

# Ubiquilin 2 enhances osteosarcoma progression through resistance to hypoxic stress

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**Abstract.** Ubiquilin 2 (UBQLN2), a member of the ubiquitin-like protein family (ubiquilins), maintains protein homeostasis. Although UBQLN2 has been implicated in the pathogenesis of neurodegenerative diseases, it is also associated with malignant tumors. Therefore, we examined whether UBQLN2 plays a role in human osteosarcoma. The human osteosarcoma cell line MG63 was transfected with UBQLN2 siRNA and cultured under hypoxic conditions. The rat osteosarcoma cell line COS1NR was inoculated into Fischer 344 rats, followed by injection of UBQLN2 siRNA with atelocollagen. An immunohistochemical analysis of UBQLN2 was performed using 34 cases of human high-grade osteosarcomas, and metastasis-free survival was estimated by the Kaplan-Meier method. Silencing of UBQLN2 by siRNA transfection under hypoxia led to activation of JNK and p38, resulting in induction of apoptosis in the osteosarcoma cell line MG63. Injection of UBQLN2 siRNA suppressed tumor growth in the rat osteosarcoma model, followed by apoptosis induction. The immunohistochemical examination revealed that high UBQLN2 expression was significantly associated with the unfavorable metastasis-free survival of osteosarcoma patients. UBQLN2 plays an important role in resistance to hypoxic stress and enhances tumor progression in osteosarcoma. UBQLN2 may be a new molecular target for chemotherapeutics and a useful clinicopathological marker in human osteosarcoma.

## Introduction

Osteosarcoma is the most common malignant bone tumor that predominantly occurs in childhood or adolescence. Current therapies include surgical tumor resection and multi-agent chemotherapy. The introduction of (neo)-adjuvant chemo-

therapy has increased the 5-year survival rate for localized disease to more than 50% compared to surgery alone. However, patients with metastases at the initial diagnosis or poor response to chemotherapy have a worse prognosis (1,2), and critical non-surgical treatments have not yet been established. The development of new therapeutic drugs with fewer side-effects is strongly expected. Recently, various biological markers in osteosarcoma have been reported and several therapeutic drugs targeting these markers have been established, such as 17-N-allylamino-17-demethoxygeldanamycin (17-AAG) (Hsp90 inhibitor) (3), trastuzumab (anti-Her2 antibody) (4), cixutumumab (IMC-A12) (anti-IGF-1R antibody) (5) and temsirolimus (mTOR inhibitor) (6). Yet, their clinical effects were found to be limited at the clinical investigation stage.

Ubiquilin (UBQLN), a member of the ubiquitin-like protein family (ubiquilins), delivers ubiquitinated proteins to the proteasome for degradation and maintains protein homeostasis. Recent evidence has shown that mutations of UBQLN1 or UBQLN2, as scaffold proteins that allow abnormally modified proteins to pass from the endoplasmic reticulum (ER) to the proteasome, are closely associated with the pathogenesis of neurodegenerative disorders such as Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (7,8). Mutations of the UBQLN2 gene suppress ubiquitin-mediated proteasomal degradation, leading to the accumulation of inclusions composed of misfolded proteins linked to the onset of ALS (8). Moreover, higher UBQLN mRNA levels are associated with shorter survival of lung cancer patients and UBQLN is an important factor in tumor progression (9).

In the present study, we aimed to clarify the roles of UBQLN2 in osteosarcoma by *in vitro* and *in vivo* experiments. UBQLN2 overexpression was found to be a poor prognostic factor for metastasis-free survival. In addition, UBQLN2 silencing through siRNA transfection effectively induced cellular apoptosis and growth suppression of osteosarcoma cells both *in vitro* and *in vivo* under hypoxic conditions. We found that osteosarcoma progression was enhanced by UBQLN2 through hypoxic stress tolerance.

## Materials and methods

**Cell culture.** The human osteosarcoma cell line MG63 was obtained from the American Type Culture Collection (ATCC;

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Rockville, MD, USA). The rat osteosarcoma cell line COS1NR was established from a chemically induced osteosarcoma in a Fischer 344 rat by 4-hydroxy quinolone 1-oxide in our laboratory (10,11). MG63 and COS1NR cells were maintained in Dulbecco's minimum essential medium (Nacalai Tesque, Kyoto, Japan) with 10% fetal bovine serum (Nichirei, Tokyo, Japan) and 50 U/ml penicillin/streptomycin (Nacalai Tesque) under 5% CO<sub>2</sub> at 37°C.

For the hypoxia experiments, the cells were cultured and treated in Forma™ Series II 3130 Water-Jacketed CO<sub>2</sub> Incubators (Thermo Fisher Scientific, Rockford, IL, USA) under 1% O<sub>2</sub>, 5% CO<sub>2</sub> and 94% N<sub>2</sub>.

