Ubiquitin-like with plant homeodomain (PHD) and RING-finger domain 1 (UHRF1) maintains methylation patterns following DNA replication and is expressed at high levels in various types of human cancer. UHRF1 has been identified as a novel oncogene involved in the pathogenesis of hepatocellular carcinoma. Previous studies have demonstrated that inhibition of the expression of UHRF1 suppresses the proliferation of cancer cells. However, the role of UHRF1 in human osteosarcoma has not been investigated. The present study examined the expression levels of UHRF1 and retinoblastoma 1 (Rb1) in human osteosarcoma cell lines by western blot analysis. Stable overexpression of UHRF1 or knockdown of Rb1 was achieved by lentiviral transfection. Subsequently, a Cell Counting Kit-8 assay and a cell invasion assay were performed to detect the biological functions of UHRF1 in vitro. The results of the present study demonstrated that UHRF1 promoted the proliferation of human osteosarcoma cells. The present study also reported that UHRF1 was able to enhance the invasion of osteosarcoma cells in Rb1-positive osteosarcoma cells, but not in Saos-2 cells with homozygous loss of Rb1. Similarly, knockdown of Rb1 in Rb1-positive osteosarcoma cells enhanced levels of invasion and eliminated the regulation of invasion by UHRF1. UHRF1 was found to inhibit the mRNA and protein expression levels of Rb1. Furthermore, deletion of Rb1 was found to suppress the expression of E-cadherin and promote epithelial-to-mesenchymal transition (EMT). In addition, the overexpression of UHRF1 inhibited the expression of E-cadherin and promoted EMT via the suppression of Rb1. These data demonstrated that UHRF1 promotes osteosarcoma cell invasion by downregulating the expression of E-cadherin and increasing EMT in an Rb1-dependent manner.

Introduction

Osteosarcoma is the most frequent type of primary bone malignant tumor in children (1). It is considered to arise from malignant mesenchymal cells, which produce osteoid or immature bone (2). Although advanced treatment for osteosarcoma consists of aggressive adjuvant chemotherapy, the five-year survival rate of patients with high-grade osteosarcoma remains <50% (3).

Ubiquitin-like with plant homeodomain (PHD) and RING-finger domain 1 (UHRF1) is a multi-domain protein, which was initially identified as a nuclear protein associated with cell proliferation (4). As a human inverted CCAAT box-binding protein, UHRF1 is involved in regulating the expression of topoisomerase IIα in proliferating cells (5). UHRF1 has been found to inhibit the mRNA and protein expression levels of Rb1 (6,7). Deletion of Rb1 was found to suppress the expression of E-cadherin and promote epithelial-to-mesenchymal transition (EMT) (8). Furthermore, UHRF1 is involved in the methylation of newly synthesized CpG sequences during DNA replication (9,10). UHRF1 binds preferentially to dimethylated and trimethylated histone 3 lysine 9 peptides, and this binding is required for the maintenance of DNA methylation (11). Through interaction with other nuclear proteins, including Tip60, histone deacetylase and G9A, UHRF1 may function as a bridge between DNA methylation and the histone methylation (12). Furthermore, significant overexpression of UHRF1 has been observed in several types of human tumor, including breast (13,14), bladder (15), prostate (16) and lung cancer (17). The overexpression of UHRF1 has also been reported to reduce the radiosensitivity of human breast cancer cells and HeLa cells to γ-irradiation (18). Previous studies have demonstrated that UHRF1 is an oncogene in hepatocellular carcinoma (19,20).
Furthermore, the overexpression of UHRF1 destabilizes and delocalizes DNA (cytosine-5')-methyltransferase 1 (DNMT1) and causes DNA hypomethylation in cancer cells (19). Despite these findings, little is known regarding the function of UHRF1 in human osteosarcoma cells. In the present study, the effects of UHRF1 on the invasion of osteosarcoma cells were examined, and the associated underlying mechanisms were investigated.

Materials and methods

Cell culture. MG-63, Saos-2, U2OS and HOS human osteosarcoma cells lines were obtained from American Type Culture Collection (Manassas, VA, USA). HEK293T cells were obtained from the Institute of Cell and Biochemistry Research of the Chinese Academy of Science (Shanghai, China). The cell lines were cultured with Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified atmosphere containing 5% CO₂ at 37°C.

