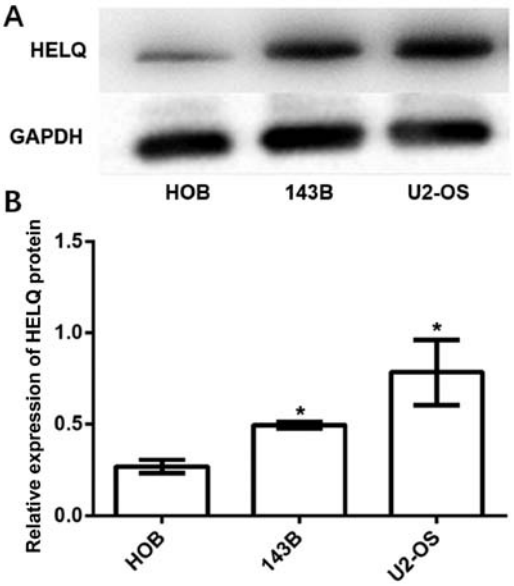

Figure 2. Western blot analysis showing the level of HELQ protein in U2-OS, 143B cells and HOB cells (n=6).

CCK8 assay. Cell viability was evaluated by a non-radioactive cell counting kit (CCK-8, TransGen) assay. U2-OS and 143B cells were seeded out in 96-well (4000/well) tissue culture plates and cultured for 24, 48 and 72 h, respectively. Then, 10 μl of CCK8 solution was added to each well, and the plates were incubated for an additional 2 h at 37˚C. Cell viability was measured as the absorbance at 450 nm with a microplate reader. The mean optical density (OD) values from triplicate wells for each treatment were used as the index of cell viability.

Comet assay. The level of DNA damage was evaluated by Comet assay. This assay was performed using a Comet

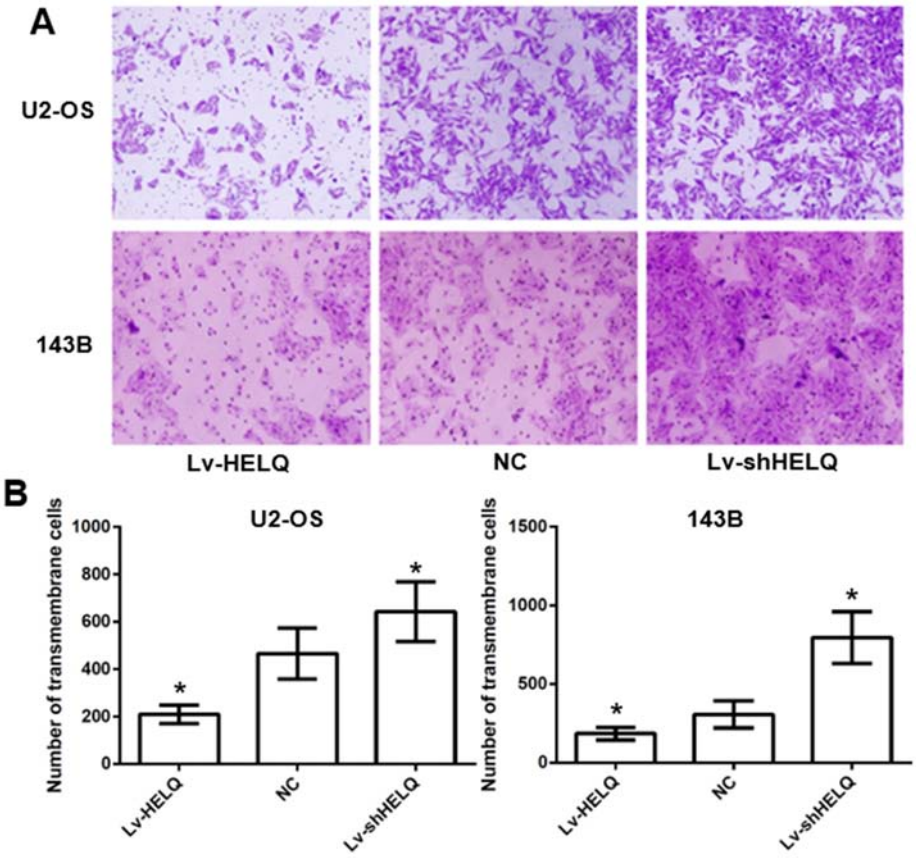

Figure 3. Transwell migration assay showing chemotactic activity of OS cells. (A) Representative images of OS cell overexpression or knock-down HELQ (x400). (B) Quantification of data in (A). (n=6). *p<0.05 vs. NC (negative control).

assay kit (Trevigen, Gaithersburg, MD, USA). All steps were processed according to the described procedures (18).

