

Curcumin may serve an anticancer role in human osteosarcoma cell line U-2 OS by targeting ITPR1

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Abstract. The present study aimed to determine the mechanisms of action of curcumin in osteosarcoma. Human osteosarcoma U-2 OS cells was purchased from the Cell Bank of the Chinese Academy of Sciences. RNA sequencing analysis was performed for 2 curcumin-treated samples and 2 control samples using Illumina deep sequencing technology. The differentially expressed genes were identified using Cufflink software. Enrichment and protein-protein interaction network analyses were performed separately using cluster Profiler package and Cytoscape software to identify key genes. Then, the mRNA levels of key genes were detected by quantitative reverse transcription polymerase chain reaction (RT-qPCR) in U-2 OS cells. Finally, cell apoptosis, proliferation, migration and invasion arrays were performed. In total, 201 DEGs were identified in the curcumin-treated group. *EEF1A1* (degree=88), *ATF7IP*, *HIF1A*, *SMAD7*, *CLTC*, *MCM10*, *ITPR1*, *ADAM15*, *WWP2* and *ATP5C1*, which were enriched in 'biological process', exhibited higher degrees than other genes in the PPI network. RT-qPCR demonstrated that treatment with curcumin was able to significantly increase the levels of *CLTC* and *ITPR1* mRNA in curcumin-treated cells compared with control. In addition, targeting *ITPR1* with curcumin significantly promoted apoptosis and suppressed proliferation, migration and invasion. Targeting *ITPR1* via curcumin may serve an anticancer role by mediating apoptosis, proliferation, migration and invasion in U-2 OS cells.

Introduction

Osteosarcoma is the most common primary bone malignancy in children and adolescents (1), which usually occurs in the metaphyseal region of tubular long bones (2). Although numerous modern therapies, including surgery, chemotherapy and radiotherapy have been used for treating osteosarcoma, the 5-year survival rate of patients with osteosarcoma is 60-70% (3). With the improvements in treatment techniques, the mortality rate of osteosarcoma is decreasing at ~1.3% per year (4). However, the etiology of osteosarcoma is unclear. Therefore, identification of the key genes associated with osteosarcoma is important for treatment of the disease.

As a type of plant polyphenol, curcumin is extracted from *Curcuma longa* (5) and exhibits anti-tumor, anti-inflammatory, antioxidant and anticoagulation capacities (6-9). Previous studies have demonstrated that curcumin serves functions in the progression of a number of types of cancer, including osteosarcoma (10), pancreatic cancer (11) and colon cancer (12). Fossey *et al* (13) revealed that FLLL32, a novel compound obtained from curcumin, inhibited signal transducers and activators of transcription 3 (*STAT3*) and induced apoptosis in osteosarcoma. Chang *et al* (14) indicated that curcumin induced apoptosis in the osteosarcoma MG63 cell line via mediating the reactive oxygen species/cytochrome *c*/caspase-3 pathway. Leow *et al* (15) demonstrated that curcumin and PKF118-310 may delay tumorigenesis and metastasis of osteosarcoma through inhibiting the Wnt/ β -catenin signaling pathway. Taken together, these studies indicate that curcumin exhibits an effect on the pathogenesis of osteosarcoma through different pathways. Despite these aforementioned data, the target genes of curcumin in osteosarcoma remain unclear.

RNA sequencing (RNA-seq), which is based on deep-sequencing technology, is a more developed approach compared with transcriptome profiling (16). Several developments in RNA-seq, including mapping of the transcription start site, characterization of small RNA and detection of strand-specific, gene fusion and alternative splicing events, have been identified (17). In the present study, RNA-seq data analysis was performed on human osteosarcoma U-2 OS cells, which were treated by curcumin or dimethyl sulfoxide (DMSO). Differentially expressed genes (DEGs) between the curcumin-treated and control groups were identified, and the functions of the DEGs were predicted by functional and pathway

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enrichment analyses. A protein-protein interaction (PPI) network involving the DEGs was also constructed to additionally determine the target genes of curcumin in osteosarcoma. In addition, the mRNA levels of the top 10 nodes in the PPI network were confirmed, and it was identified that the mRNA levels of clathrin heavy chain (*CLTC*) and inositol 1,4,5-triphosphate receptor type 1 (*ITPR1*) in curcumin-treated cells were significantly increased. In addition, the effects of curcumin and *ITPR1* on proliferation, apoptosis, migration and invasion in human osteosarcoma U-2 OS cells were investigated.

Materials and methods

Cell cultivation and curcumin treatment. The human osteosarcoma U-2 OS cell line was purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The U-2 OS cells were inoculated in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.) and cultured at 37°C in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.). When the passaged cells reached 80-90% confluence, the cells were digested using pancreatin (Gibco; Thermo Fisher Scientific, Inc.). The digested cells were centrifuged at 1,000 × g and 4°C for 5 min, and then supernatant was discarded. Subsequently, the cells were preserved in frozen stock solution containing 10% DMSO (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 40% FBS and 50% RPMI-1640 medium, and stored in program frozen box.

The U-2 OS cells were seeded in 6-well plates (2 × 10⁶ cell/well) and incubated in 5 ml serum-free medium in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C overnight. Curcumin (Sigma-Aldrich; Merck KGaA, 15 μmol/l) was dissolved in DMSO. Subsequently, U-2 OS cells were treated with 15 μM curcumin at 37°C in a 5% CO₂ incubator for 48 h (curcumin group). Concurrently, in the control groups, U-2 OS cells were treated with an equal volume of DMSO. Finally, the cells were washed twice with cold PBS.