Reagents. The mouse anti-human monoclonal antibodies targeting UHRF1 (cat no. ab57083; 1:2,000 dilution) and GAPDH (cat no. ab9484; 1:5,000 dilution) were purchased from Abcam (Cambridge, MA, USA). Human Rb1-specific rabbit polyclonal antibody (cat no. 9313; 1:2,000 dilution) was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The mouse anti-human E-cadherin monoclonal antibody (cat no. sc-21791; 1:2,000 dilution) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). The plentifullenti-UHRF1 lentiviral expression vector and the control vector were purchased from OriGene Technologies, Inc. (Beijing, China). PLKO.1 lentiviral vectors containing short hairpin (sh)RNA inserts against Rb1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The target sequences were as follows: shRb-A, 5'-GCCTTTGATTCGTTCCTTCTT-3' and shRb-B, 5'-TGGAAATGTTGCCCGAGAA-3'.

Transfection and lentiviral transduction. Transfection of the cells was performed using FuGENE transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany). The cell lysates were then centrifuged at 11,500 x g for 10 min at 4°C. Protein concentrations were estimated using the Quick Start™ Bradford Protein Assay (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The recombinant lentivirus was subsequently harvested, filtered through Millipore Millex-HV 0.45 µM polyvinylidene difluoride filters (Millipore, Billerica, MA, USA) and transduced into the target cells (MG-63, Saos-2 and U2OS cells at 60% confluence) with 8 µg/ml polybrene (Sigma-Aldrich). After 48 h of incubation, the cells were selected with fresh puromycin-containing media (2.0 µg/ml; Sigma-Aldrich). Following puromycin selection for 48 h, the expression levels of UHRF1 and Rb1 were quantified using western blot analysis.

Cell Counting kit-8 (CCK-8) assay. Cell proliferation was determined using a CCK-8 assay (Dojindo, Kumamoto, Japan). Briefly, the cells were plated in 96-well plates at 2,500 cells/well and cultured in DMEM. Every 24 h, 10 µl CCK-8 was added to each well containing 100 µl DMEM once. The plates were then incubated for a further 2 h at 37°C. The cell growth was monitored every 24 h for 7 days using the CCK-8 assay. Absorbance was measured at 450 nm using a microplate reader (Infinite Pro 2000; Tecan GmbH, Grödig, Austria).

Cell invasion assay. To assess the role of UHRF1 in cell invasion, a total of 1 x 10⁵ cells were suspended in 100 µl DMEM supplemented with 10% FBS, and were seeded into the upper compartment of Matrigel-coated (BD Biosciences, Franklin Lakes, NJ, USA) Transwell chambers (24-well; 8 µm; Merck Millipore, Darmstadt, Germany). The cells were incubated for 24 h at 37°C in a 5% CO₂ chamber. The cells, which did not invade through the pores were removed using a cotton swab. The cells on the lower surface of the membrane were stained with a hematoxylin and eosin staining kit (Baixu of Biotechnology, Shanghai, China) and counted with a microscope (Leica DM 5000 B; Leica Microsystems, Wetzlar, Germany). Results are presented as the average number of cells in five randomly selected fields.

Western blot analysis. The cells were suspended in lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China) containing a mixture of protease and phosphatase inhibitors (both from Roche Diagnostics GmbH, Mannheim, Germany). The cell lysates were then centrifuged at 11,500 x g for 10 min at 4°C. Protein concentrations were estimated using the Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The samples were separated by SDS-PAGE (4-29% gradient; Bio-Rad Laboratories, Inc.) and then blotted onto a polyvinylidene difluoride membrane (Merck Millipore). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with 1% Tween 20 (TBST; Sigma-Aldrich) for 1 h at room temperature prior to incubation with specific primary antibodies overnight at 4°C. Following three washes with TBST, the membrane was incubated with peroxidase-conjugated secondary antibodies [anti-mouse immunoglobulin (Ig)G (cat no. 7076; 1:5,000 dilution) and anti-rabbit IgG (cat no. 7074; both from Cell Signaling Technology, Inc.)] for 30 min at 37°C and then washed three times with TBST. The bound antibodies were detected using chemiluminescent horseradish peroxidase substrate (Merck Millipore) and images were captured using an LAS-4000 digital imaging system (GE Healthcare Life Sciences, Little Chalfont, UK).