Western blot analysis. Total protein from the OS cells was extracted using RIPA lysis buffer containing 6 μg/ml PMSF. Protein concentration was determined by Bradford assay. Equal amounts of protein were electrophoresed by 8% SDS-PAGE and transferred onto a pure Nitrocellulose blotting membrane (0.22 ml). Membranes were blocked with 5% skim milk for 1 h at room temperature, then blocked with primary antibodies (mouse anti-HELQ IgG, 1:200, Santa Cruz Biotechnology, Inc., sc-81095, Shanghai, China; rabbit anti-CHK1 IgG, 1:1000, Abcam, ab47574, UK; rabbit anti-RAD51 IgG, 1:800; Abcam, ab63801, UK) overnight at 4˚C. Membranes were washed before incubated with appropriate peroxidase-conjugated secondary antibodies (anti-rabbit and anti-mouse, 1:2000). The immune complexes were detected with pro-light HRP kit (Tiangen Biotech Co., Ltd., Beijing, China). GAPDH (1:2000, Santa Cruz Biotechnology, Inc.) protein expression was used as a normalization control for protein loading. All experiments were repeated by six times over multiple days.

Statistical analysis. Data are expressed as mean ± SD. One-way ANOVA was used in multiple-sample analysis. A value of $p<0.05$ was considered to indicate a statistically significant difference. All analyses were performed with SPSS Version 13.0 (SPSS Inc, Chicago, IL, USA).

Results

Expression of HELQ mRNA is lower in OS cell lines than in osteoblast cell line. HELQ mRNA expression in OS cell lines (U2-OS and 143B cells) and osteoblast cell line (HOB cells) were detected by real-time PCR analysis. The results showed that expression of HELQ mRNA was significantly lower in U2-OS and 143B cells compared to HOB cells (Fig. 1).

Expression of HELQ protein is higher in OS cell lines than in osteoblast cell line. HELQ protein expression in OS cell lines (U2-OS and 143B cells) and osteoblast cell line (HOB cells) were detected by western blot analysis. The results showed that expression of HELQ protein was significantly higher in U2-OS and 143B cells compared to HOB cells (Fig. 2).

HELQ retrograde expression regulates U2-OS and 143B cell invasion ability in vitro. Transwell invasion assays were performed to measure the invasion of U2-OS and 143B cells. Fig. 3 showed decreased transmembrane cells (U2-OS: 209.2±38.6 cells/membrane; 143B: 185.3±40.4 cells/membrane) in HELQ-lentivirus-transfected cells and increased transmembrane cells (U2-OS: 642.3±125.6 cells/membrane; 143B: 795.7±164.4 cells/membrane) in shRNA-HELQ-lentivirus-transfected cells, compared with the negative control (U2-OS: 465.4±107.8 cells/membrane; 143B: 307.1±86.9 cells/membrane), indicating that HELQ could inhibit OS cells invasion *in vitro*.

