MCUR1 Promotes Osteosarcoma Cell Growth Through AKT/p53 Pathway

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Abstract

Background: Osteosarcoma (OS) is one of the most aggressive malignant bone tumor worldwide. This study focuses on investigating the mechanism underlying the mitochondrial calcium uniporter regulator 1 (MCUR1)-mediated osteosarcoma (OS) cell growth.

Methods: A total of 49 patients diagnosed as OS in our hospital were included in the current study. The expression of MCUR1 in human OS tissues and various OS cell lines was detected by immunohistochemical staining (IHC) and western blot analysis, respectively. The effect of MCUR1 on AKT/p53 pathway and its correlation with miR-506-3p were investigated by a series of experiments including MTS, QRT-PCR, Western blot and IHC.

Results: Our data showed that MCUR1 was highly overexpressed in OS tumor tissues compared with the para-carcinoma tissues (P<0.01). The Kaplan-Meier analysis showed that high expression level of MCUR1 was linked with poor prognosis of OS patients. Additionally, knockdown of MCUR1 enhanced OS cell apoptosis and decreased the growth of OS cells compared with the corresponding controls (P<0.05). Meanwhile, the expression level of p-AKT was decreased, whereas the protein expression level of p53 was increased in OS cells with MCUR1 downregulation. We also found that the IHC scores of MCUR1 were inversely correlated with that of p53 (r = -0.304, P=0.034). Likewise, the expression of MCUR1 and miR-506-3p in OS tissues were also inversely correlated (r=-0.304, P=0.034).

Conclusions: MCUR1 is overexpressed in OS cells and its expression is regulated by miR-506-3p. MCUR1 facilitates the progression of OS through activating AKT/p53 pathway. The data in the current study suggests that MCUR1 may serve as a new target for the diagnosis and treatment of OS.

Background

Osteosarcoma (OS) is one of the most common primary malignant bone tumor and aggressive malignant tumor worldwide. It is very likely to occur in childhood and adolescence [1]. Although chemotherapy in combination with surgery can improve the prognosis of patients with OS, no significant breakthrough has been achieved in OS treatment during the past decade due to the heterogeneity of OS [2]. Thus, there is an urgent need to discover new drugs to treat OS. Also, due to the lack of knowledge regarding the biological changes in the occurrence and development of OS, limited progress has been made in the early diagnosis and developing effective treatment strategies of OS [3, 4]. Thus, it is necessary to investigate the mechanism underlying the pathogenesis of OS.

Mitochondria are important organelles that not only play essential roles in providing energy to eukaryotic cells, but also participate in regulating the calcium signaling in the cells [5]. Alternations in the calcium ion uptake by mitochondria harbor important regulatory roles in ATP production, glycolipid metabolism, autophagy as well as in determining the cell fate [6]. Mitochondrial calcium uniporter regulator 1 (MCUR1) belongs to an essential component of a mitochondrial uniporter channel complex that mediates mitochondrial Ca^{2+} uptake, thus playing imperative roles in regulating the homeostasis of calcium in

mitochondria [7, 8]. To investigate the biological functions of MCUR1 in OS growth, this study examined the expression level of MCUR1 in patients carrying OS. Moreover, the biological roles of MCUR1 in OS cell growth and apoptosis were also studied in vitro. Additionally, the possible causes for elevated expression of MCUR1 in OS tissues and the mechanism underlying MCUR1-promoted OS cell growth were discussed as well. Taken together, this study will provide molecular basis and novel strategies for OS diagnosis, prognosis and treatment.

Methods

Basic Information of Participants

Tumor tissues and matched adjacent non-cancerous tissues were acquired from 49 patients who underwent surgery at Xijing Hospital affiliated with Air Force Military Medical University from 2016 to 2020. The inclusion criteria for OS patients were set as follows: (1) post-operative pathological examination confirmed the diagnosis of OS; (2) no treatment of chemotherapy or radiotherapy before surgery; (3) the completeness of clinical and follow-up data; (4) no history of other malignancy. The last follow-up date was November 2020 and the clinical information of each patient was summarized in Table 1. The median follow-up duration was 18 months (from 3.5 to 32.5 months).

Table 1
Correlation analysis of MCUR1 expression level and pathological characteristics in OS patients

Characteristic	*n*	MCUR1 protein levels		χ^2	*P*
	(49)	Low (*n* = 24)	High (*n* = 25)		
Age (year)				0.163	0.686
≤ 18	32	15	17		
> 18	17	9	8		
Gender				0.017	0.897
Female	22	11	11		
Male	27	13	14		
Tumor size (cm)				7.411	0.006
< 5	21	15	6		
≥ 5	28	9	19		
Tumor stage				4.601	0.032
I + II	21	14	7		
III + IV	28	10	18		
Lymph node metastasis				3.600	0.058
No	28	17	11		
Yes	21	7	14		
Distant metastasis				3.760	0.052
No	30	18	12		
Yes	19	6	13		
TNM (AJCC, 8.0): Tumor-Nodes-Metastases					