All studies were approved by the Scientific and Ethical Committee of the 309th Hospital of Chinese People's Liberation Army (Beijing, China) and performed in accordance with the ethical standards.

RNA sequencing data. Total RNA was isolated from the U-2 OS cells using TRIzol® (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol and quantified by spectrophotometry. Then, a transcriptome library was constructed using NEBNext® Ultra™ RNA Library Prep kit for Illumina® (cat. no. E7530; New England Biolabs, Inc., Ipswich, MA, USA) following the manufacturer's protocol. RNA was separated into RNA fragments (~200 nt), followed by double-stranded cDNA being synthesized and end-repaired. Then, adaptor ligation and polymerase chain reaction (PCR) enrichment (denaturation step: 98°C for 30 sec; 12 cycles: 98°C for 10 sec and annealing temperature 65°C for 75 sec; final extension step: 65°C for 5 min) were performed using the primers and reagents supplied with the NEBNext® Ultra RNA Library Prep kit. The quality of the library was analyzed using

Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA), and RNA was sequenced on HiSeq 2500 (Illumina, Inc., San Diego, CA, USA).

Data quality control and screening of DEGs. Subsequent to quality controls of the reads, they were mapped to the reference human genome (hg19) using TopHat (18) and assembled by Bowtie 1 software (19). Combined with sequence alignment results from TopHat and hg19 annotation information downloaded from the University of California, Santa Cruz (UCSC) database (<https://www.astro.ucsc.edu/>) (20), the DEGs between curcumin and control groups were identified using Cufflink software (21). $P < 0.01$ and $|\log_2 \text{fold change (FC)}| \geq 1$ were set as cut-off criteria.

Functional and pathway enrichment analysis. Gene Ontology (GO, <http://geneontology.org/>) analysis develops a series of controlled and structured vocabularies for annotating functions of genes and their products (22). The Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.kegg.jp/>) is a biological database, which stores genomic, chemical and systemic functional information (23). Using the cluster profiler (24) package in R, GO functional and KEGG pathway enrichment analyses were performed for upregulated and downregulated genes, respectively. The raw P-value was adjusted by the Benjamini-Hochberg method (25), and the adjusted P-value (also known as false discovery rate, FDR) < 0.05 was used as a threshold.

Construction of PPI network. Human Protein Reference Database (HPRD) includes curated proteomic information and describes human PPIs (26). Using HPRD Release 9 (<http://www.hprd.org/query>) (26), pairs of interacting human proteins were downloaded, and the self-interacting protein pairs were removed. The DEGs were mapped with the downloaded PPI pairs, and those pairs involving the identified DEGs were extracted. Finally, the PPI network was visualized using Cytoscape software (version 2.8.0; www.cytoscape.org) and named as the DEG PPI network (27). Proteins are represented by nodes, the degree is the number of nodes which interact with the node in question, and the higher the degree the more important the protein in the PPI network.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis. The cells treated with curcumin were dissolved in TRIzol® reagent (Takara Bio, Inc.) for extraction of total RNA. Primers for key genes are summarized in Table I. Using SYBR® Premix Ex Taq™ (Applied Biosystems; Thermo Fisher Scientific, Inc.), RT-qPCR amplification was performed according to the following thermocycling conditions: 50°C for 3 min, 95°C for 3 min, followed by 40 cycles of 95°C for 10 sec and 58°C for 30 sec. Gene expression was quantified and calculated by the comparative threshold (Cq) cycle method ($2^{-\Delta\Delta Cq}$) (28).

RNA interference assay. The cells in the siRNA group were digested by pancreatin at 37°C for 5 min, and then complete medium was added to neutralize the pancreatin. Subsequently, the cells were centrifuged at 1,000 × g for 5 min at 4°C, and the supernatant was discarded. Subsequent to being counted,

Table I. Primer sequences for specific genes.

Name of primer	Primer sequences (5'-3')
EEF1A1-hf	TGCCTGGGTCTTGATAAAC
EEF1A1-hr	GCCTGAGATGTCCTGTAAT
ATF7IP-hf	TTCCGCCCAAAAGATTCAGA
ATF7IP-hr	CTGCTTCAAGTTGCTGACGATC
HIF1A-hf	ACTTCTGGATGCTGGTGATT
HIF1A-hr	GTTCAAACCTGAGTTAATCCC
SMAD7-hf	ACCTTAGCCGACTCTGCGAACT
SMAD7-hr	TTTCAGCGGAGGAAGGCACA
CLTC-hf	TTGAATACGGTTGCTCTTGT
CLTC-hr	ATGCCAGTCAGAAGTAACCA
MCM10-hf	CTTTGAATACGGTTGCTCTT
MCM10-hr	GTACGGTAATTGATAATCTGG
ITPR1-hf	CCTGGTTGATGATCGTTGTGTT
ITPR1-hr	GCTTTTGGGCAGAGTAGCGGTT
ADAM15-hf	ATCCCTGCTGTGATTCTTTGACC
ADAM15-hr	TGGGCATAGGAGGCACAACG
WWP2-hf	CCCCGAATCCCAACACGACT
WWP2-hr	TCCCATCCAGCAGGCAGAGC
ATP5C1-hf	AAAAGCGAGGTTGCTACACT
ATP5C1-hr	ATGACTGACGCATCTCCAAA
GAPDH-hf	TGACAACCTTGGTATCGTGGAAGG
GAPDH-hr	AGGCAGGGATGATGTTCTGGAGAG

