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ORIGINAL ARTICLE

Low Arid1a Expression Correlates with Poor Prognosis and Promotes Cell Proliferation and Metastasis in Osteosarcoma

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Abstract AT-rich interactive domain-containing protein 1A (ARID1A) has been shown to function as a tumour suppressor in various malignancies. However, the biological role of ARID1A in osteosarcoma is not clear. The present study aimed to investigate the expression pattern, prognostic value and the biological role of ARID1A in human osteosarcoma. ARID1A expression in 53 osteosarcoma surgical specimens was examined by quantitative real-time polymerase chain reaction, and its clinical significance was analysed. The role of ARID1A in cell proliferation, apoptosis, and metastasis were examined. ARID1A mRNA expression were significantly down-regulated in osteosarcoma tumours from that in matched adjacent non-tumour tissues. ARID1A expression was significantly inversely correlated with tumour stage and distant metastasis, as well as poor overall survival in patients with osteosarcoma. Furthermore, ARID1A mRNA was down-regulated in four human osteosarcoma cell lines MG-63, U2OS, HOS and Saos-2. Restoring of ARID1A expression in MG-63 and U2OS cells significantly inhibited cell proliferation and metastasis *in vitro*. Collectively, our data demonstrate that ARID1A may serve as a tumour suppressor in osteosarcoma progression, and represent a valuable prognostic marker and potential therapeutic target for osteosarcoma.

Keywords ARID1A · Osteosarcoma · Tumour suppressor · Proliferation · Metastasis Prognosis

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Introduction

Osteosarcoma (OS) is an aggressive malignant mesenchymal neoplasm among children and young adults [19]. Currently, osteosarcoma is very difficult to treat, patients who developed lung metastases frequently have a worse prognosis, and their five year survival rate are far lower [1]. At present, there are few prognostic factors used in the clinic for osteosarcoma. Several biomarkers are reported in the literature to be associated with patients' prognosis and metastasis, such as tyrosine kinase receptors EGFR [12], cell cycle kinase inhibitor p27 [14], cytochrome P450 CYP3A4/5 [5], and Cathepsin D [7]. However, none of them is currently evaluated as a routine in the clinic. Therefore, to understand the molecular pathogenesis of this disease is essential to design new, effective therapeutic strategies to improve patient survival.

BRG1-associated factor 250a (BAF250a), which encoded by AT-rich interactive domain-containing protein 1A (ARID1A), is one of the accessory subunits of the switching defective/sucrose non-fermenting (SWI/SNF) complexes [23]. Recently, loss of ARID1A protein and ARID1A mutations have been detected in a wide variety of human cancers, as revealed by several genome-wide sequencing studies [10, 11, 22, 27]. Although many of the functions of ARID1A remain unknown, it is believed that ARID1A is a potential candidate tumor suppressor gene [9, 24, 28]. However, the clinical significance and the biological function of the ARID1A in osteosarcoma remain undefined.

In the present study, we analyzed the expression status of ARID1A in osteosarcoma tissues and cell lines. Meanwhile, we identified the relationships between ARID1A expression

and clinical factors and evaluated its prognostic value in osteosarcoma patients. Additionally, we evaluated the functional role of ARID1A in tumor growth, apoptosis and metastasis by *in vitro* experiments. Our data demonstrate that ARID1A may serve as a tumour suppressor in osteosarcoma progression, and represent a valuable prognostic marker and potential therapeutic target for osteosarcoma.

Material and Methods

Clinical Samples

The tissue samples and the clinical data were obtained from the Department of Gastroenterology and Hepatology, Chinese PLA General Hospital between 2009 and 2010. 53 snap-frozen tissue samples were used. The histology of each sample was reviewed by a pathologist to confirm the diagnosis before being used in this work. The study was approved by the Ethics Committee of Chinese PLA General Hospital. Clinical characteristics of all patients are given in Table 1.