Cell Lines and Reagents

The normal human osteoblast cell line hFOB and OS cell lines U2OS、SAOS2、HOS、MG63 and 143B were purchased from American Type Culture Collection (ATCC). Fetal bovine serum was from Gibco (USA) and Lipofectamine™ 2000 was from Invitrogen (USA). MCUR1 siRNAs (MCUR1 siRNA sequence #1: CUUCGACACUCAUGCCUUA;#2: GGAAAUCACUUUUCAGCAA) and negative control shRNA were synthesized by GenePharma (Shanghai, China). MCUR1 antibody was purchased from Sigma (USA) and

β-actin antibody was from Proteintech Group (Wuhan, China). AKT□p-AKT and p53 antibodies were from Cell Signaling (USA). MTS assay kit was from Promega (USA) and Annexin V-FITC kit was from BestBio (Shanghai, China).

Cell Culture and Transfection

The OS SAOS2 cells were routinely cultured at 37℃ and 5% CO_2 in McCoy's 5A medium supplemented with 10% fetal bovine serum. SAOS2 cells were seeded in 6-well plates for transfection when the confluence of the cells reaches 80%. siRNAs targeting MCUR1 or control siRNA were employed to transfect SAOS2 cells using Lipofectamine™ 2000 following the manufacture's protocols. Transfected cells were harvested 48 hr later for further experiments.

Immunohistochemical Staining (IHC)

IHC analysis was carried out according to standard protocols described previously [9]. In brief, paraffin-embedded tissues were sectioned and treated with boiling citrate buffer (pH = 6.0) under high pressure for antigen retrieval. Primary antibody was then used to bind the protein of interest on the sections for overnight at 4℃, followed by color development with the addition of 3,5-diaminobenzidine (DAB) substrate and counterstaining with hematoxylin. The IHC score was determined following a previous study [10] with following modifications: six random microscopic visual fields per slide (400-fold magnification) were selected to count the proportion and intensity of positive cells; a total score < 6 and > 6 were defined as low expression and high expression of p53 or MCUR1, respectively.

Western Blot Analysis

Western blot experiments were carried out according to standard protocols [11]. In brief, total proteins were extracted using the radioimmunoprecipitation (RIPA) buffer. The concentration of total protein was measured using the Nanodrop. The proteins were then separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was then incubated with a specific primary antibody overnight, followed by incubation with a peroxidase-labelled secondary antibody. The enhanced chemiluminescence system was then used to develop immunoblots.

Cell Viability and Apoptosis Assays

Cell viability and apoptosis were determined by the MTS assay kit and ANXA5/annexin V-FITC Apoptosis Detection Kit, respectively. For the MTS assay, $1x10^3$ SAOS2 cells were plated a 96-well culture plate. Cell viability was determined by adding 20μL of MTS reagent and incubated for 2 h. The spectrophotometer was employed to determine the absorbance of the cells at 490 nm. Each sample was analyzed in triple. For the apoptosis assay, 500 μL binding buffer was used to resuspend the SAOS2 cells with a concentration of 10^6 cells/mL. Cells were mixed and incubated at room temperature in the dark for 15 min after the addition of 5 μL ANXA5-FITC and 5 μL PI. A flow cytometer was utilized to analyze the samples. Each sample was analyzed in triple.

Statistical Analysis

SPSS 17.0 software (SPSS, Chicago, IL, USA) was utilized for all statistical analyses and *P*-value < 0.05 was considered statistically significant. All experiments were conducted in triplicate, where appropriate. Significant differences between two groups were determined according the result obtained from Student t-test. The differences between tumor tissue and adjacent nontumor tissues were analyzed using the Paired two-tailed t-tests. The correlations between measured variables were analyzed by Pearson or Spearman correlation.

Results

The Expression of MCUR1 in OS Tissues

To explore the functional role of MCUR1 in the tumorigenesis of OS, we investigated the expression level of MCUR1 in OS tissues. The immunohistochemical (IHC) analysis revealed that MCUR1 appeared to be predominantly localized in the cytoplasm (Fig. 1A). Moreover, IHC score of 49 paired OS and adjacent nontumor tissues showed that the expression level of MCUR1 was significantly upregulated in OS tissues compared to nonmalignant tissues (6.286 ± 3.725 *vs* 1.939 ± 1.625, t = 8.514, *P* < 0.001) (Fig. 1B). The expression level of MCUR1 was further examined in a series of OS cell lines (SAOS2、MG63、HOS、143B、U2OS) and normal human osteoblast cell line (hFOB). qRT-PCR and Western blot analysis indicated that MCUR1 was overexpressed in OS cells at both mRNA and protein levels, respectively, compared with normal human osteoblast cells (Fig. 1C and 1D).

MCUR1 Expression and Pathological Features of OS Patients

Patients with OS were categorized into two groups including MCUR1 high-expression group and MCUR1 low-expression according to the median level of MCUR1. By analyzing the pathological data, we found that the protein expression level of MCUR1 were associated with the tumor size and tumor stage (*P* < 0.05). However, no statistical differences exist in lymph node metastasis and distant metastasis between the MCUR1 low-expression group and MCUR1 high-expression group (*P* > 0.05) (Table. 1).