EEF1A1, eukaryotic translation elongation factor 1 α 1; ATF7IP, activating transcription factor 7 interacting protein; HIF1A, hypoxia-inducible factor-1 α ; SMAD7, SMAD family member 7; CLTC, clathrin heavy chain; MCM10, minichromosome maintenance 10 replication initiation factor; ITPR1, inositol 1,4,5-triphosphate receptor type 1; ADAM15, a disintegrin and metalloprotease domain 15; WWP2, WW domain containing E3 ubiquitin protein ligase 2; ATP5C1, ATP synthase, H⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1; h, human; f, forward; r, reverse.

the cells were seeded in 6-well plates (8x10⁵ cell/well) and cultured in 2 ml antibiotic-free complete medium in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C overnight. The *ITPR1* small interfering (siRNA) were synthesized with the forward, 5'-AGACAGAAAACAGGAAAUUTT-3' and reverse primer, (5'-AAUUUCCUGUUUCUGUCUCA-3'). The *ITPR1* siRNA (final concentration, 66 nM/l) and Lipofectamine[®] 2000 reagent (1:25 vol/vol; Invitrogen; Thermo Fisher Scientific, Inc.) diluted with Opti-MEM medium (Gibco; Thermo Fisher Scientific, Inc.) were mixed and allowed to rest for 20 min. Subsequently, the cells were added to the siRNA mixture (500 μ l/well) and Opti-MEM medium was replaced with complete medium following transfection for 6 h at room temperature. In addition, the cells were cultivated in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C for 48 h. The cells were then collected for RT-qPCR assay. The cells in the negative control group were treated with scrambled siRNA (final concentration, 66 nM/l) (Shanghai Biotend Biological Technology, Co., Ltd., Shanghai, China) as control and cultured like the cells in the siRNA group. The cells in the control group

Table II. Results from quality control and assembly of sequencing data.

ID	Group	Number of cleaned paired-end reads	Number of aligned pair reads
14710C-1	Control	11,660,638	8,129,145
14710C-2	Control	12,351,701	8,531,536
14710C-5	Curcumin	13,820,514	8,368,710
14710C-6	Curcumin	11,927,581	8,797,461

were treated without siRNA but otherwise culture the same as the cells in the siRNA group.

Cell proliferation assay. Subsequent to being digested and counted, the cells were seeded in a 96-well plate (4x10⁴ cell/well) and cultured in 100 ml antibiotic-free RPMI-1640 medium supplemented with 10% FBS (both FBS; Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C overnight. Then, the cells were separately transfected at room temperature with the siRNA mixture, negative control siRNA and Lipofectamine 2000 reagent (Invitrogen; ThermoFisher Scientific Inc.) for 6 h. Subsequent to treatment with curcumin (Sigma-Aldrich; Merck KGaA), the cells were incubated with cell counting kit-8 at 37°C (CCK8; 10 μ l/well; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) for 2 h. Finally, the absorption values under 450 nm were measured using an Epoch[™] Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA) to calculate viability.

Flow cytometry. The U-2 OS cells were digested and counted, followed by seeding in a 6-well plate (8x10⁵ cell/well) and cultivation in 2 ml antibiotic-free RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ at 37°C overnight. Following transfection at room temperature for 6 h, the cells were treated with curcumin. The cells were digested by pancreatin, washed by PBS and resuspended in 1X binding buffer (BD Biosciences, San Jose, CA, USA). Subsequently, 100 μ l of the above solution was transferred to flow cytometry tube, and stained with 5 μ l fluorescein isothiocyanate-Annexin V (BD Biosciences) and 5 μ l propidium iodide (50 μ g/ml; BD Biosciences) in the dark for 15 min at 25°C. Additionally, 400 μ l 1X binding buffer (BD Biosciences) was added prior to flow cytometry using FCS Express 4 (De Novo Software, Glendale, CA).

Wound healing assay. Lines were drawn on the base of 6-well plate with an equal interval of ~0.5-1 cm. Then, the digested and counted cells were seeded in a 6-well plate (8x10⁵ cell/well) and cultured in 2 ml RPMI-1640 medium without serum (Gibco; Thermo Fisher Scientific, Inc.) in a 5% CO₂ at 37°C overnight. Then, the cells were separately transfected at room temperature with the siRNA mixture, negative control siRNA and Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific Inc.) for 6 h. Afterwards, the cells were wound by drawing lines using a pipetting needle. After this, the cells were washed with PBS and then there were several channels

Table III. GO terms enriched for upregulated and downregulated genes.