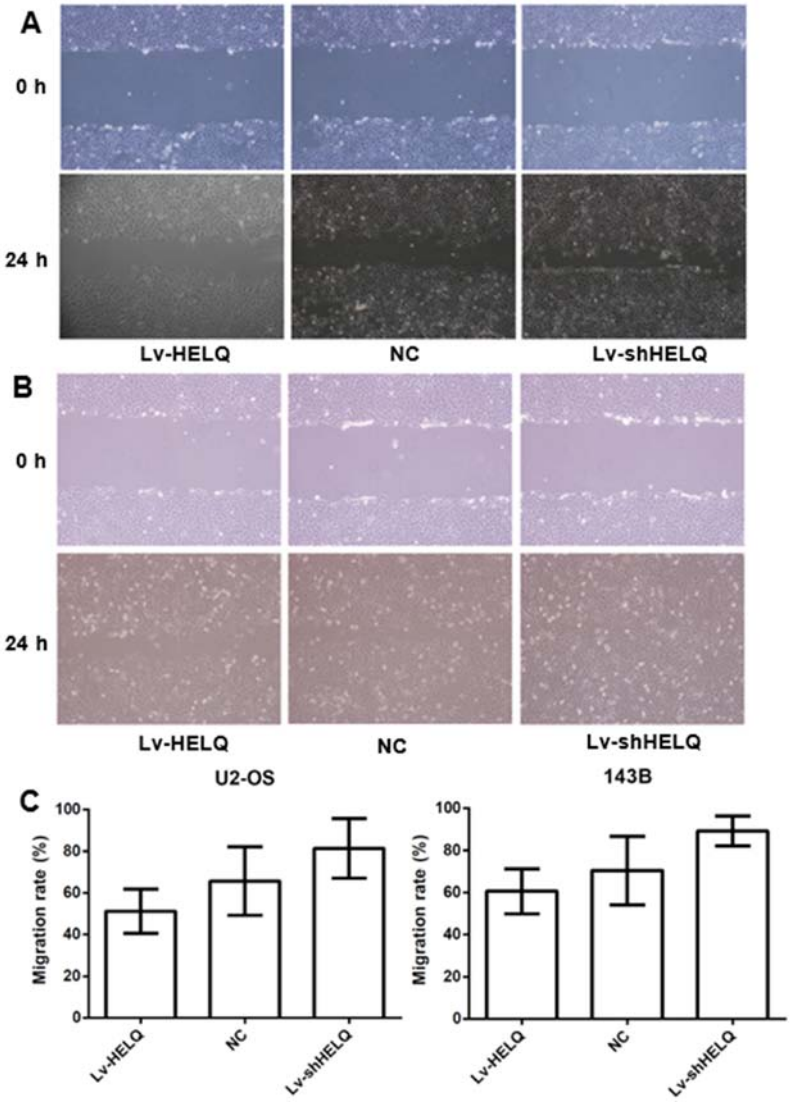

Figure 4. Scratch wound healing assay showing locomotion of OS cells. Representative images of U2-OS cells (A) or 143B cells (B) by wound healing assay were displayed (x400). (C) Quantification of data in (A and B). (n=6). $^{*}p<0.05$ vs. NC (negative control).

HELQ retrograde expression regulates U2-OS and 143B cell migration ability in vitro. Wound healing assay revealed the inhibited migration in HELQ-lentivirus-transfected cells (U2-OS: 51.2±10.6%, 143B: 60.6±10.7%) and enhanced migration in shRNA-HELQ-lentivirus-transfected cells (U2-OS: 81.4±14.3%, 143B: 89.2±7.1%), compared with the negative control (U2-OS: 65.7±16.4%, 143B: 70.5±16.3%) (Fig. 4). The results indicated that HELQ could inhibit OS cells migration.

HELQ retrograde expression regulates U2-OS and 143B cell proliferation. The CCK8 assay was used to assessed the roles of HELQ in OS cell proliferation. Downregulating HELQ expression by shRNA-lentivirus enhanced the proliferation abilities of U2-OS and 143B cells. Moreover, overexpression of HELQ with transfection of HELQ-lentivirus significantly suppressed the proliferation of U2-OS and 143B cells (Fig. 5). The results suggested that activation of HELQ signaling inhibits OS cell proliferation.

HELQ is critical to DNA repair. The comet assay, also known as the single cell gel electrophoresis assay, is a very sensitive and rapid quantitative technique used to detect DNA damage at the individual cell level (19,20). As illustrated in Fig. 6, the DNA damage levels, measured by %DNA in tail, were higher in the shRNA-HELQ-lentivirus group (U2-OS: 86.1632±11.3856%, 143B: 83.5961±12.7842%) but lower in the HELQ-lentivirus group (U2-OS: 45.8098±15.7096%, 143B: 54.1610±14.4469%) as compared to negative control (U2-OS: U2-OS: 64.4241±16.3866%, 143B: 71.0989±17.4967%). The results demonstrated that HELQ promoted DNA repair after damage in OS cells.