**Antibodies and chemicals.** Antibodies against phosphorylated (p)-p38 and p-c-Jun NH<sub>2</sub>-terminal kinase (JNK) were supplied by Cell Signaling (Boston, MA, USA). An antibody against UBQLN2 was obtained from Abnova Corporation (Taipei, Taiwan). Antibodies against p38, JNK and actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). An antibody against hypoxia-inducible factor (HIF)-1 $\alpha$  was obtained from Novus Biologicals (Littleton, MA, USA). An antibody against Ki-67 was obtained from Thermo Fisher Scientific. An antibody against vascular endothelial growth factor (VEGF) was obtained from Bioss Antibodies (Woburn, MA, USA). The JNK inhibitor SP600125 and p38 inhibitor SB203580 were obtained from Calbiochem (San Diego, CA, USA).

**Preparation of cell lysates and western blot analysis.** MG63 cells were washed with phosphate-buffered saline (PBS) and suspended in lysis buffer (40 mmol/l HEPES pH 7.4, 10% glycerol, 1% Triton X-100, 0.5% NP-40, 150 mmol/l NaCl, 50 mmol/l NaF, 20 mmol/l  $\beta$ -glycerophosphate, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride and 0.1 mmol/l vanadate) containing a protease inhibitor mixture (aprotinin, leupeptin and pepstatin). Cell lysates were cleared by centrifugation at 15,000 rpm for 30 min. Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific). MG63 cell lysates were resolved in SDS-polyacrylamide gels (Wako, Osaka, Japan) and transferred onto polyvinylidene difluoride membranes (Millipore, Temecula, CA, USA), followed by blocking with 5% skimmed milk at room temperature for 1 h. The membranes were then incubated with the individual primary antibodies described above overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology Inc.). We detected the resulting peroxidase activity on X-ray film (Amersham Hyperfilm MP; GE Healthcare, Amersham, UK) using an enhanced chemiluminescence detection system (Western Lightning; Perkin Elmer, Waltham, MA, USA).

**Small interfering RNA (siRNA) transfection for UBQLN2.** For transfections, 8 $\times$ 10<sup>4</sup> MG63 cells/well were seeded in 6-well dishes and transfected with 165 nmol/l of siRNA against UBQLN2 (Qiagen, Venlo, The Netherlands). Transfections were carried out using Lipofectamine RNAiMAX (Life Technologies, Foster City, CA, USA) in accordance with the manufacturer's protocol. The UBQLN2 siRNA sequence was designed after selection of appropriate DNA target sequences

and was as follows: 5'-TCCCATAAAGAGACCCTAATA-3'. The UBQLN2 siRNA sequence for the rat experiments was also designed after selection of appropriate DNA target sequences and was as follows: 5'-AACCATCGCGGCCATGTCAAA-3'.

**Preparation of total RNA and RT-PCR.** Total RNA was extracted using an RNeasy Mini kit (Qiagen). Template cDNA was synthesized from 1  $\mu$ g of total RNA using a PrimeScript RT reagent kit (Perfect Real-Time), and RT-PCR was performed using SYBR Premix Ex Taq II (Tli RNaseH Plus) (both from Takara, Shiga, Japan). The PCR conditions were 95°C for 30 sec followed by 55–63°C for 30 sec, for a total of 35–45 cycles. The amount of actin mRNA (sense, 5'-ATGGGTC AGAAGGATTCCTATGT-3' and antisense, 5'-GAAGGCTC AAACATGATCTGGG-3') was used to standardize the quantity of UBQLN2 mRNA (sense, 5'-GCTGAATGAACTGCTG GTTGGG-3' and antisense, 5'-CATAGGACCCACTGGCCC TG-3').

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) assay.** DNA cleavage, a characteristic of apoptosis, was detected using the TUNEL assay (Apop Tag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Millipore). After siRNA transfection, the cells were washed with PBS and fixed with CytoRich Red (Becton-Dickinson, Franklin Lakes, NJ, USA) at room temperature for 30 min. The fixed cells were washed with distilled water, deposited on a slide, further fixed in 95% ethanol and stained with the above kit. At least 600 cells from three different fields were examined in each experiment, and cell death was expressed as the percentage of TUNEL-positive cells.

Formalin-fixed and paraffin-embedded 5- $\mu$ m-thick sections of all rat tumor samples were deparaffinized in xylene, dehydrated in a graded ethanol series and subsequently rinsed with distilled water. Antigen retrieval was performed with proteinase K (Dako, Glostrup, Denmark). Identification of apoptotic cells by TUNEL staining was performed using the same kit. The apoptotic index (per microscopic field at x400 magnification) was calculated as follows: Number of apoptotic cells  $\times$  100/total number of cells.

**Cell proliferation assay.** Cells were stimulated with various reagents for a specified period, followed by addition of MTS reagent, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulphonyl)-2H-tetrazolium, inner salt (Promega, Tokyo, Japan). After a 2-h incubation period, the optical absorbance at 490 nm was measured using a microplate reader (Multiskan FC; Thermo Fisher Scientific). Cell viability was expressed as the mean ratio  $\pm$  SD of the absorbance values after control RNA and UBQLN2 siRNA transfections. All experiments were performed in triplicate.