Table 1 Association between ARID1A expression and clinicopathological features of 53 osteosarcoma patients

Characteristics	Cases (53)	ARID1A expression		*P* value
		Low (35)	High (18)	
Age (years)				
≤ 18	30	20	10	0.912
> 18	23	15	8	
Gender				
Male	33	23	10	0.470
Female	20	12	8	
Tumor site				
Femur/Tibia	43	30	13	0.234
Others	10	5	5	
Tumor stage				
I + II	28	14	14	0.011
III	25	21	4	
Tumor diameter (cm)				
≤ 6	38	23	15	0.215
> 6	15	12	3	
Distant metastasis				
Yes	19	17	2	0.008
No	34	18	16	
Metastasis situation				
Pulmonary metastasis	15	10	5	0.603
Other	4	2	2	
Differentiation status				
High	26	15	11	0.208
Low	27	20	7	

Cell Culture

The cell lines used in this study were: human osteosarcoma cell lines (MG-63, U2OS, HOS and Saos-2), human normal osteoblastic cell line hFOB 1.19, human normal osteoblast cells NHOst, They were all purchased from American Type Culture Collection biobank (ATCC; Rockville, MD, USA) and maintained in our lab. Cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, and with 100 U/ml penicillin and 100 U/ml streptomycin at 37 °C with 5% CO_2 in a humidified incubator. Cells were split using trypsin-EDTA 1:3 to 1:10, depending of their growth rate, two to three times a week.

mRNA Extraction and Real-Time Quantitative PCR

Total RNA was extracted from tumor tissues and cell lines with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The concentration and quality of the mRNA obtained was monitored using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from mRNA using M-MLV reverse transcriptase (Invitrogen). The resulting cDNA was then subjected to real-time quantitative PCR for evaluation of the relative mRNA levels of ARID1A and β-actin with the following primers: ARID1A forward: 5′-CTTCAACCTCAGTCAGCTCCCA-3′ and reverse: 5′-GGTCACCCACCTCATACTCCTTT-3′; β-actin forward: 5′- GTGGACATCCGCAAAGAC-3′, and reverse 5′-AAAGGGTGTAACGCAACTA -3′. Gene-specific amplification was performed on ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The expression level of ARID1A was normalized to the expression of the housekeeping gene β-actin using the comparative threshold cycle ($2^{-\Delta Ct}$) method.

Construction of ARID1A Overexpressing Cell Lines

The full-length cDNA sequences of the human ARID1A cDNA were purchased from Sino Biological (Beijing, China). The full length cDNA of ARNT2 was cloned into pcDNA3.1 vector, the recombinant plasmid pcDNA3.1-ARID1A and control plasmid were transferred into MG-63 or U2OS cells using Lipofectamine™2000 (Invitrogen). After transfection, the cells were diluted at the ratio of 1:5 into 12-well plate and the medium was changed with medium containing G418 (600 μg/mL). The stable cell lines were obtained after 28 days screening by G418. Stably transfectant clones were validated by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting for the level of target gene expression.

MTS Assay

Cells were plated in triplicate in 100 μl of medium in a 96-well plate at 5×10^3 cells. The cells were grown in the usual conditions and their proliferation was assessed every 12 h from day 1 to day 3 using the MTS assay CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) as instructed by the manufacturer. Briefly, 20 μl of MTS solution was added to each well and they were incubated at 37 °C and 5% CO2 for 1 h. The reaction was then blocked by adding 25 μl of 10% SDS to the cells, and the absorbance at 490 nm was measured using a microplate reader (Bio-Tek Company, Winooski, VT, USA).

Cell Apoptosis Assay

Cell apoptosis was evaluated by flow cytometry using an Annexin V-FITC Apoptosis Detection Kit (KeyGen Biotech Co., Nanjing, China). Briefly, cells were harvested, washed in PBS, and resuspended in 500 μL binding buffer. A volume of 5 μL Annexin V-FITC and 5 μL propidium iodide was added and mixed gently, and the cells were stained in the dark for 10 min at room temperature. The cells were analyzed immediately by FACS Calibur.