The Relationship Between MCUR1 Expression and Prognosis of OS Patients

The Kaplan-Meier analysis of 49 patients with OS showed that there was a higher overall 3-year survival rate in OS patients with low MCUR1 expression (87.5%) compared with those with high MCUR1 expression (35.4%) (log-rank *P* = 0.027). The median overall survival was 29 months (min 15.9- max 42.1 months) for OS patients with high MCUR1 expression. However, the overall survival was remarkably improved in OS patients with MCUR1 low-expression (Fig. 2).

Knockdown of MCUR1 Inhibits SAOS2 Cell Growth and Increases Cell Apoptosis

The R2 (Genomics Analysis and Visualization Platform) was employed to analyze the KEGG pathways which are closely related with MCUR1. The data implied that MCUR1 may possess important roles in apoptosis (Fig. 3A). To verify this hypothesis and to examine the effect of MCUR1 on OS cell growth, we

have successfully established MCUR1 knocked-down SAOS2 cells by transfecting siRNA targeting MCUR1. As shown in Fig. 3B and 3C, both the qRT-PCR and Western blot data showed that the expression of MCUR1 was remarkably lower in siMCUR1 group compared to the corresponding controls. MTS assays were then carried out to measure the cell viability of SAOS2 cells. The result indicated that knock-down of MCUR1 suppressed the growth SAOS2 cells compared to the control cells ($P < 0.01$) (Fig. 3D). To investigate the effect of MCUR1 knockdown on the apoptosis of SAOS2 cells, the rate of SAOS2 cell apoptosis was measured by flow cytometry. Our data showed that the rate of SAOS2 cell apoptosis was higher in MCUR1-knocked down SAOS2 cells (30.867 ± 3.729)%(32.633 ± 4.365)% compared with that of control cells (14.300 ± 2.066)% (Fig. 3E). These results suggested that knock-down of MCUR1 inhibited SAOS2 cell growth and increased the rate of SAOS2 cell apoptosis.

The Mechanism Underlying the MCUR1-Mediated SAOS2 Cell Apoptosis

Western blot experiments showed that the protein expression level of AKT was not obviously changed in MCUR1 knocked-down SAOS2 cells compared to the corresponding controls. However, the protein expression level of phospho-AKT (p-AKT) was decreased, whereas the protein levels of p53 were remarkably increased (Fig. 4A). As displayed in Fig. 4B, the mRNA level of p53 was not obviously changed in MCUR1 knocked-down SAOS2 cells compared with the control cells according to the qRT-PCR analysis. Furthermore, the IHC analysis revealed that the expression of p53 protein was inversely correlated with the protein level of MCUR1 in OS tissues (Fig. 4C and 4D). Collectively, these data indicated that MCUR1 promoted SAOS2 cell apoptosis may be through activating AKT/p53 pathway.

Regulation of MCUR1 in OS Tissues

MicroRNA usually plays significant roles in regulating the gene expression [12]. Thus, the MicroRNA Data Integration Portal (mirDIP) was used to explore the possible microRNA that may be involved in mediating the expression of MCUR1 in OS. The data indicates that hsa-miR-506-3p is likely to harbor a key role in regulating MCUR1 (Fig. 5A). Thus, qRT-PCR assays were carried out to verify this hypothesis. The result showed that the upregulation of miR-506-3p resulted in a decrease of mRNA and protein levels of MCUR1 in SAOS2 cells compared with the corresponding controls (Fig. 5B and 5C). Moreover, Pearson correlation analysis revealed that the mRNA level of MCUR1 was inversely correlated with the level of miR-506-3p, suggesting that miR-506-3p may participate in regulating the expression of MCUR1 in SAOS2 cells (Fig. 5D).

Discussion

Mitochondria are important organelles that not only supply energy to eukaryotic organisms, but also play essential roles in various cellular pathways; including but not limited to cell proliferation, apoptosis, autophagy, carbon metabolism, lipid metabolism and amino acid metabolism [13, 14]. Moreover, mitochondria are also bioenergetic and biosynthetic organelles that enable cells to adapt to various stresses such as oxidative stress and apoptosis [15]. Thus, mitochondria are reported to be imperative regulators of tumorigenesis, of which process generally requires adaption to cellular and environmental