**In vivo UBQLN2 treatment in syngeneic rats.** The animal experiments were approved by The Institutional Animal Care and Use Committee at Nara Medical University. COS1NR cells (5 $\times$ 10<sup>6</sup>) in 100  $\mu$ l of PBS were inoculated into subcutaneous tissue in the back of 7-week-old male Fischer 344 rats. When each tumor diameter reached 2 mm, we started injections of 20  $\mu$ mol/l of control RNA or 20  $\mu$ mol/l of UBQLN2 siRNA with atelocollagen (AteloGene; Koken Co., Ltd., Tokyo, Japan)

mixture into groups of 5 rats every week for a total of three times. The growth rate of each tumor was evaluated twice a week. The sizes of the tumors were calculated by the formula for volume (V):  $V = 0.2618 \times L \times W \times (L+W)$  (12). The rats were euthanized at 5 weeks after the first injection, and the subcutaneous tumors were excised, weighed, and fixed in 10% formalin for histological, immunohistochemical and TUNEL assay assessments.

**Tissue samples and immunohistochemistry.** After obtaining approval from our Institutional Review Board (authorization no. 575), we searched the surgical pathology database of Nara Medical University from 1984 to 2012, and identified 34 cases of human high-grade osteosarcomas and 6 cases of low-grade osteosarcomas. As benign counterparts, 11 specimens of reactive bone (including fracture callus, reactive new bone formation and osteomyelitis) were also retrieved. The diagnosis of osteosarcoma was made according to the latest edition of the World Health Organization classification (13). The clinicopathological data of the high-grade osteosarcoma cases are summarized in Table I. The surgical staging was based on the 7th edition of the American Joint Committee on Cancer (AJCC) Staging Manual (14). Clinical details and follow-up information were obtained by reviewing the medical charts. All primary tumors were treated either surgically or with carbon ion radiotherapy, accompanied by neoadjuvant and adjuvant chemotherapy. The median follow-up period after surgery was 6 years (range, 1-16 years). Metastasis-free survival was defined as the interval from diagnosis to discovery of a metastasis or the last follow-up examination.

Immunohistochemical staining for UBQLN2 was performed in all cases. All of the osteosarcoma samples were obtained by open biopsy. Tumor tissues from each case were fixed in 10% neutral-buffered formalin and embedded in paraffin. Specimens were cut at 4- $\mu$ m intervals and mounted for immunohistochemical analyses and histopathological analysis by conventional hematoxylin and eosin (H&E) staining. The H&E-stained sections served as a guide for the immunohistochemical analyses. Immunohistochemistry was performed using a Histofine Simple Stain kit (Nichirei) according to the manufacturer's instructions. After deparaffinization in xylene and sequential hydration in 100 and 95% ethanol, the sections were heated at 120°C for 10 min in 10 mM sodium citrate buffer (pH 6.0). The sections were cooled down, incubated in 3% H<sub>2</sub>O<sub>2</sub> solution for 5 min to block endogenous peroxidase activity, rinsed in PBS for 5 min, and incubated with the primary antibody against UBQLN2 (1:500 dilution) for 1 h at room temperature. After three washes with PBS, the sections were incubated with secondary antibodies for 30 min at room temperature. Diaminobenzene was used as the chromogen, with hematoxylin as a nuclear counterstain. The stained sections were dehydrated, cleared and mounted. The intensity of immunohistochemical staining was evaluated at x400 magnification with a microscope (BX51; Olympus, Tokyo, Japan). The levels of immunostaining were graded by scoring the percentages of positivity into two groups: negative (<60%) and positive ( $\geq$ 60%).

**Statistical analysis.** Data were statistically analyzed using the Student's t-test, with the Mann-Whitney U test used for non-

Table I. Clinicopathological characteristics of the high-grade osteosarcoma cases.

Characteristics	Data
Age (years)	
<40	30
$\geq$ 40	4
Median	20.1
Range	7-51
Gender	
Male	24
Female	10
Site	
Femur	13
Tibia or fibula	12
Others	9
Surgical stage	
IA	0
IB	0
IIA	7
IIB	24
III	0
IVA	3
IVB	0
Surgery	
Amputation	12
Limb salvage	18
Total	34

parametric analyses. Metastasis-free survival was estimated by the Kaplan-Meier method. The log-rank test was used to evaluate the differences between survival curves. All analyses were performed with IBM SPSS version 20.0 (IBM Co., Armonk, NY, USA). Values of  $P < 0.05$  were considered to indicate a statistically significant result.

## Results

**Expression of UBQLN2 in human osteosarcoma.** UBQLN2 expression was positive in 44% (15/34) of the high-grade osteosarcomas (Fig. 1A) and 33% (2/6) of the low-grade osteosarcomas in contrast to no expression in reactive bone (0/11). Positivity for UBQLN2 ( $>60\%$  UBQLN2-positive tumor cells) was significantly associated with unfavorable metastasis-free survival of the osteosarcoma patients as well as AJCC surgical stage (IIB, III and IVA/B) in the univariate prognostic analyses ( $P < 0.05$ ; Fig. 1B, Table II).