Go term	Description	Gene number	FDR	Gene symbol
Upregulated DEGs				
GO: 8150	Biological process	94	3.75x10 ⁻⁶	<i>EEF1A1, ATF7IP, SMAD7, CLTC, MCM10</i>
GO: 16043	Cellular component organization	41	1.95x10 ⁻²	<i>ATF7IP, SMAD7, CLTC, CDH4, RTN3</i>
GO: 71840	Cellular component organization or biogenesis	41	2.08x10 ⁻²	<i>ATF7IP, SMAD7, CLTC, CDH4, RTN3</i>
Downregulated DEGs				
GO: 8150	Biological process	64	8.18x10 ⁻⁴	<i>HIF1A, ADAM15, ARFRP1, AKAP9, ABI2</i>
GO: 50896	Response to stimulus	40	5.62x10 ⁻³	<i>HIF1A, ADAM15, ARFRP1, AKAP9, MALT1</i>
GO: 51716	Cellular response to stimulus	34	5.62x10 ⁻³	<i>HIF1A, ARFRP1, AKAP9, MALT1, MGLL</i>
GO: 9987	Cellular process	59	5.62x10 ⁻³	<i>HIF1A, ADAM15, ARFRP1, RBM5, PFDN6</i>
GO: 7165	Signal transduction	29	1.03x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, MALT1, RAP1GAP2</i>
GO: 23052	Signaling	30	1.79x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, MALT1, RAP1GAP2</i>
GO: 44700	Single organism signaling	30	1.79x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, MALT1, RAP1GAP2</i>
GO: 7154	Cell communication	30	1.89x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, MALT1, RAP1GAP2</i>
GO: 71704	Organic substance metabolic process	46	1.89x10 ⁻²	<i>HIF1A, ADAM15, ARFRP1, GSTO2, ADK</i>
GO: 44238	Primary metabolic process	45	1.89x10 ⁻²	<i>HIF1A, ADAM15, ARFRP1, GSTO2, ADK</i>
GO: 50789	Regulation of biological process	43	2.04x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, ABI2, RBM5</i>
GO: 50794	Regulation of cellular process	41	2.55x10 ⁻²	<i>HIF1A, ARFRP1, AKAP9, ABI2, RBM5</i>
GO: 44699	Single-organism process	53	2.98x10 ⁻²	<i>HIF1A, ADAM15, ARFRP1, AKAP9, ABI2</i>
GO: 1568	Blood vessel development	6	4.65x10 ⁻²	<i>HIF1A, ADAM15, ANKRD17, RAPGEF1, NPRL3, NRP1</i>
GO: 43170	Macromolecule metabolic process	38	4.98x10 ⁻²	<i>HIF1A, ADAM15, ABI2, RBM5, PFDN6</i>

GO, Gene Ontology; DEGs, differentially expressed genes; FDR: false discovery rate; EEF1A1, eukaryotic translation elongation factor 1 α 1; ATF7IP, activating transcription factor 7 interacting protein; HIF1A, hypoxia-inducible factor-1 α ; SMAD7, SMAD family member 7; CLTC, clathrin heavy chain; MCM10, minichromosome maintenance 10 replication initiation factor; ITPR1, inositol 1,4,5-triphosphate receptor type 1; ADAM15, a disintegrin and metalloprotease domain 15; WWP2, WW domain containing E3 ubiquitin protein ligase 2; ATP5C1, ATP synthase, H⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1; RTN3, reticulon 3; GSTO2, glutathione S-transferase Omega 2; ADK, adenosine kinase; MALT1, mucosa-associated lymphoid tissue lymphoma translocation protein 1; RAP1GAP2, RAPI GTPase activating protein 2; AKAP9, A-kinase anchoring protein 9; MGLL, monoglyceride lipase; PFDN6, prefoldin subunit 6; RBM5, RNA binding motif protein 5; ABI2, Abl interactor 2; NRP1, neuropilin 1; NPRL3, nitrogen permease regulator 3-like protein 3.

on the surface of cultured cells. Subsequent to treatment with 15 μ mol/l curcumin, images of movement of the cells were captured every 12 h and measured by Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD, USA).

Transwell assay. Subsequent to dilution with PBS (1:3 dilution), 50 μ l Matrigel (1 mg/ml; Corning Incorporated, Corning, NY, USA) was solidified in a 24-well polycarbonate membrane following standing for 1 h at 37°C and resuspended following addition of 200 μ l RPMI-1640 without serum (Gibco; Thermo Fisher Scientific, Inc.). The cells in each well of cell petri dish were added with 600 μ l 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and then were cultured in a 6-well plate (8x10⁵ cell/well) in 2 ml RPMI-1640 medium with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in 5% CO₂ at 37°C overnight. Following this, the transferred and treated cells

were digested by pancreatin (Gibco: Thermo Fisher Scientific, Inc.) and cultivated in the upper wells (1x10⁵ cell/well) in a 5% CO₂ incubator (Thermo Fisher Scientific, Inc.) at 37°C for 12 h. Finally, the liquids in upper wells were discarded, and the cells that had not passed through the membranes were wiped off. The cells were stained with 0.1% crystal violet staining at room temperature for 20 min and observed under an inverted light microscope in 3 fields of view at magnification, x200 (Olympus Corporation, Tokyo, Japan).

Statistical analysis. Using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA), comparisons between groups were made using one-way ANOVA followed by a least significant difference test. All results are presented as the mean \pm standard error. P<0.05 was considered to indicate a statistically significant difference.