Transwell Assay

Cell migration was assessed using a 24-well transwells assay (Minipore, Billerica, MA, USA) with 8-μm pore size. Cells were plated at a density of 2×10^4 cells in 100 μL DMEM with 1% FBS on the upper chamber in each transwell. The bottom of the well contained 1 ml of DMEM with 10% FBS. After an overnight incubation at 37 °C and 5% CO2, the wells were rinsed with PBS and the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. The transwells were washed with PBS and the cells were then permeabilised with 0.5% triton-X in PBS for 3 min. They were then washed with PBS and stained with Crystal violet for 5 min. After washes, the transwells were mounted under a coverslip and the migrating cells were observed under an inverse-light microscope. The number of migrating cells was then counted in four different areas for each well.

Protein Extraction and Western Blotting

20–50 μg of total protein from osteosarcoma cell lines and their derivates was extracted and separated by 10% SDS-PAGE, after which the protein was transferred onto polyvinylidene fluoride membranes, and then the membranes were incubated overnight at 4 °C with primary antibody against ARID1A, Bcl-2, Bax, E-cadherin, N-cadherin, and β-actin (all from Cell Signaling Technology, Beverly, MA, USA). The membranes were then washed three times with TBST (tris-buffered saline with tween-20) and probed with the horseradish peroxidase (HRP)-conjugated seconded IgG antibody at 37 °C for 1 h. The bands were visualized using BioImaging Systems (UVP Inc., Upland, CA, USA).

Statistical Analysis

All experiments were repeated as independent replicates. Error bars represent the standard deviation from the mean. Mann-Whitney U test (for unpaired continuous data), Student's t-test