**Silencing of UBQLN2 induces apoptosis in human osteosarcoma cells under hypoxic conditions.** Human osteosarcoma MG63 cells expressing UBQLN2 were used in the present study. These cells expressed both UBQLN2 mRNA and protein, which were strongly reduced under both normoxia and hypoxia (1% O<sub>2</sub>) after UBQLN2 siRNA transfection (Fig. 2A). MG63 cells were cultured for 48 h under normoxia after

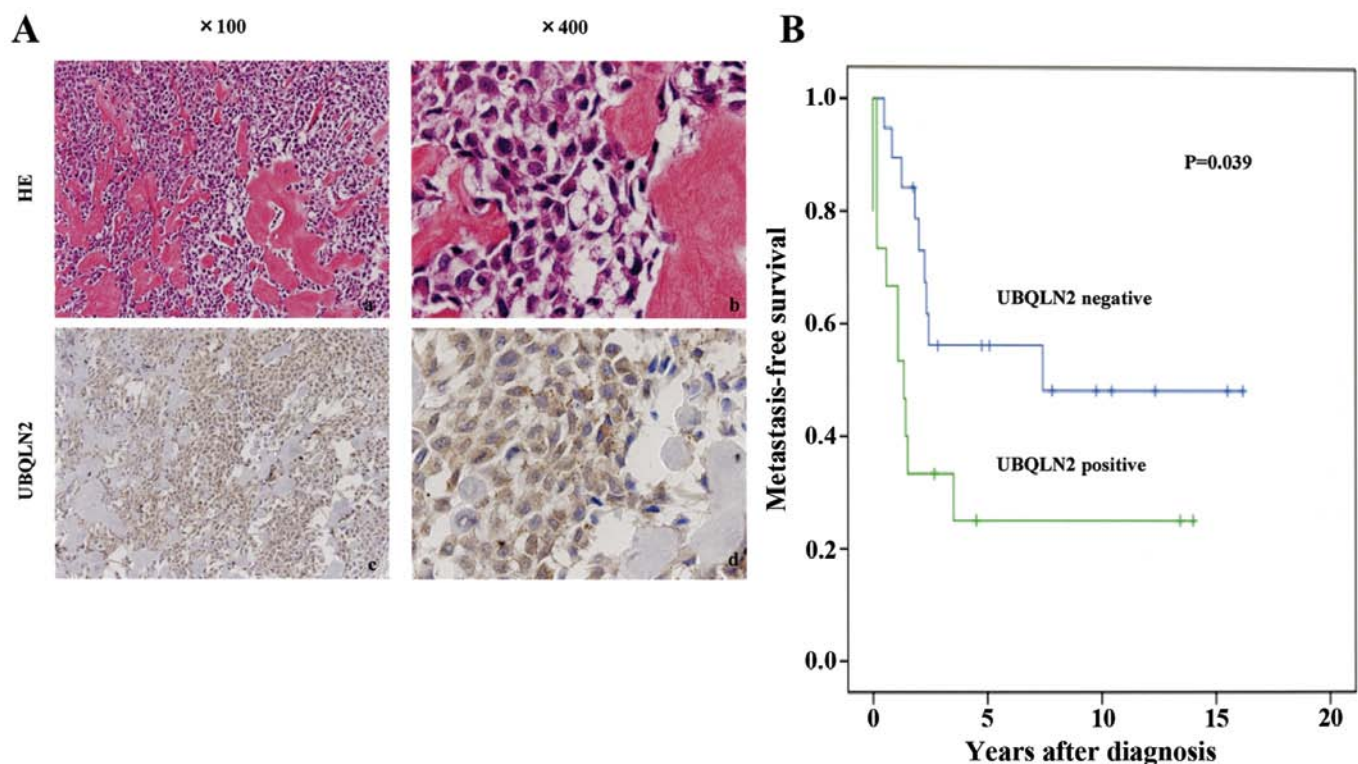


Figure 1. Immunohistochemical analysis of UBQLN2 in human osteosarcoma indicates a correlation with unfavorable metastasis-free survival. (A) Photomicrographs showing staining with hematoxylin and eosin (HE) and immunohistochemical staining with an anti-UBQLN2 antibody in high-grade osteosarcoma specimens. Expression of UBQLN2 was positive in 65% of the tumor cells. (B) Kaplan-Meier analysis of metastasis-free survival in osteosarcoma cases according to the expression of UBQLN 2.

Table II. Univariate analysis for metastasis-free survival.

Variable	No. of patients	5-year metastasis-free survival (%)	P-value
Age (years)			0.49
<40	30	41.6	
≥40	4	50.0	
Gender			0.62
Male	24	40.7	
Female	10	46.7	
Site			0.85
Extremity	30	41.7	
Trunk	4	50.0	
AJCC staging			0.007
IA.B, IIA	7	100.0	
IIB, III, IVA.B	27	26.6	
UBQLN2			0.039
Negative	19	56.1	
Positive	15	25.0	

UBQLN2 gene silencing. However, the cell growth was not significantly affected. In contrast, the cell survival was strongly suppressed by UBQLN2 gene silencing under hypoxic conditions (1% O<sub>2</sub>) (Fig. 2B). As shown in Fig. 2C, the percentage of apoptotic cells was significantly increased in response to

UBQLN2 gene silencing as assessed by TUNEL assays (0.9% for control RNA transfection vs. 9.4% for UBQLN2 siRNA transfection; P=0.04).