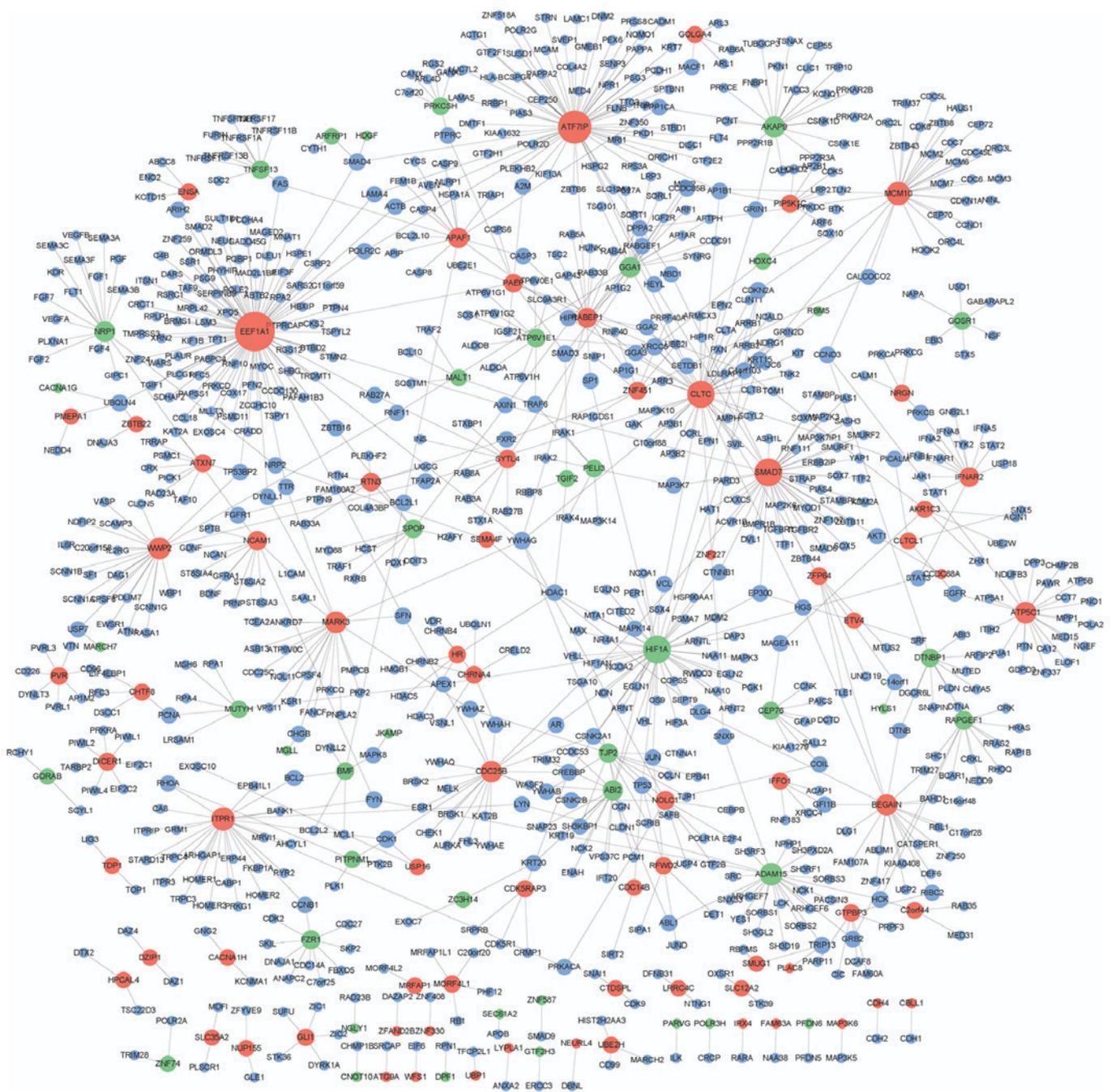


Figure 1. Protein-protein interaction network that involves the identified differentially expressed genes. Red and green nodes represent upregulated and downregulated genes, respectively. Blue nodes indicate the genes with no significant change in expression.

Results

Data quality control and analysis of DEGs. Quality control of the reads was performed and then assembled using Bowtie 1 software. The results are summarized in Table II. With $P < 0.01$ and $\log_2 FCI \geq 1$ as thresholds, the DEGs between the curcumin and control group were identified by Cufflink software. Compared with the control group, a total of 201 DEGs were identified in the curcumin-treated group, including 114 upregulated and 87 downregulated genes.

Functional and pathway enrichment analyses. The upregulated genes were significantly enriched in 3 GO terms, including

‘biological process’ ($FDR = 3.75 \times 10^{-6}$), ‘cellular component organization’ ($FDR = 1.95 \times 10^{-2}$) and ‘cellular component organization or biogenesis’ ($FDR = 2.08 \times 10^{-2}$; Table III). The downregulated genes including hypoxia-inducible factor-1 α (*HIF1A*) were significantly enriched in signal transduction ($FDR = 1.03 \times 10^{-2}$), cell communication ($FDR = 1.89 \times 10^{-2}$) and cellular process ($FDR = 5.62 \times 10^{-3}$; Table III). In addition, there were no KEGG pathways significantly enriched for the upregulated or downregulated genes. Importantly, the GO term ‘biological process’ was enriched for upregulated genes [eukaryotic translation elongation factor 1 α 1 (*EEF1A1*), activating transcription factor 7 interacting protein (*ATF7IP*), SMAD family member 7 (*SMAD7*), *CLTC*, microchromosome

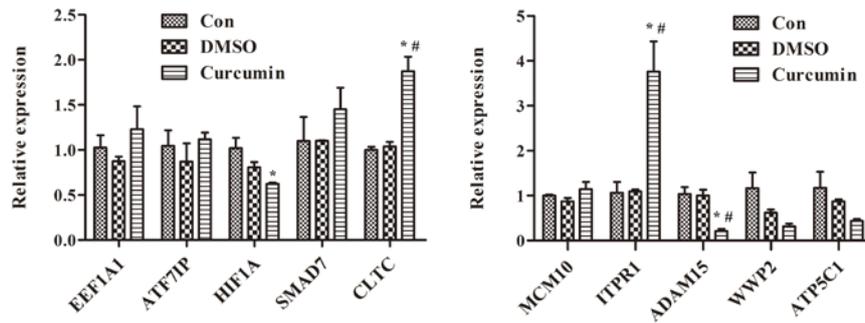


Figure 2. Treatment with curcumin significantly increases the mRNA levels of *CLTC* and *ITPR1* and decreases the mRNA levels of *ADAM15* in curcumin-treated cells compared with dimethyl sulfoxide-treated and control cells, * $P < 0.01$ compared with control cells, # $P < 0.01$ compared with dimethyl sulfoxide-treated cells. *EEF1A1*, eukaryotic translation elongation factor 1 α 1; *ATF7IP*, activating transcription factor 7 interacting protein; *HIF1A*, hypoxia-inducible factor-1 α ; *SMAD7*, SMAD family member 7; *CLTC*, clathrin heavy chain; *MCM10*, minichromosome maintenance 10 replication initiation factor; *ITPR1*, inositol 1,4,5-triphosphate receptor type 1; *ADAM15*, a disintegrin and metalloprotease 15; *WWP2*, WW domain containing E3 ubiquitin protein ligase 2; *ATP5C1*, ATP synthase, H⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1; Con, control; DMSO, dimethyl sulfoxide.