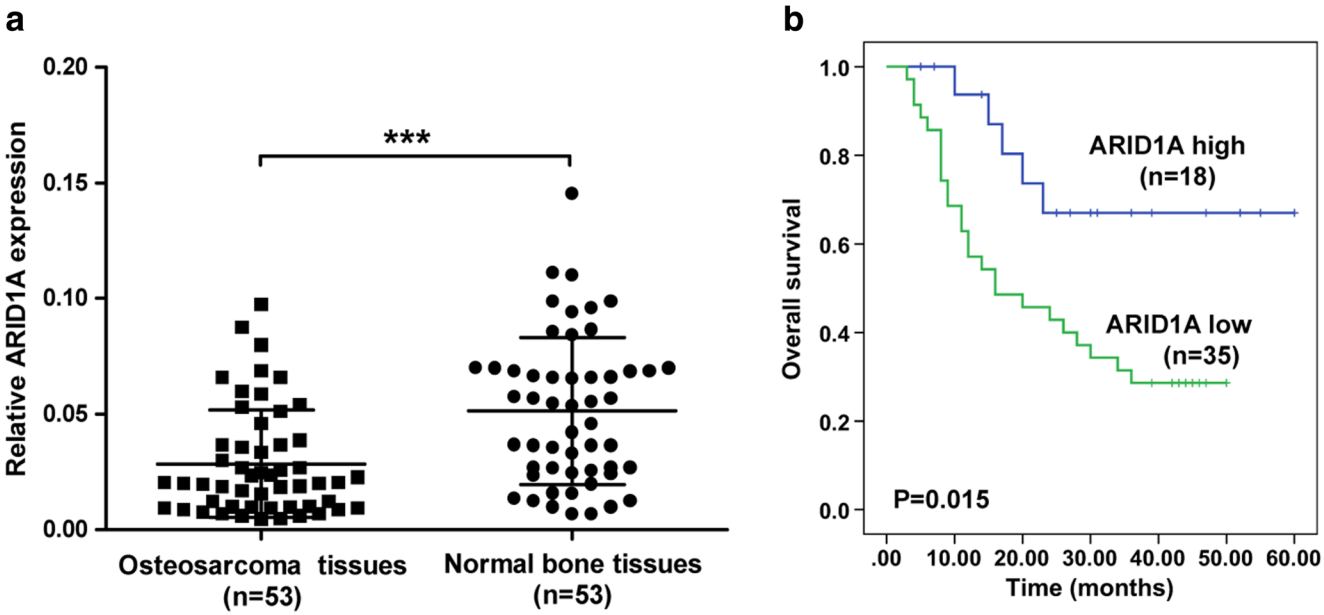

Fig. 1 **a** Expression of ARID1A in 53 cases of osteosarcoma tissues and matched adjacent normal bone tissues as assessed by quantitative RT-PCR. **b** Kaplan-Meier survival curve for overall survival according to ARID1A expression from 53 cases of osteosarcoma patients. The *P*-value was calculated using the log-rank test. $^{***}P < 0.001$

Table 2 Univariate and multivariate analyses of overall survival in osteosarcoma patients

Factors	Univariate			Multivariate		
	Hazard Ratio	95%CI	*P*	Hazard Ratio	95%CI	*P*
Age, years (≤18/>18)	0.815	(0.478–1.091)	0.691			
Gender (male/Female)	0.934	(0.503–1.134)	0.873			
Tumor diameter (≤6/>6)	1.369	(0.920–1.834)	0.204			
Differentiation status (High/Low)	1.454	(0.742–2.408)	0.097			
Tumor stage (I,II/III)	4.391	(2.353–7.108)	<0.001	2.301	(1.881–6.144)	0.009
Distant metastasis (Yes/No)	5.720	(1.931–6.309)	0.002	1.624	(1.183–4.289)	0.018
Metastasis situation (Pulmonary/other)	1.267	(0.655–1.630)	0.677			
ARID1A expression (Low/High)	0.594	(0.406–1.229)	0.001	0.819	(0.432–1.322)	0.011

(for unpaired continuous and normally distributed data), chi-square test or Fisher exact test (for unpaired categorical data), and log-rank test (for Survival analysis) were used in this study as indicated. The software SPSS version 19.0 (SPSS Inc., Chicago, US) was used for statistical calculations, and differences were considered statistically significant if $p < 0.05$.

Results

ARID1A Expression is Down-Regulated in Tumor Tissues and Correlated with Poor Prognosis of Osteosarcoma

As shown in Fig. 1a, the ARID1A expression level was significantly lower in tumor tissues compared with the adjacent non-tumor tissues (0.028 ± 0.003 vs. 0.051 ± 0.004, $P < 0.001$). ARID1A expression at levels less than the mean expression level were assigned to the low expression group ($n = 35$), and those samples with expression above the mean value were assigned to the high expression group ($n = 18$). As shown in Table 1, low expression of ARID1A expression was significantly correlated with tumor stage and distant metastasis ($P = 0.011$ and $P = 0.008$, respectively).

The overall survival time in patients with high and low ARID1A expression were (45.8 ± 5.2) and (24.6 ± 3.1) months, respectively. Overall survival of patients with a high ARID1A level was significantly longer than survival of those with a negative ARID1A level ($P = 0.015$, Fig. 1b). Univariate analyses showed that tumor stage, distant metastasis and ARID1A expression were significantly associated with overall survival (Table 2), Additionally, multivariate Cox regression analysis confirmed that ARID1A expression(HR = 0.819, 95% confidence interval [CI]: 0.432–1.322, $P = 0.011$) was an independent prognostic factors for overall survival of osteosarcoma patients (Table 2).