*JNK and p38 activation contributes to apoptosis induced by UBQLN2 downregulation under hypoxia.* Since stress kinases, including JNK and p38, are known to play essential roles in the apoptosis induced by various extrinsic stimuli in cancer cells (15), we examined whether mitogen-activated protein kinase (MAPK) activation contributed to the cytotoxicity induced by UBQLN2 knockdown under hypoxic conditions. Western blot analyses revealed that both p38 and JNK were activated in MG63 cells under hypoxic conditions following UBQLN2 siRNA transfection, in contrast to only p38 activation under normoxic conditions (Fig. 3A). Treatment with p38 or JNK inhibitors cancelled the cytotoxic effects of UBQLN2 knockdown under hypoxic conditions in the MG63 cells (Fig. 3B). TUNEL assays produced similar findings (data not shown). Thus, activation of both p38 and JNK appeared to be required for apoptosis induction by UBQLN2 gene silencing. We examined whether the master of hypoxia, HIF-1 $\alpha$ , was inhibited by silencing of UBQLN2 (data not shown). These findings showed that activation of the stress kinases JNK and p38 was essential for the apoptosis induced by silencing of UBQLN2 under hypoxia, and was independent of HIF-1 $\alpha$  stabilization.

*Growth of osteosarcoma is suppressed by UBQLN2 siRNA transfection in vivo.* The experimental protocol for this part

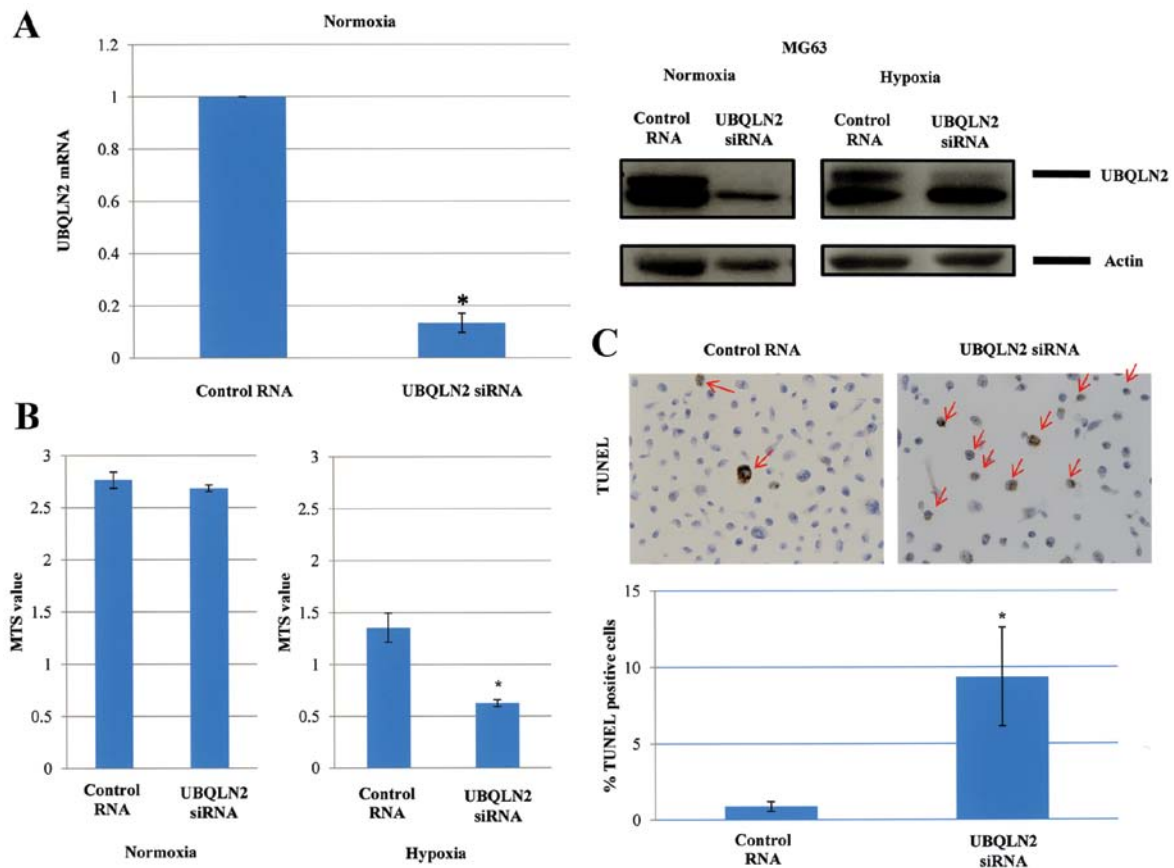


Figure 2. Downregulation of UBQLN2 in hypoxia induces apoptosis in human osteosarcoma cells. (A) MG63 cells were transfected with 165 nmol/l of UBQLN2 siRNA or control RNA. After transfection for 48 h under normoxia, the expression of UBQLN2 mRNA was examined by RT-PCR. After transfection for 24 h under normoxia and a further 24 h under normoxia or hypoxia, the expression of UBQLN2 protein was examined by western blotting. (B) Cell growth rates were examined after control RNA or UBQLN2 siRNA transfection for 24 h under normoxia and a further 24 h under normoxia or hypoxia. (C) After control RNA or UBQLN2 siRNA transfection for 24 h under normoxia and a further 24 h under hypoxia, the transfected cells were analyzed by TUNEL staining. Actin was evaluated as an internal control. Data are means  $\pm$  SD. \* $P < 0.05$ .