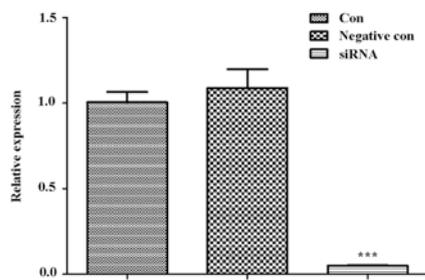


Figure 3. *ITPR1* is significantly decreased in cells transferred with *ITPR1* siRNA sequences in comparison with negative control cells and control cells. *** $P < 0.001$ when comparing the siRNA group with the negative control and control cells (compared separately). *ITPR1*, inositol 1,4,5-triphosphate receptor type 1; si, small interfering; con, control.

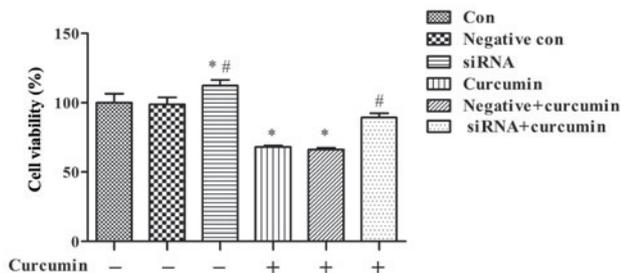


Figure 4. Treatment with curcumin significantly suppresses proliferation of U-2 OS cells, and the effect of curcumin-mediated suppression decreases following knockdown of inositol 1,4,5-triphosphate receptor type 1. * $P < 0.05$, compared with negative control cells and control cells; # $P < 0.01$, compared with negative and curcumin-treated cells. si, small interfering; con, control.

maintenance 10 (*MCM10*), *ITPR1*, WW domain containing E3 ubiquitin protein ligase 2 (*WWP2*) and ATP synthase, H⁺ transporting, mitochondrial F1 complex, gamma polypeptide 1 (*ATP5C1*), and downregulated genes [*HIF1A* and a disintegrin and metalloprotease 15 (*ADAM15*)].

Analysis of PPI network. A total of 39,240 PPI pairs were downloaded from HPRD Release 9. The DEG.PPI network had 929 interactions and 913 node genes, including 73 upregulated, 43 downregulated and 797 genes with no significant change

in expression (Fig. 1). In particular, *EEF1A1* (degree=88), *ATF7IP* (degree=64), *HIF1A* (degree=44), *SMAD7* (degree=43), *CLTC* (degree=42), *MCM10* (degree=28), *ITPR1* (degree=27), *ADAM15* (degree=24), *WWP2* (degree=22) and *ATP5C1* (degree=21) exhibited higher degrees in the DEG.PPI network.

Effects of curcumin treatment on the mRNA levels of *EEF1A1*, *ATF7IP*, *HIF1A*, *SMAD7*, *CLTC*, *MCM10*, *ITPR1*, *ADAM15*, *WWP2* and *ATP5C1* in U-2 OS cells. The mRNA levels of *EEF1A1*, *ATF7IP*, *HIF1A*, *SMAD7*, *CLTC*, *MCM10*, *ITPR1*, *ADAM15*, *WWP2* and *ATP5C1*, which were the top 10 nodes with higher degrees in the PPI network, were measured. The results of RT-qPCR demonstrated that treatment with curcumin significantly increased the mRNA levels of *CLTC* and *ITPR1* compared with DMSO-treated and control cells ($P < 0.01$; Fig. 2). Additionally, the differential expression of *ITPR1* in curcumin-treated cells was more marked compared with *CLTC*.

RNA interference assay of *ITPR1*. With *ITPR1* siRNA sequences, RNA interference assay was performed in U-2 OS cells. Then, the cells were collected for RT-qPCR analysis. The result of RT-qPCR demonstrated that *ITPR1* was significantly decreased in cells transferred with *ITPR1* siRNA sequences compared with the negative control cells and control cells ($P < 0.001$; Fig. 3).

Effects of curcumin treatment and *ITPR1* expression on proliferation in U-2 OS cells. The cell proliferation assay indicated that curcumin treatment was able to significantly suppress the proliferation of U-2 OS cells than the control group not treated with curcumin ($P < 0.05$; Fig. 4), and the effect of curcumin-mediated suppression decreased subsequent to *ITPR1* knockdown ($P < 0.01$ vs. negative + curcumin; Fig. 4).