changes [15, 16]. These evidence suggests that it is necessary to understand the biology of mitochondria in cancer in order to develop novel cancer treatment strategies [17]. It has been well documented that calcium ion, one of the most important second messenger molecules, harbors vital roles in a broad spectrum of physical processes including cell proliferation, apoptosis, autophagy and metabolism [18]. In recent years, accumulating evidence indicates that deregulation of mitochondrial calcium uniporter complex is associated with various types of cancers including breast cancer [19], hepatocellular carcinoma (HCC) [20], colon cancer [9] and pancreatic cancer [21]. Since mitochondrial calcium uniporter (MCU) complex are mainly responsible to regulate calcium homeostasis in mitochondria, it is thus suggested that altered calcium ion level is linked to tumor growth, progression and metastasis [22]. MCUR1 was found to be localized in the inner membrane of mitochondria and involved in calcium ion transport in a MCU-dependent manner [8]. Additionally, several studies indicate that MCUR1 can effectively increase calcium ion uptake into the mitochondria and plays an essential role in maintaining intracellular Ca^{2+} homeostasis [7, 8]. Consistently, Tomar et.al found that ablation of MCUR1 in mouse cardiomyocytes and endothelial cells can interrupt the mitochondrial calcium uptake process. Moreover, ablation of MCU and MCUR1 in vascular endothelial causes an adverse effect on mitochondrial bioenergetics, cell proliferation, and migration [23]. Another study suggests that knockdown of MCUR1 inhibits the oxidative phosphorylation, reduces ATP production and promotes AMP-dependent autophagy [8]. A previous study in our research group indicates that MCUR1 is overexpressed in HCC to increase the calcium ion uptake into mitochondria, which then results in enhanced cell survival and proliferation of HCC cells [20]. Taken together, the above studies have shown that MCUR1 is important for mitochondrial functions and fundamental cellular processes. Deregulation of MCUR1 can result in deterioration in calcium balance and mitochondrial dysfunction, which ultimately affecting cell growth and apoptosis.

Our data indicated that MCUR1 was upregulated in OS tumor tissues compared with the paired nonmalignant tissues. Similarly, we found that MCUR1 was also overexpressed in OS cell lines compared to normal human osteoblast cells. The high protein expression level of MCUR1 in OS tissues led to a poor prognosis of patients with OS, implying that MCUR1 may possess a pivotal role in OS tumoriogenesis. A growing body of evidence suggests that calcium homeostasis is associated with AKT/p53 signaling pathway, which plays a role in apoptosis [24, 25]. Consistently, our results showed that knockdown of MCUR1 in OS cells decreased the protein level of phospho-AKT and increased the protein level of p53. In the meantime, the protein expression of MCUR1 in OS tissues was inversely correlated with the protein expression level of p53. Similarly, a previous study indicates that phospho-AKT can lead to p53 degradation through p-MDM2 [26]. Collectively, the above results implied that MCUR1 may inhibit OS cell apoptosis through activating AKT/p53 pathway. MicroRNA has been proven to play vital roles in mediating the gene expression [12]. A series of experiments indicated that the expression of miR-506-3p was inversely correlated with the expression of MCUR1. This finding was consistent with a previous study, which indicated that miR-506-3p was downregulated in OS tissues [21]. Our study suggested that miR-506-3p possessed a pivotal role in mediating the expression of MCUR1. Due to the limited number of patient samples in this study, our conclusions in this study need to be further validated with more patient samples in the future.

Conclusions

In conclusion, MCUR1 expression was upregulated in OS tissues and elevated expression of MCUR1 was associated with poor prognosis of OS patients. MCUR1 may be regulated by miR-506-3p and promotes OS progression through activating the AKT/p53 signaling pathway.

Abbreviations

OS: Osteosarcoma; MCUR1: mitochondrial calcium uniporter regulator 1; IHC: immunohistochemical; MTS:3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium: QRT-PCR: Quantitative reverse transcription PCR; p53: protein 53; siRNA: small interfering RNA; DAB: 3,5-diaminobenzidine; RIPA: radioimmunoprecipitation; MDM2: mouse double minute-2 homolog; PVDF: polyvinylidene fluoride; MCU: mitochondrial calcium uniporter.

Declarations

Acknowledgements

Not applicable

Authors' Contributions

Study concepts and design: All authors; Data acquisition: WYY, TR, HZH and TYY; Data Analysis and interpretation: WYY, CYF, ZHX and YP; Statistical analysis: TR, HZH and ZHX; Manuscript preparation: WYY, TR and YP; Manuscript editing and approval of the final article: All authors.

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Availability of data and materials

The datasets will be available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The Ethics Committee of Air Force Military Medical University approved this study and each participant signed an informed consent form.

Consent for publication

Not applicable

Competing Interests

The authors declare that they have no conflicts of interest to report regarding the present study.