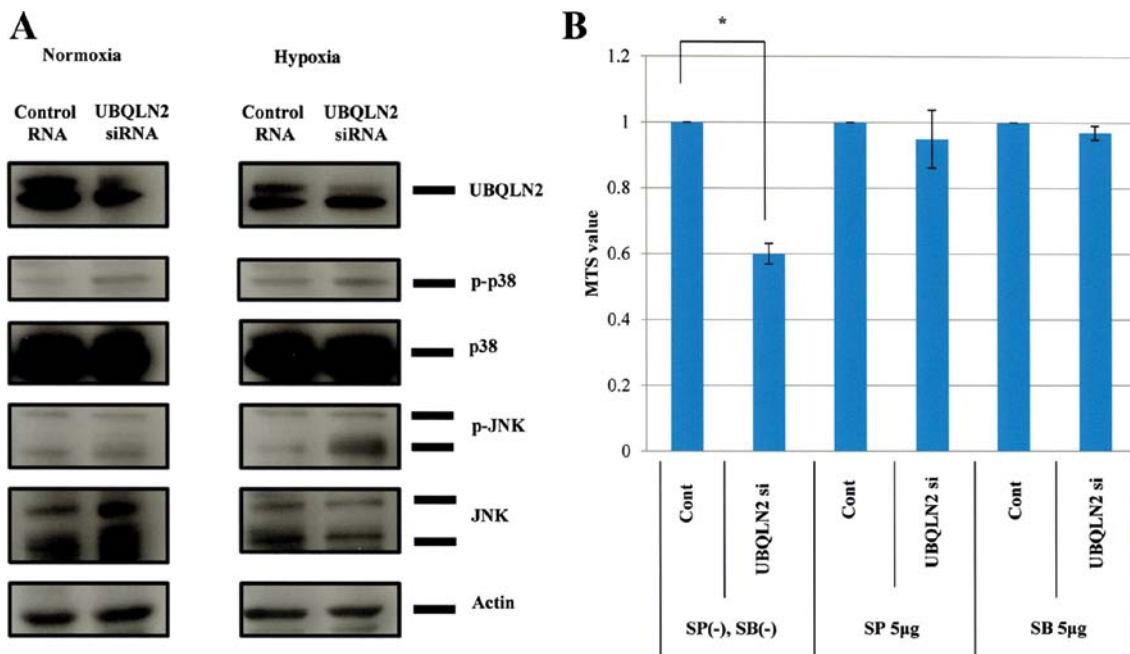


Figure 3. Both JNK and p38 are activated by UBQLN2 gene silencing under hypoxia, whereas only p38 is activated under normoxia. (A) MG63 cells were collected after control RNA or UBQLN2 siRNA transfection for 24 h under normoxia and a further 24 h under normoxia or hypoxia, and examined for their expression of phosphorylated p38 or JNK by western blotting. (B) MG63 cells were treated with 5  $\mu$ mol/l of SP600125 (SP) or SB203580 (SB) for 1 h before transfection with control RNA or UBQLN2 siRNA, subsequently cultured for 24 h under normoxia and a further 24 h under hypoxia, and subjected to MTS assays. Actin was evaluated as an internal control. Data are means  $\pm$  SD. \* $P < 0.05$ .