HELQ regulates the CHK1-RAD51 signaling pathway. To investigate the effect of HELQ on CHK1-RAD51 signaling pathway, the expression levels of HELQ, CHK1 and RAD51 proteins were measured with western blot analysis. Results indicate that HELQ, CHK1 and RAD51 protein expression levels were higher in cells transfected with HELQ-lentivirus and lower in cells transfected with shRNA-HELQ-lentivirus, compare to the negative control (Fig. 7). These observations demonstrate that altered HELQ expression could impact CHK1 and RAD51 protein expression in U2-OS and 143B

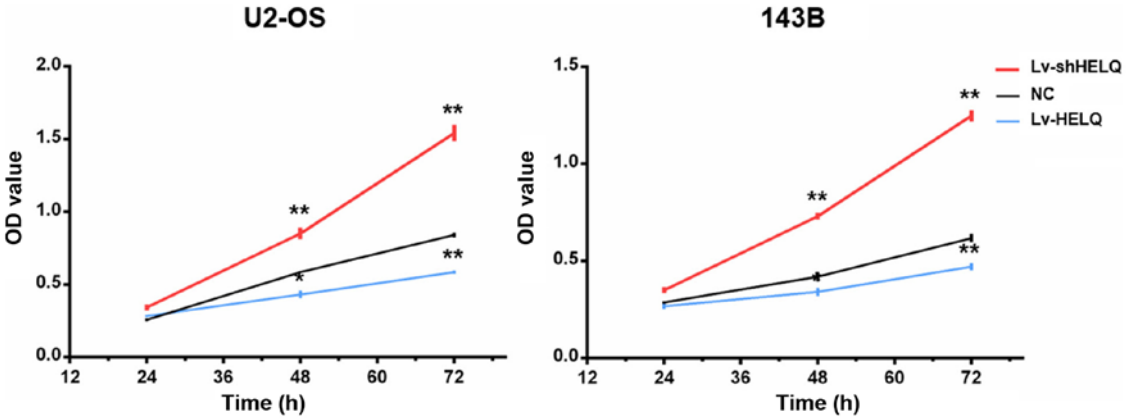

Figure 5. CCK8 assay evaluating the proliferation of U2-OS and 143B cells after transfection with shRNA-HELQ-lentivirus, HELQ-lentivirus or negative control (n=6).

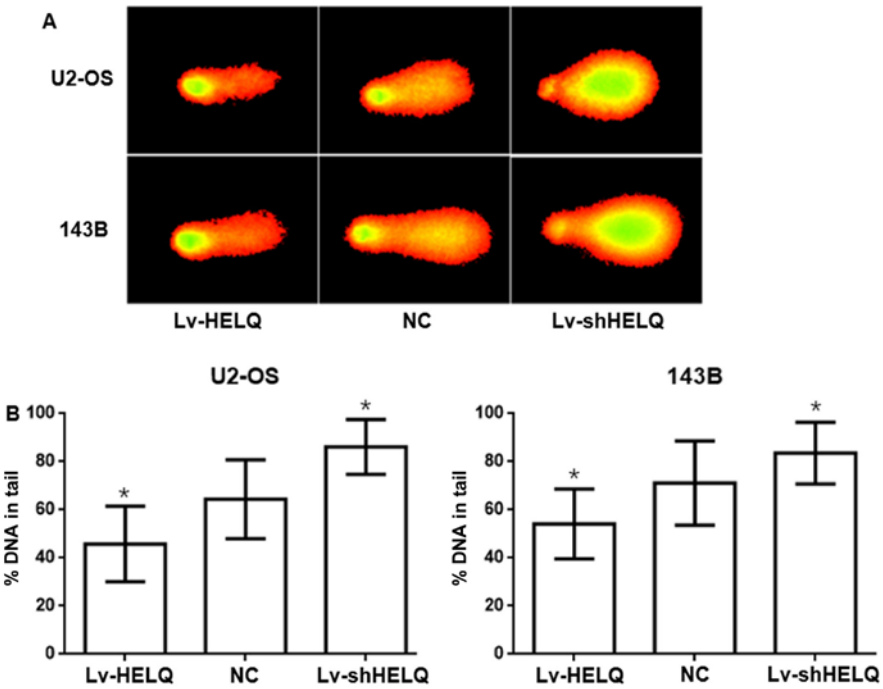

Figure 6. Comet assay detecting DNA damage levels in U2-OS and 143B cells. Representative images of n=6 individual samples per group are shown.

cells, suggesting that HELQ may regulate the CHK1-RAD51 pathway.

Discussion

HELQ family members contribute to the repair of replication-blocking lesions such as DNA interstrand cross-links (11). HELQ is a superfamily II DNA helicase, conserved from archaea through to humans (21,22). Studies have established that human HELQ is an ATP-dependent enzyme that unwinds DNA with a 3' to 5' polarity (23,24). HELQ helicase-deficient mice exhibit subfertility, germ cell attrition, ICL sensitivity and tumor predisposition (12). Previous study reported that single nucleotide polymorphisms at loci within or near HELQ that are associated with increased risks for several different cancers including upper aerodigestive tract cancers, ovarian cancers, head and neck cancers (25-28). In the present study, our results showed that the migration, invasion and proliferation were significantly suppressed by activation of HELQ signaling in OS cells, indicating that modulating HELQ could effectively reverse the malignant phenotype of OS cells.