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Figures

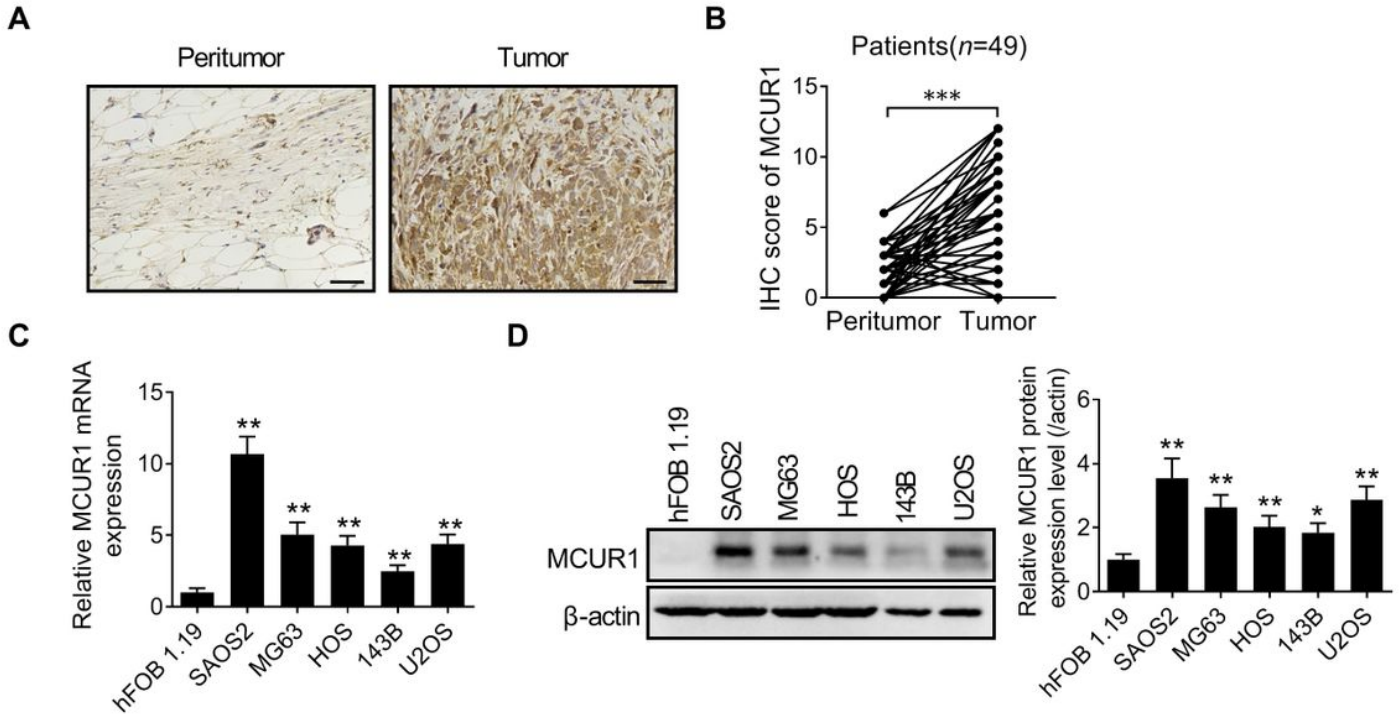

Figure 1

Expression of MCUR1 in OS tissues and OS cell lines. (A) Representative IHC staining images of MCUR1 in OS peritumor and tumor tissues. (B) IHC score of MCUR1 in OS peritumor and tumor. (C) qRT-PCR analysis for the mRNA level of MCUR1 in various OS cell lines and normal human osteoblast cells. (D) Western blot analysis of protein level of MCUR1 in various OS cell lines and normal human osteoblast cells. **P<0.01; ***P<0.001.