Effects of curcumin treatment and *ITPR1* expression on apoptosis of U-2 OS cells. Flow cytometry was used to analysis the apoptotic rate of U-2 OS cells, and the results indicated that treatment with curcumin was able to significantly promote apoptosis of U-2 OS cells compared with the control not treated with curcumin ($P < 0.05$; Fig. 5), while the apoptotic

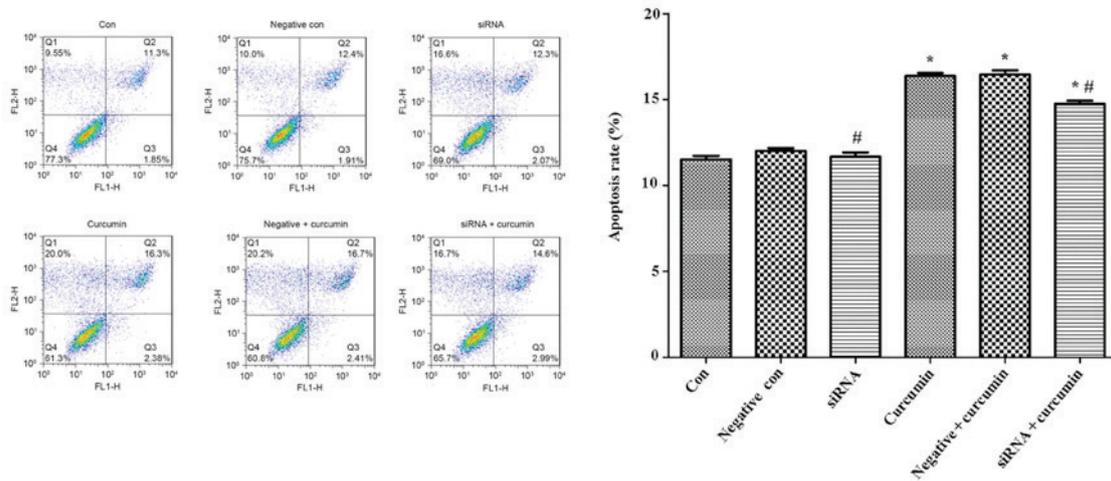


Figure 5. Treatment with curcumin significantly promotes apoptosis of U-2 OS cells, while the apoptotic rate of U-2 OS cells transfected with inositol 1,4,5-triphosphate receptor type 1 siRNA sequences were significantly decreased. *P<0.05, compared with negative control cells and control cells; #P<0.05, compared with negative and curcumin-treated cells. si, small interfering; con, control.

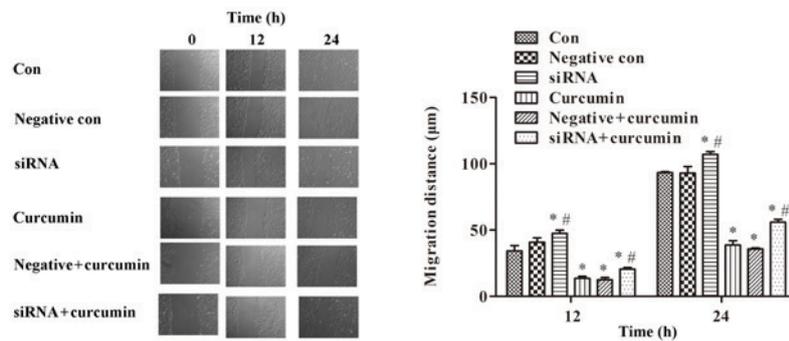


Figure 6. Treatment with curcumin significantly inhibits cell migration, while *ITPR1* interference promotes migration of U-2 OS cells (magnification, x200). *P<0.01, compared with negative control cells and control cells (Note: for siRNA treated cells at 12 h, the *P<0.01 is compared with control cells); #P<0.05, compared with negative and curcumin-treated cells. si, small interfering; con, control. *ITPR1*, inositol 1,4,5-triphosphate receptor type 1.

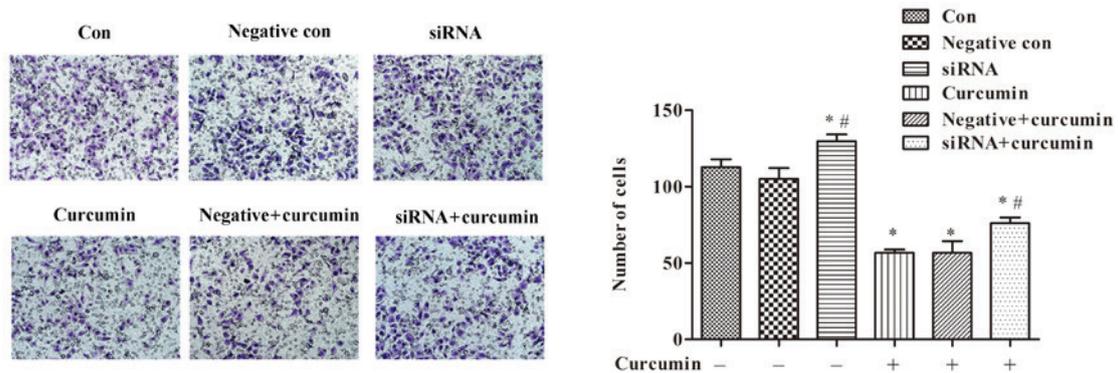


Figure 7. Treatment with curcumin significantly inhibits invasion of U-2 OS cells, and inositol 1,4,5-triphosphate receptor type 1 interference relieves the inhibitory effect of curcumin on cell invasion (magnification, x200). *P<0.05 compared with negative control cells and control cells; #P<0.05 compared with negative and curcumin-treated cells. si, small interfering; con, control.

rate of U-2 OS cells transfected with *ITPR1* siRNA sequences was significantly decreased (P<0.05; Fig. 5).

Effects of curcumin treatment and ITPR1 expression on migration of U-2 OS cells. A wound healing assay was performed to detect the migration of U-2 OS cells. The results

indicated that treatment with curcumin significantly inhibited cell migration (P<0.01; Fig. 6). Compared with the control group, *ITPR1* interference promoted migration of U-2 OS cells (P<0.01; Fig. 6). Meanwhile, *ITPR1* interference significantly promoted migration of U-2 OS cells compared with curcumin-treated cells (P<0.05; Fig. 6).