Statistical analysis. Values are expressed as the mean ± standard deviation. GraphPad 6.01 Prism software (GraphPad, Inc., La Jolla, CA, USA) was used for statistical analyses. Each experiment was repeated three times. P < 0.05 was considered to indicate a statistically significant difference.

Results

UHRF1 promotes the proliferation of human osteosarcoma cells. UHRF1 has been reported to be overexpressed in various types of cancer (13). The present study examined the expression levels of UHRF1 in four human osteosarcoma cell lines. As shown in Fig. 1A, the expression of UHRF1 was detected in all of the cell lines. Overexpression of UHRF1 can increase cell proliferation (14,21), therefore, the effects of UHRF1 on the proliferation of human osteosarcoma cells were also
investigated. UHRF1 was stably overexpressed in the MG-63 and Saos-2 osteosarcoma cell lines (Fig. 1B). As expected, overexpression of UHRF1 increased the proliferation rate of the MG-63 and Saos-2 osteosarcoma cell lines (Fig. 1C).

**UHRF1 promotes the invasion of MG-63 and U2OS human osteosarcoma cells, but not Saos-2 cells.** In order to investigate the function of UHRF1 on the invasion of osteosarcoma cells, Transwell invasion assays were used with three human osteosarcoma cell lines **in vitro**. The overexpression of UHRF1 significantly increased the invasion of MG-63 and U2OS human osteosarcoma cells (Fig. 2). By contrast, the overexpression of UHRF1 had no significant effect on the invasion of the Saos-2 cells (Fig. 2). Homozygous deletion of the Rb1 gene has been
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UHRF1 promotes the invasion of human osteosarcoma cells in an Rb1-dependent manner. To test the hypothesis that Rb1 may be involved in the regulation of the invasion of osteosarcoma cells by UHRF1, the expression levels of Rb1 were quantified in the osteosarcoma cell lines. As expected, the MG-63 and U2OS cells exhibited normal expression levels of Rb1. The expression of Rb1 in the Saos-2 cells containing the homozygous deletion of Rb1 was not detected (Fig. 3A). To further investigate the mechanism underlying the effect of UHRF1 on the regulation of osteosarcoma cell invasion, the expression of Rb1 was stably knocked down in the MG-63 cells (Fig. 3B). The stable knockdown of Rb1 resulted in UHRF1 being overexpressed in the MG-63 cells (Fig. 3C). Following the knockdown of Rb1 in the Rb1-positive MG-63 cells, the invasion of the cells increased. By contrast, the induced overexpression of UHRF1 had no effect on the invasion of the MG-63 cells following stable Rb1 knockdown (Fig. 3D). These results indicated that Rb1 appeared to be responsible for the regulation of cell invasion by UHRF1.

UHRF1 suppresses the expression of E-cadherin and promotes epithelial-mesenchymal transition (EMT) through the inhibition of Rb1. UHRF1 can inhibit the expression levels of Rb1 at the protein and mRNA levels (6). Furthermore, the inhibition of Rb1 suppresses the expression of E-cadherin and increases EMT (8). The present study hypothesized that UHRF1 may inhibit the expression of Rb1 and thereby suppress the expression...
of E-cadherin. E-cadherin is known to suppress the invasion of cancer cells (25). Therefore, the UHRF1-mediated promotion of invasion may be the result of E-cadherin inhibition. The results of the present study demonstrated that the expression levels of E-cadherin were significantly lower in the Saos-2 cells, compared with the other Rb1-positive cells (Fig. 4A). Furthermore, overexpression of UHRF1 in the MG-63 and U2OS cells inhibited the expression of Rb1 and E-cadherin (Fig. 4B). The loss of E-cadherin is the initial or primary cause for EMT (26). In the present study, the UHRF1-overexpressing MG-63 cells exhibited marked changes in cell morphology, with transformation of the cobblestone-like epithelial cells to an elongated fibroblast-like morphology, and with pronounced cellular scattering that indicated EMT (Fig. 5). Similarly, knockdown of Rb1 in the MG-63 cells also showed EMT characteristic patterns (Fig. 5). However, UHRF1 had no effect on the cell morphology of the Saos-2 cells with the Rb1 deletion (Fig. 5). These results further supported the hypothesis that UHRF1 inhibits the expression of E-cadherin and promotes EMT through the suppression of Rb1.