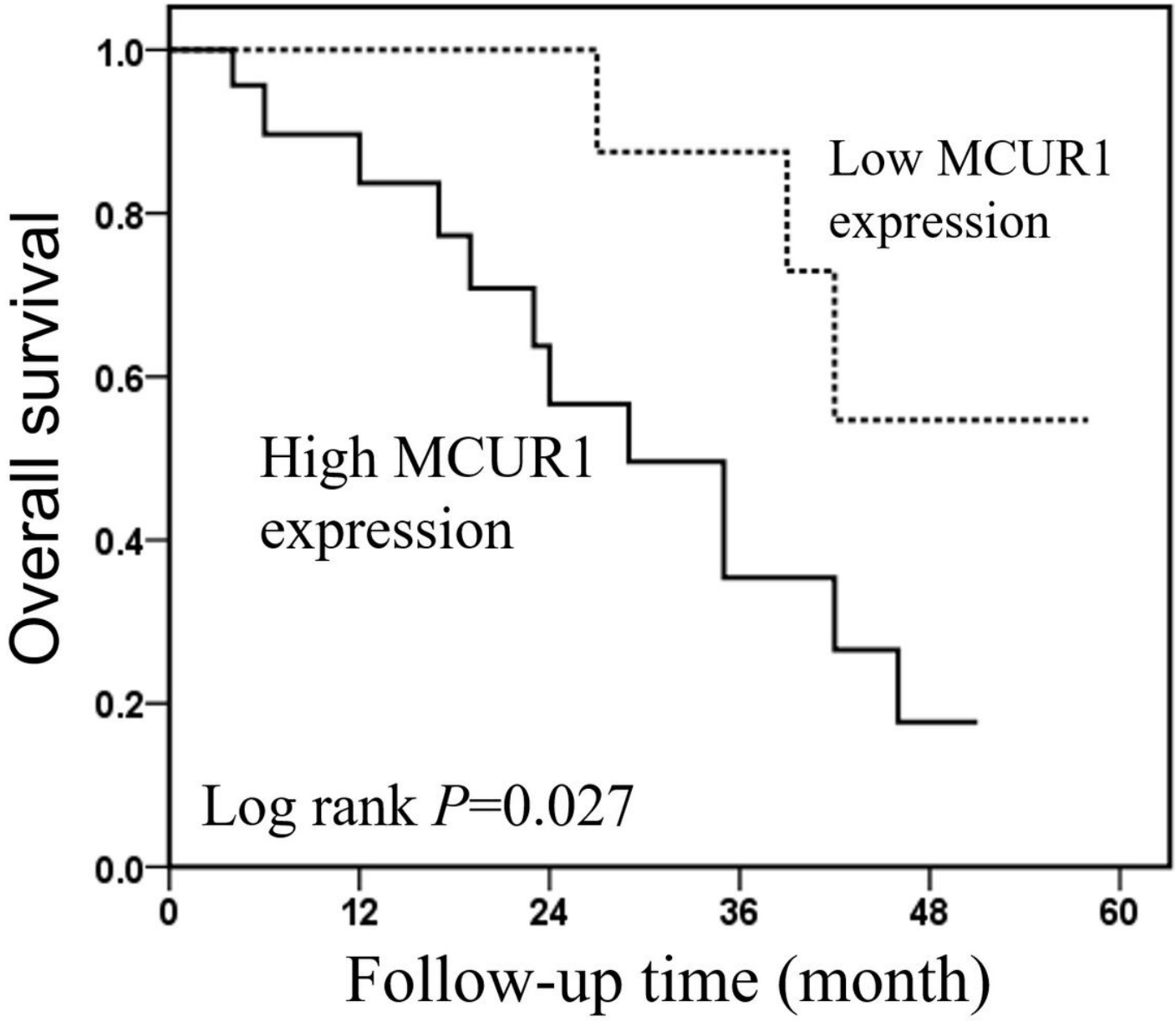

Figure 2

Kaplan-Meier plot of overall survival of OS patients by MCUR1 expression.