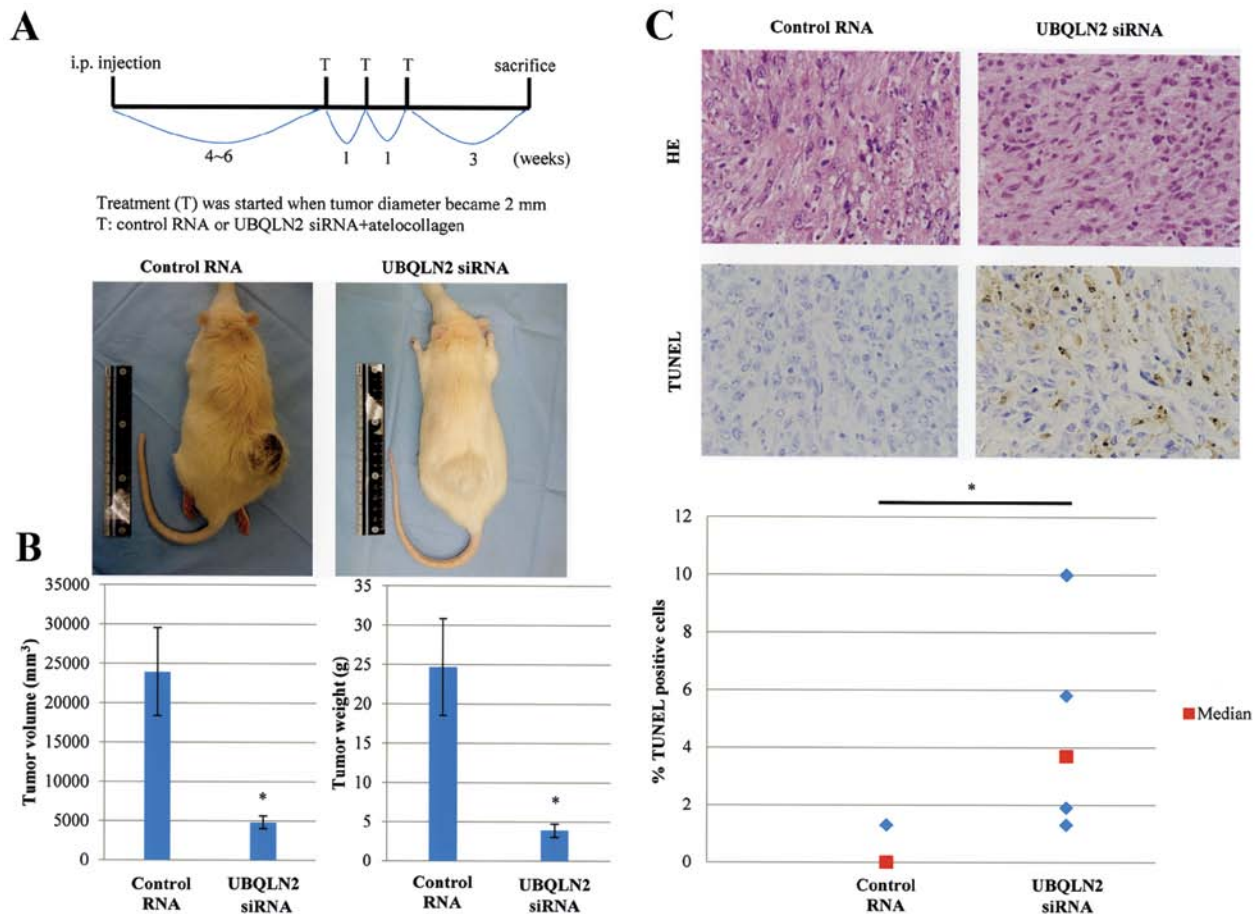


Figure 4. Silencing of UBQLN2 suppresses tumor growth and induces apoptosis *in vivo* in a rat osteosarcoma model. (A) Rats were injected with control RNA or UBQLN2 siRNA and an atelocollagen mixture once a week for a total of three times after subcutaneous inoculation of COS1NR cells. (B) Tumor size and weight were analyzed. Data are means  $\pm$  SD of 5 rats/group. \* $P < 0.05$ . (C) Hematoxylin and eosin (HE) staining and TUNEL assay results. Representative images are shown for each transfection group. Magnification,  $\times 400$ . TUNEL-positive cells were calculated as the number of positive cells  $\times$  100/total number of cells counted under  $\times 400$  magnification in 10 randomly selected areas in each tumor sample. \* $P < 0.05$ .

of the present study is illustrated in Fig. 4A. Briefly, COS1NR cells were inoculated into subcutaneous tissue in the back of Fischer 344 rats. When each tumor diameter reached 2 mm (at 4–6 weeks after inoculation), injection of either 20  $\mu$ mol/l of control RNA or 20  $\mu$ mol/l of UBQLN2 siRNA with atelocollagen mixture was initiated every week for a total of three times. The rats were euthanized at 5 weeks after the first injection, and evaluated for the volume and weight of their tumors. UBQLN2 downregulation was found to inhibit tumor growth. Significant differences in the tumor volume ( $P = 0.01$ ) and tumor weight ( $P = 0.01$ ) at euthanasia were observed between the groups treated with control RNA and UBQLN2 siRNA (Fig. 4A and B). TUNEL assays revealed a significant increase in the number of cells undergoing apoptosis after UBQLN2 knockdown (3.7% for UBQLN2 siRNA vs. 0% for control RNA;  $P = 0.009$ ; Fig. 4C). There were no differences in Ki-67, HIF-1 $\alpha$  or VEGF expression between control RNA and UBQLN2 siRNA injections (data not shown).

## Discussion

In the present study, we demonstrated for the first time that UBQLN2 protects osteosarcoma cells against hypoxic stress-induced cytotoxicity, independently from HIF-1 $\alpha$ -mediated

signaling. Hypoxia is known to activate two opposite cytotoxic and cytoprotective pathways. The representative cytotoxicity is apoptosis via hypoxia-induced mitochondrial membrane permeability, leading to release of cytochrome *c* into the cytoplasm and apoptosome formation in which caspase-9 is activated. Stress kinases, particularly MAPKs such as JNK, have been shown to play important roles in hypoxia-induced apoptosis (16). On the other hand, the cytoprotective effect of hypoxia is mainly mediated by HIF-1 $\alpha$ , which allows tumor cells to adapt to the hypoxic microenvironment and to acquire invasive and metastatic biological properties (17). Our results showed that silencing of UBQLN2 induced apoptosis in human osteosarcoma MG63 cells under hypoxic conditions without any change in HIF-1 $\alpha$  expression *in vitro*, and that osteosarcoma growth was suppressed and apoptosis was induced by UBQLN2 siRNA transfection while HIF-1 $\alpha$  expression remained unchanged *in vivo*. Therefore, UBQLN2 enhanced osteosarcoma progression by inhibiting the apoptotic pathway, yet not the HIF-1 $\alpha$ -mediated pathway. Immunohistochemical and prognostic analyses for UBQLN2 performed in human high-grade osteosarcomas demonstrated unfavorable metastasis-free survival with UBQLN2 overexpression. While no or reduced HIF-1 $\alpha$  expression in primary osteosarcoma was found in this study (data not shown), HIF-1 $\alpha$