ARID1A Inhibits Osteosarcoma Cell Proliferation and Metastasis *in vitro*

The quantitative RT-PCR and Western blotting showed that the expression of ARID1A was lower in osteosarcoma cells (MG-63, U2OS, HOS and Saos-2) and higher in hFOB 1.19 and NHOst cells (Fig. 2a). We then overexpressed ARID1A in

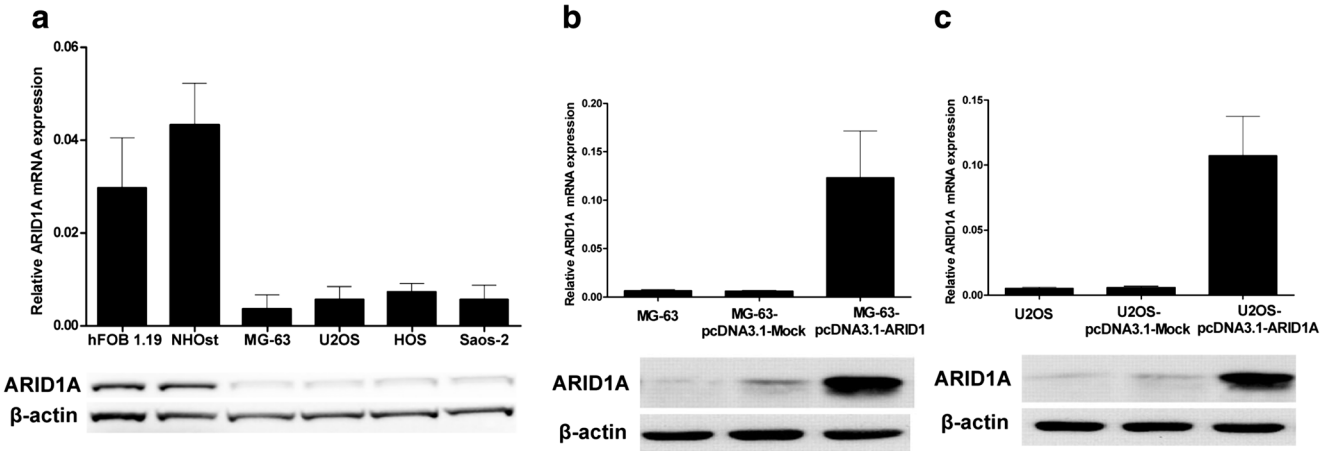

Fig. 2 **a** Expression of ARID1A in osteosarcoma cell lines (MG-63, U2OS, HOS and Saos-2) and human normal osteoblastic cell line hFOB 1.19, human normal osteoblast cells NHOst, as measured by quantitative RT-PCR and western blot. **b** and **c** Quantitative RT-PCR and western blot confirmed ARID1A expression in stably transfected MG-63and U2OS cells and their derivates