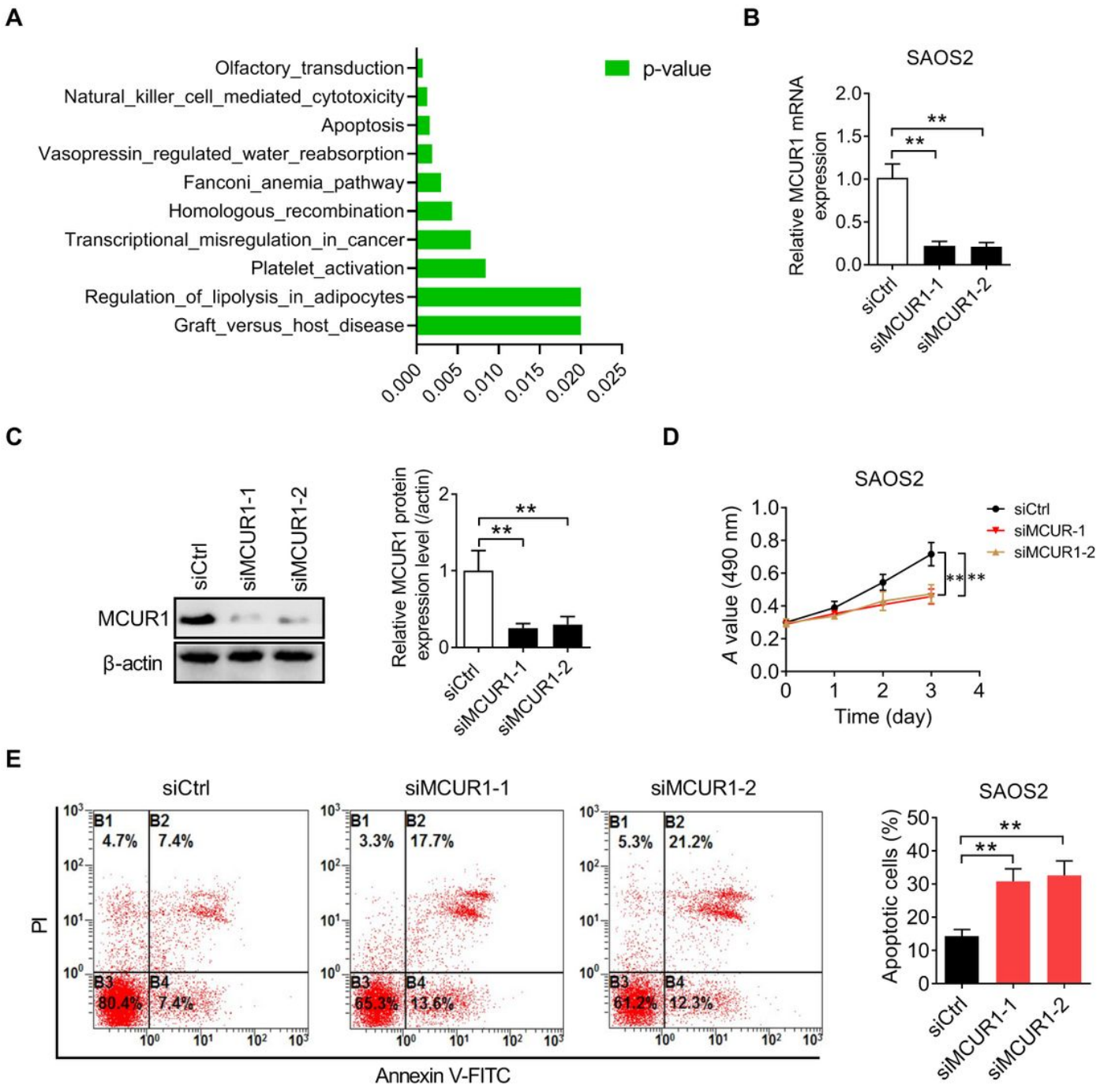

Figure 3

Effect of MCUR1 knockdown on SAOS2 cell growth. (A) Top ten predicted significant KEGG pathways of MCUR1 using R2 (Genomics Analysis and Visualization Platform) in human OS tissues; (B) qRT-PCR analysis for MCUR1 mRNA level in SAOS2 cells with treatment as indicated; (C) Western blot analysis for MCUR1 mRNA level in SAOS2 cells with treatment as indicated. (D) MTS analysis for cell growth of SAOS2 cells with treatment as indicated. (E) Analysis of cell apoptosis by flow cytometry in SAOS2 cells with treatments as indicated. **P<0.01

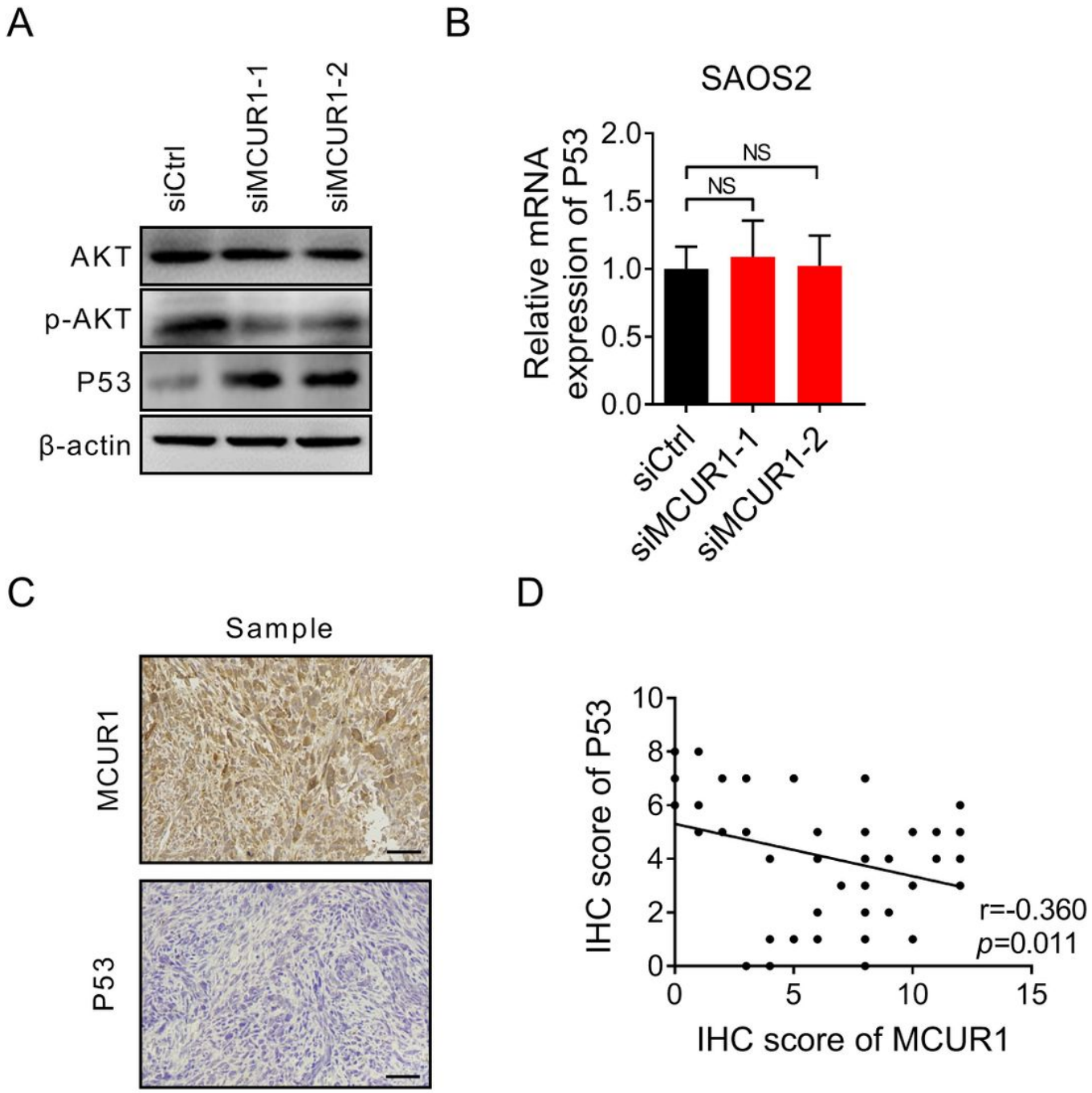

Figure 4

The effects of MCUR1 on AKT/p53 pathway. (A) Western blot analysis for the protein expression levels of AKT, p-AKT and p53 in SAOS2 cells with treatment as indicated; (B) qRT-PCR analysis for p53 expression in SAOS2 cells with treatment as indicated; (C) Representative IHC images of MCUR1 and p53 in human OS tissue; (D) The correlation was determined between the protein expression levels of MCUR1 and p53 in 49 OS tissues according to the IHC staining.