Discussion

Osteosarcoma arises from malignant mesenchymal cells, which produce osteoid or immature bone, and is the most frequent type of primary bone malignant tumor (27). Despite advanced treatment for osteosarcoma, which combines chemotherapy, surgery and occasionally radiotherapy, the five-year survival rate for patients with high-grade osteosarcoma remains <50% (3). Therefore, novel targeted molecular therapeutic techniques for osteosarcoma are required.

UHRF1, also known as inverted CCAAT box-binding protein of 90 kDa, contains different domains, including an E3-ligase RING domain, a SET and RING-associated (SRA) domain, a PHD finger domain and a tandem tudor domain (28). The SRA domain of UHRF1 specifically binds to hemi-methylated CpG following DNA replication, and recruits DNMT1 to methylate the newly synthesized DNA strand (29). UHRF1 is overexpressed and associated with tumor stages, and predicts poor prognosis in various types of cancer (15,21). UHRF1 has previously been identified as an oncogene in hepatocellular carcinoma (19,20). Furthermore, the overexpression of UHRF1 destabilizes and delocalizes DNMT1, and causes DNA hypomethylation in cancer cells (19). Although several studies have demonstrated the function of UHRF1 in tumorigenesis and tumor progression (14,17,20), little is known regarding the function of UHRF1 in human osteosarcoma cells. In the present study, the expression of UHRF1 was detected in all of the human osteosarcoma cell lines examined. Furthermore, the overexpression of UHRF1 increased the proliferation rates of the osteosarcoma cell lines.

EMT, during which epithelial cells are transdifferentiated to a mesenchymal state, is considered to be important in the initiation of the invasion and metastasis of cancer cells (30,31). Loss of E-cadherin is considered to be the most fundamental event during EMT (32). Deregulation in the expression of several genes or microRNAs has been reported to downregulate the expression of E-cadherin (33,34). As an oncogene, UHRF1 is able to bind to methylated DNA and recruit transcriptional repressors to suppress the transcription of several tumor suppressor genes (35,36). Therefore, UHRF1 may regulate the transcription of E-cadherin. The deletion of Rb1 is associated with downregulation of the expression of E-cadherin and increased EMT (8). UHRF1 binds to the Rb1 gene promoter and inhibits the expression of Rb1 (6). The results of the present study demonstrated that UHRF1 promoted the invasion of osteosarcoma cells of the MG-63 and U2OS cell lines, but not of the Saos-2 cell line. Saos-2 cells undergo homozygous deletion of the Rb1 gene (22,23), therefore the present study hypothesized that Rb1 may be involved in the regulation of the invasion of osteosarcoma cells by UHRF1. Further investigations demonstrated that knockdown of the expression of Rb1 in the Rb1-positive cells eliminated the regulation of invasion by UHRF1. Furthermore, the expression levels of E-cadherin were consistent with the Rb1 status. The results of the present study also demonstrated that the overexpression of UHRF1 significantly downregulated the expression levels of Rb1 and E-cadherin in the Rb1-positive cells. Similarly, overexpression of UHRF1 in the MG-63 cells

Figure 5. UHRF1 promotes epithelial-mesenchymal transition. Top row, morphological changes induced by overexpression of UHRF1 or knockdown of retinoblastoma 1 in MG-63 cells; lower row, morphological changes induced by overexpression of UHRF1 in Saos-2 cells. Magnification, x100. UHRF1, ubiquitin-like with plant homeodomain and RING-finger domain 1; sh, short hairpin RNA.
resulted in marked changes in cell morphology, indicating EMT, which promotes human osteosarcoma cell invasion by downregulating the expression of E-cadherin and increasing MT, in an Rb1-dependent manner.

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References