Modulation of CHK1 and RAD51 is also tightly regulated in DNA repair and HELQ function. HELQ can promote CHK1 activation, CHK1 phosphorylation was significantly reduced in HELQ$^{-/-}$ cells (10). The Chk1 protein kinase is activated in response to damaged DNA and stalled replication forks and acts as a central effector of the DNA damage and replication checkpoint responses in vertebrate cells (29). Supernumerary centrosomes in human lymphoblastoid cells exposed to ionizing radiation were eradicated by treatment with 2 mM caffeine or by the depletion of Chk1, suggesting that Chk1 may be involved in promoting centrosome amplification induced by DNA damage (30). CHK1 physically interacts with Rad51 to regulate homologous recombination

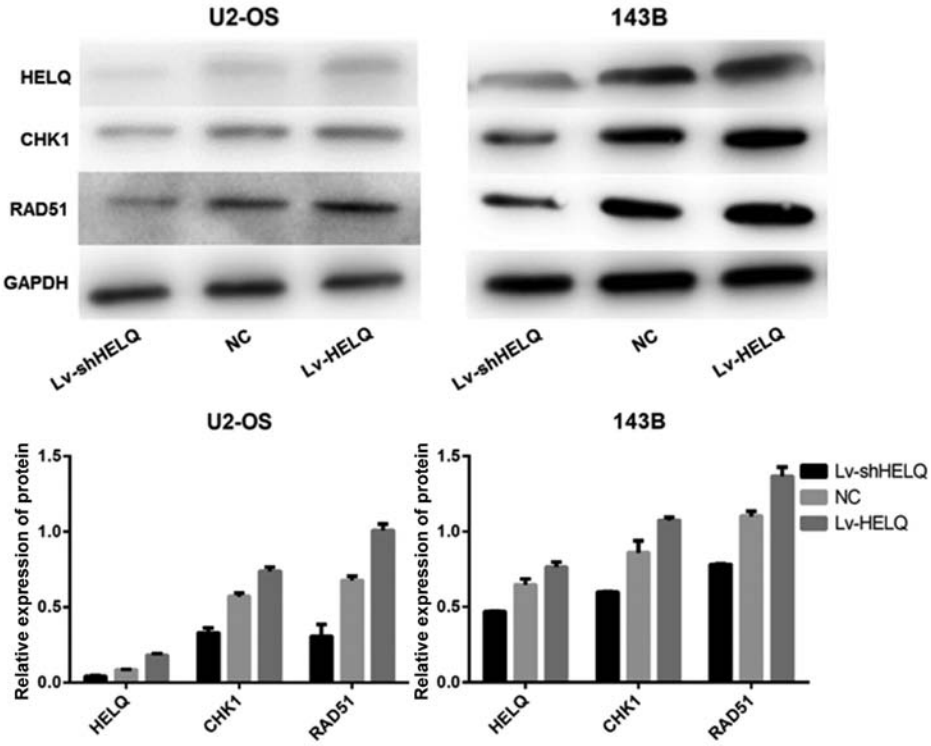

Figure 7. Western blot analysis showing expression of HELQ, CHK1 and RAD51 protein in U2-OS and 143B cells. (n=6).

and defects in Chk1-Rad51 signaling result in HR defective and chromosome instabilities, consequently leading to tumorigenesis (14). Parplys *et al* have described a role for RAD51 in driving genomic instability caused by impaired replication and intra-S-phase mediated CHK1 signaling by studying an inducible RAD51 overexpression model as well as ten breast cancer cell lines (31). Our results revealed that HELQ is involved in the DNA damage of osteosarcoma via the CHK1-RAD51 signaling pathway.

In conclusion, our present study demonstrates a role of HELQ signaling in repairing DNA damage and modulating OS cell phenotype via targeting the CHK1-RAD51 pathway after tumorigenesis. Targeting HELQ and CHK1-RAD51 pathway may be a potential strategy for treating OS metastases. Further research is required to identify the detailed roles of HELQ in osteosarcoma.

Acknowledgements

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