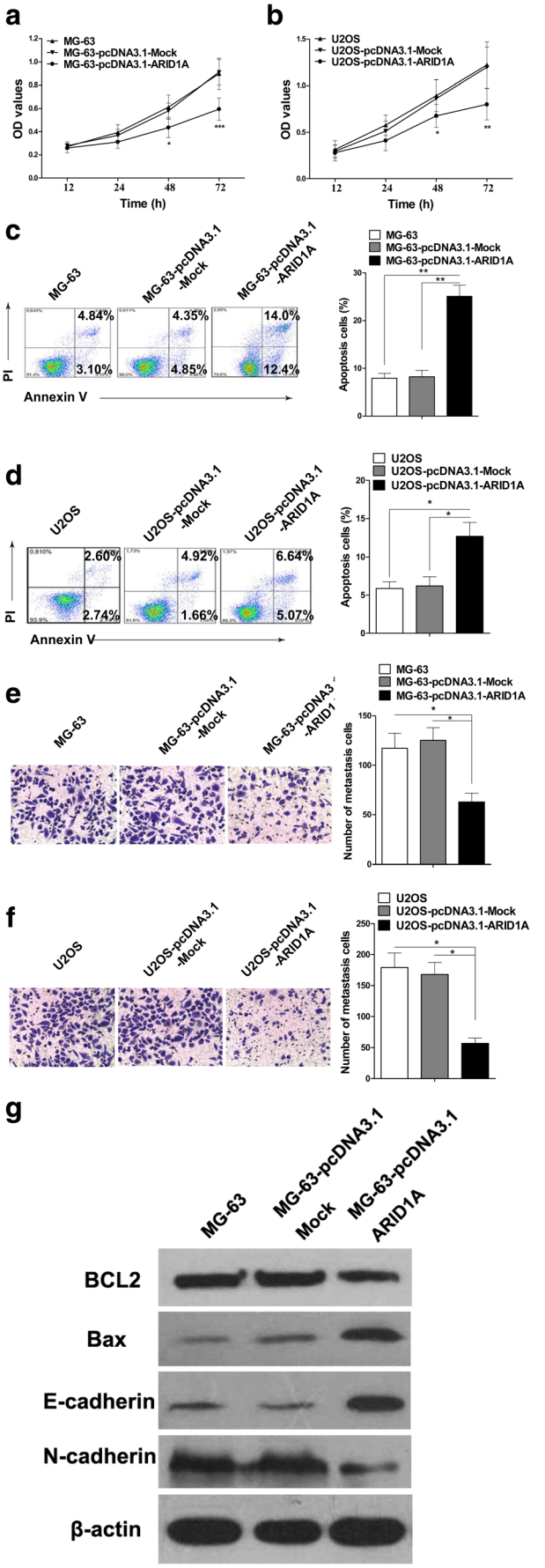

Fig. 3 ARID1A inhibits osteosarcoma cell proliferation and metastasis *in vitro*. **a** and **b**. The cell viability influenced by ARID1A was assessed by time course MTS assay. **c** and **d** The cell apoptosis influenced by ARID1A was assessed by Annexin V/PI apoptosis detection assay. **e** and **f** The cell metastasis influenced by Transwell assay. **g** Cell apoptosis and metastasis-related proteins including Bcl-2, Bax, E-cadherin and N-cadherin in ARID1A overexpressed MG-63 cells were measured by western blot. Data shown are mean ± SD from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$

MG-63 and U2OS cells by pcDNA3.1 plasmid. Data showed the ARID1A protein level was significantly up-regulated in MG-63 (Fig. 2b) or U2OS (Fig. 2c) transfection cells than their Mock control cells. We next evaluated the role of ARID1A on cell proliferation, apoptosis and metastasis of osteosarcoma. The cell viability in the ARID1A over-expression MG-63 or U2OS cells was significantly lower than that in the pcDNA3.1 control cells (Fig. 3a and b). The percentage of apoptotic cells in the ARID1A over-expression MG-63 or U2OS cells were significantly higher than those in the pcDNA3.1 control cells (Fig. 3c and d). Furthermore, over-expression of ARID1A led to decreased in vitro cell metastasis ability compared with those transfected with control plasmid (Fig. 3e and f).

ARID1A Suppresses Proliferation and Migration of Osteosarcoma Cell Via Inhibiting Bcl-2 and N-cadherin Expression

To investigate the potential mechanism by which ARID1A affected cell proliferation and invasion, we explored the change of cell apoptosis and metastasis-related molecules in the stable transfectants. As shown in Fig. 3g, Bax and E-cadherin expression were significantly elevated, while Bcl-2 and N-cadherin expression were markedly decreased in ARID1A overexpressed MG63 cells.