Effects of curcumin treatment and ITPR1 expression on invasion of U-2 OS cells. Additionally, a Transwell assay was utilized to investigate the effects of curcumin treatment and *ITPR1* expression on invasion of U-2 OS cells. The results of the Transwell assay suggested that treatment with curcumin was able to significantly inhibit invasion of U-2 OS cells compared with the control not treated with curcumin ($P < 0.05$; Fig. 7). In addition, *ITPR1* interference relieved the inhibitory effect of curcumin treatment on cell invasion ($P < 0.05$; Fig. 7).

Discussion

In the present study, RNA-sequencing was used to perform transcriptomic analysis of human osteosarcoma U-2 OS cells treated with curcumin or DMSO. A total of 201 DEGs were identified in the curcumin-treated group, including 114 upregulated and 87 downregulated genes. Functional enrichment indicated that downregulated *HIF1A* was involved in biological processes, including signal transduction, cell communication and cellular process. *HIF1A* serves a key role in progression, invasion and metastasis of a number of types of human cancer, including osteosarcoma (29). It has been demonstrated that small hairpin RNA (shRNA)-mediated knockdown of *HIF1A* was able to efficiently inhibit the hypoxia transduction pathway and block the growth of osteosarcoma cells (30). *HIF1A* functions in osteosarcoma progression and the oxygen-dependent degradation of *HIF1A* may be critical for osteosarcoma (31). *HIF1A* is highly relevant to the primary occurrence or recurrence, size, clinical stage, pathological grade and angiogenesis of the osteosarcoma of the jaw, and may serve as a therapeutic target for the disease (32). Therefore, the present study hypothesized that *HIF1A* may also be involved in osteosarcoma.

EEF1A1 is overexpressed in methotrexate-treated osteosarcoma Saos-2 cell line, and may promote cell growth through the increase of protein translation (33). Concurrently, the inhibition of *EEF1A1* may protect myotubes from apoptosis (34). Blanch *et al* (35) revealed that *EEF1A1* exhibited effects on tumor protein (*p*) 53 and *p73* through regulating human homologue of Mdm2 (*HDM2*) in a number of types of cancer, including osteosarcoma (35). Furthermore, several studies have demonstrated that the p53-family proteins participate in tumorigenesis via the mediation of the genes associated with cell cycle progression and apoptosis (36,37). In osteosarcoma Saos2 cells, *SMAD7* may suppress bone morphogenetic protein 2 (BMP2)-induced differentiation and BMP/SMAD signaling through interacting with nuclear factor- κ B (38). Previous studies have reported that *SMAD7* overexpression may inhibit tumor progression and lung metastasis of osteosarcoma (39,40). Taken together, these data suggested that *EEF1A1* and *SMAD7* may participate in osteosarcoma. In the PPI network of the present study, *EEF1A1* (degree=88), *ATF7IP* (degree=64), *HIF1A* (degree=44), *SMAD7* (degree=43), *CLTC* (degree=42), *MCM10* (degree=28), *ITPR1* (degree=27), *ADAM15* (degree=24), *WWP2* (degree=22) and *ATP5C1* (degree=21) exhibited the highest degrees. These genes were involved in the GO term 'biological process', indicating that they may be involved in the anticancer role of curcumin in human osteosarcoma U-2 OS cells through biological processes.

To additionally investigate the mechanisms of action of curcumin on human osteosarcoma U-2 OS cells, the mRNA levels of *EEF1A1*, *ATF7IP*, *HIF1A*, *SMAD7*, *CLTC*, *MCM10*, *ITPR1*, *ADAM15*, *WWP2* and *ATP5C1* in U-2 OS cells were analyzed. RT-qPCR demonstrated that treatment with curcumin significantly increased the mRNA levels of *CLTC* and *ITPR1* in curcumin-treated cells, while the mRNA levels of *ADAM15* dramatically decreased in curcumin-treated cells. Notably, the differential expression of *ITPR1* was more significant compared with *CLTC*. Then, the effects of curcumin treatment and *ITPR1* expression on proliferation, apoptosis, migration and invasion in U-2 OS cells were investigated. The results indicated that treatment with curcumin was able to significantly promote apoptosis and suppress proliferation, migration and invasion. It was also indicated that *ITPR1* may contribute to the effects mediated by curcumin. A previous study had suggested that curcumin suppressed proliferation, invasion, survival, metastasis and angiogenesis of a number of types of cancer via interacting with cell signaling proteins including enzymes and inflammatory cytokines (41). Lee *et al* (42) with other previous studies demonstrated that treatment with curcumin promoted G1/S and G2/M cell cycle arrest and that activation of the caspase-3 signaling pathway induced apoptosis in human osteosarcoma cells (14,43). Li *et al* (44) indicated that curcumin inhibited proliferation of osteosarcoma via the inactivation of the Notch-1 signaling pathway. The *ITPR1* gene encodes the intracellular calcium release channel type 1 InsP3R that can bind sensitized cells and cytochrome c to apoptotic stimuli (45-47). In osteoblastic cells, including G-292 osteosarcoma cells, exposure to interleukin-1 β , tumor necrosis factor α and other apoptotic stimuli increase mRNA and protein levels of *ITPR1* (48). These findings suggest that curcumin may serve an anticancer role in human osteosarcoma U-2 OS cells by regulating *ITPR1*.

In conclusion, a total of 201 DEGs were identified in the curcumin-treated group compared with the control group. Curcumin may serve an anticancer role by regulating apoptosis, proliferation, migration and invasion in human osteosarcoma U-2 OS cells. In addition, *ITPR1* may be involved in the mechanism of action of curcumin treatment of osteosarcoma.

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