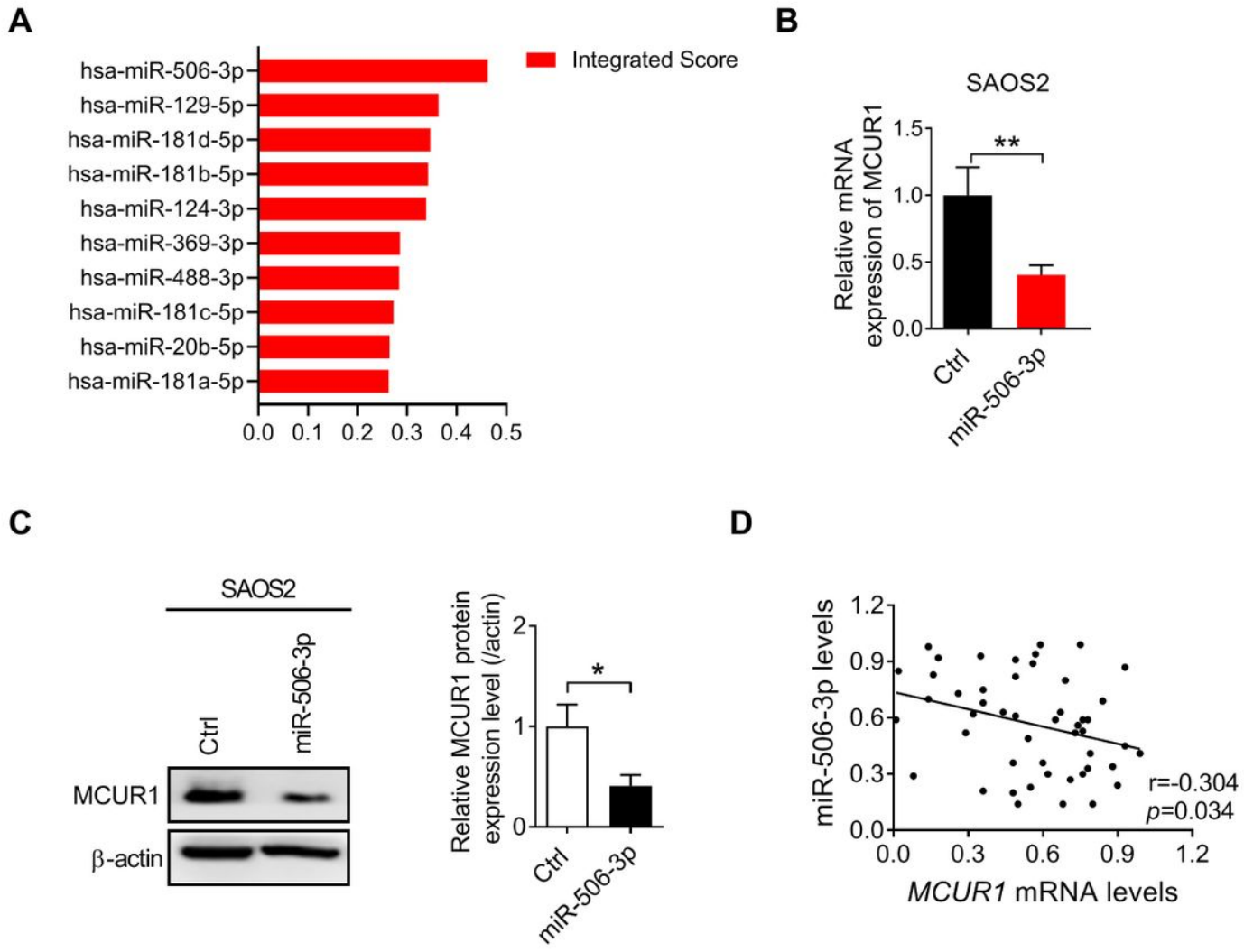

Figure 5

The correlation between MCUR1 expression and miR-506-3p. (A) Top ten predicted miRNAs targeting MCUR1 using microRNA Data Integration Portal (mirDIP); (B) qRT-PCR analysis for the expression of MCUR1 in SAOS2 cells transfected with the miR-506-3p mimics; (C) Western blot analysis for MCUR1 expression in SAOS2 cells transfected with the miR-506-3p mimics; (D) The correlation of mRNA levels between MCUR1 and miR-506-3p was determined.