is overexpressed in metastatic osteosarcoma rather than in the primary tumors (18). Frequent expression of two HIF-1 $\alpha$  downstream molecules, VEGF and platelet-derived growth factor (PDGF), is correlated with inferior event free-survival in osteosarcoma (19,20). With regard to malignant tumor effects related to UBQLN, it stabilizes BCL2L10/BCLb, one of the six anti-apoptotic members of the BCL2 family, inhibits apoptosis, and contributes to the survival of human lung cancer cells (9). Thus, the function of UBQLN in cancer cells is the same as that in osteosarcoma, in that it inhibits hypoxia-induced apoptosis in tumor cells.

UBQLN, a member of the ubiquitin-like protein family, is characterized by the presence of N-terminal ubiquitin-like and C-terminal ubiquitin-associated domains, and delivers ubiquitinated proteins to the proteasome for degradation. In accordance with this function, the ubiquitin-like domain of UBQLN binds to subunits of the proteasome, and its ubiquitin-associated domain binds to polyubiquitin chains that typically accelerate protein degradation mediated by the proteasome (21). Moreover, recent studies have shown that UBQLN regulates endoplasmic reticulum (ER)-associated protein degradation (ERAD), by which misfolded proteins are translocated from the ER to the cytoplasm for proteasomal degradation (22,23). In addition, UBQLN was shown to be linked to macroautophagy, an alternative degradation pathway by which cellular cargos are sequestered in double-membrane structures called autophagosomes and subsequently fuse with lysosomes harboring the acid hydrolases involved in protein degradation (24,25). UBQLN maintains protein homeostasis and aids cell survival through the degradation of misfolded or damaged proteins, which accumulate upon stimulation with various stresses, including hypoxia and/or starvation. Our finding that UBQLN2 inhibits hypoxic stress-induced apoptosis in osteosarcoma cells can also be deduced from the above reports. Mutations of the UBQLN2 gene suppress ubiquitin-mediated proteasomal degradation, leading to the accumulation of inclusions composed of misfolded proteins linked to the onset of ALS (8). Chronically reduced vascular perfusion by aging or other factors can produce chronic or episodic deficits in oxygen (hypoxia). This hypoxic stress may induce neuronal cell death and lead to the occurrence of ALS (26). Thus, UBQLN2 inhibits hypoxic stress-induced apoptosis in neuronal cells and prevents the occurrence of ALS. It is of great interest that a number of common signals involved in cell survival actually function in both neuronal and osteosarcoma cells.

In the present study, JNK and p38 were activated in MG63 cells under hypoxia following UBQLN2 siRNA transfection, while p38, but not JNK was activated under normoxia. In addition, either the JNK or the p38 inhibitor was able to significantly suppress the apoptosis induction. Thus, activation of both JNK and p38 is required for induction of apoptosis in these cells. Our results indicate that UBQLN2 may inhibit JNK activation and apoptosis in response to hypoxic stress, thereby successfully enhancing the malignant potential of osteosarcoma cells. JNK plays a critical role in both death receptor- and mitochondrial-mediated apoptotic pathways by upregulating pro-apoptotic genes via transactivation of specific transcription factors or by modulating the phosphorylation of pro-apoptotic and anti-apoptotic proteins in mitochondria (27).

Similar to JNK, p38 seems to sensitize cells to apoptosis via upregulation of pro-apoptotic proteins and downregulation of survival pathways (28). Our results were thus in line with these previous reports. We are currently investigating how the expression and/or activity of upstream kinases that do not activate p38, but do activate JNK, are modified by UBQLN2 gene silencing in osteosarcoma cells under hypoxic conditions.

Solid tumors including osteosarcoma are well-known to be exposed to hypoxia due to an inadequate oxygen supply, particularly when the tumors outgrow their blood supply (29,30). Coagulative tumor necrosis, which is caused by an inadequate blood supply and hypoxic conditions, is closely related to advancement of malignant tumors and poor response to chemotherapy, including cases of osteosarcoma (31). Since UBQLN2 protected osteosarcoma cells against hypoxia-induced apoptosis, a drug that inhibits UBQLN2 could lead osteosarcoma cells under hypoxia toward apoptosis. Thus, we can provide new methods for treating osteosarcoma that kill osteosarcoma cells under normoxia using conventional anticancer agents and surgery or kill these cells under hypoxia showing high invasion, metastatic potential and resistance to chemotherapy using UBQLN2 inhibitors. From the finding that UBQLN2 downregulation under normoxia did not inhibit the growth of osteosarcoma cells, UBQLN2 inhibitors will not affect normal tissues under normoxia and if used as drugs would confer few side-effects.

In summary, UBQLN2 is essential for resistance to hypoxic stress and acquisition of high malignant potential in osteosarcoma, and silencing of UBQLN2 enhances hypoxia-induced apoptosis *in vitro* and *in vivo*. In addition, UBQLN2 overexpression is significantly associated with unfavorable metastasis-free survival. UBQLN2 could represent a new molecular target and a useful clinicopathological marker in osteosarcoma.

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