Discussion

In the present study, we confirmed that ARID1A was down-regulated in osteosarcoma tissues than corresponding normal tissues, which was in accordance with previous findings in other malignancies [13, 17, 27]. Up to now, there is controversy regarding the relevance of ARID1A mutation or protein loss to survival. Some studies found that ARID1A mutation or loss was a predictor of worse survival in cervical cancer and gastric cancer [4, 20]. Others found no association between ARID1A mutation or loss and survival in clear cell carcinoma of the endometrium and ovarian clear cell adenocarcinomas [6, 26]. There are also studies indicating that ARID1A mutation or loss was related to a better survival in endometrial carcinoma and colorectal cancer [2, 21]. In our study, downregulaton of ARID1A was significantly correlated with

advanced TNM stage; distant metastasis as well as poor overall survival, suggesting that downregulaton of ARID1A may be associated with tumor growth and tumor metastasis of osteosarcoma. Our observations that the downregulaton of ARID1A expression in osteosarcoma is associated with more malignant phenotypes and a worse prognosis, indicate that ARID1A may play a tumor suppressor role in osteosarcoma carcinogenesis.

In addition, we found that the expression of ARID1A was also decreased in osteosarcoma cell lines. Restoring ARID1A expression in MG63 or U2OS cells significantly inhibited cell proliferation, promoted cell apoptosis and inhibited cell metastasis *in vitro*. Consistent with our findings, knockdown of ARID1A enhanced gastric cancer proliferation, whereas overexpression of ARID1A in mutant cell lines dampened cell proliferation [29]. Moreover, knockdown studies in a variety of cell types, which achieved only partial depletion of ARID1A, showed increased cell proliferation and colony formation, impaired differentiation, as well as decreased apoptosis [8, 16]. These data, together with ours, indicate that ARID1A functions as a gatekeeper tumor suppressor gene in osteosarcoma. Hence, as an important tumor suppressor, targeting ARID1A hold great therapeutic potential for cancer treatment. The involvement of ARID1A in maintaining genomic stability makes tumors with ARID1A mutations potential candidates for therapeutics based on synthetic lethality, A recent study demonstrated a synthetic lethality by targeting EZH2 histone methyltransferase activity in ARID1A-mutated ovarian cancer cells [3]. Evidence also suggests that ARID1A mutation may often co-occurs with PIK3CA mutation in ovarian carcinoma [10] and gastric carcinomas [29], suggesting that PI3K inhibitors may have a synergistic effect on tumor development with ARID1A mutation status.

The molecular mechanisms by which ARID1A inhibits cancer cell proliferation and metastasis remain unclear. Precious studies identified a significant correlation between ARID1A loss and the presence of activating mutations in PIK3CA and PTEN in ovarian clear cell carcinomas and endometrioid carcinomas [15, 26, 29]. Guan et al. reported that tumor suppressor role of ARID1A mainly involved negative regulation of cell-cycle progression and two p53-regulated genes, CDKN1A and SMAD3 [8]. Recently, Wu et al. have reported a tendency toward mutual exclusivity between ARID1A loss and telomerase reverse transcriptase (TERT) promoter mutation in ovarian clear cell carcinoma [25]. In addition, ARID1A also enhanced TERT transcription and maintains telomere length, loss of ARID1A protein induced tumor cells promoted tumor growth by enhancing the TERT transcriptional activity [18], suggesting that ARID1A may also play a role in telomere biology. In the present study, we found that ARID1A overexpression could decreased Bcl-2 protein expression and increased Bax expression, and reduced N-cadherin level and increased E-cadherin levels, suggesting that ARID1A may regulate cell proliferation and metastasis of osteosarcoma through activation of intrinsic apoptotic pathway, and a switch from N-cadherin to E-cadherin.

In conclusion, our study demonstrated that ARID1A was downregualted in osteosarcoma and its downregualtion was correlated with poor overall survival of osteosarcoma patients. ARID1A can inhibit tumor growth and metastasis possibly through modulating Bcl-2 and N-cadherin expression. Our data demonstrate that ARID1A may serve as a tumor suppressor in osteosarcoma progression and represent a valuable prognostic marker and potential therapeutic target for osteosarcoma.

Compliance with Ethical Standards

Competing interests The authors declare that they have no competing interests.

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