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ORIGINAL ARTICLE

ARID1A regulates E-cadherin expression in colorectal cancer cells: a promising candidate therapeutic target

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Abstract

Background Metastasis is a major cause of death in Colorectal cancer (CRC) patients, and the Epithelial–mesenchymal transition (EMT) has been known to be a crucial event in cancer metastasis. Downregulated expression of AT-rich interaction domain-containing protein 1A (ARID1A), a bona fide tumor suppressor gene, plays an important role in promoting EMT and CRC metastasis, but the underlying molecular mechanisms remain poorly understood. Here, we evaluated the impact of *ARID1A* knockdown and overexpression on the expression of EMT-related genes, *E-cadherin* and *β-catenin*, in human CRC cells.

Methods and results The expression levels of *ARID1A, E-cadherin and β-catenin* in CRC cell lines were detected via real-time quantitative PCR (qPCR) and western blot. *ARID1A* overexpression and shRNA-mediated knockdown were performed to indicate the effect of *ARID1A* expression on *E-cadherin* and *β-catenin* expression in CRC cell lines. The effect of *ARID1A* knockdown on the migration ability of HCT116 cells was assessed using wound-healing assay. We found that the mRNA and protein expression of adhesive protein E-cadherin was remarkably downregulated in response to shRNA-mediated *ARID1A* knockdown in HCT116 and HT29 cells. Conversely, overexpression of *ARID1A* in SW48 cells significantly increased E-cadherin expression. In addition, *ARID1A* silencing promoted the migration of HCT116 cells. *ARID1A* knockdown and overexpression did not alter the level of *β-catenin* expression.

Conclusions Our study demonstrates that E-cadherin levels were closely correlated with *ARID1A* expression. Thus, *ARID1A* downregulation may promote CRC metastasis through decreasing EMT-related protein E-cadherin and promoting epithelial cell movement. *ARID1A* could represent a promising candidate therapeutic target for CRC.

Keywords Colorectal cancer · ARID1A · E-cadherin · β-catenin

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide, and is a major cause of cancer mortality, accounting for approximately one-tenth of all tumor-related deaths in the world annually [1, 2]. Metastasis is the primary cause of treatment failure and death in CRC [3, 4]. Thus, a better understanding of the molecular mechanism underlying colorectal cancer metastasis may lead to new therapeutic strategies.

AT-rich interactive domain-containing protein 1A (*ARID1A*), a bona fide tumor suppressor gene, encodes a key subunit of the SWItch/Sucrose Non-Fermentable (SWI/SNF) chromatin-remodeling complex. The SWI/SNF complex modulates the DNA accessibility to co-regulatory and transcriptional machinery and acts as a transcriptional regulator of

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genes involved in many important cellular processes, including proliferation, development, differentiation and DNA damage repair [5]. As a subunit of SWI/SNF complex, ARID1A is assumed to play a crucial role in SWI/SNF complex targeting to enhancers and in maintaining their chromatin accessibility as it has DNA binding activity. Upon ARID1A inactivation, defects in targeting SWI/SNF and control of enhancer activity impair development and differentiation programs and cause extensive dysregulation of gene expression, thus driving tumor formation [5]. For instance, in ARID1A-deficient colon cancer, SWI/SNF is lost from thousands of enhancers with corresponding effects on gene expression [6].

ARID1A is one of the most frequently mutated tumor suppressors in various human cancers [7]. As data have emerged from the Cancer Genome Atlas project (TCGA), mutations in *ARID1A* have been found at high frequencies in CRC. With an estimated mutation rate of 10–40%, *ARID1A* is the third most significantly mutated gene in human CRC, following APC and P53 [6, 8]. Furthermore, mutations in ARID1A are generally inactivating (insertion/deletion) a characteristic of many tumor suppressors [7]. Apart from inactivating mutations, aberrant DNA methylation, has also been reported as an important mechanism for suppressing ARID1A expression in CRC [9]. Available data support an important role of ARID1A inactivation in promoting formation and metastasis of CRC. *ARID1A* knockout in mice per se, drives the formation of invasive colon tumors [6]. In addition, in a clinical study of CRC, Kishida et al. revealed that negative *ARID1A* expression was correlated with early onset and lymphatic invasion [10]. It has been found that *ARID1A* knockout can enhance the migratory activity of cancer cells by promoting Epithelial mesenchymal transition (EMT) [11–14]. EMT has been known to be a crucial event in cancer invasion and metastasis [15, 16]. During EMT, cancer cells lose their cell–cell adhesions and polarity and thereby acquire enhanced invasive and migratory properties [16, 17]. However, the role of ARID1A in expression of genes associated with EMT and cell–cell adhesion in CRC remains poorly understood. Therefore, in this study, we aimed to investigate the impact of *ARID1A* knockdown and overexpression on the expression of EMT-related genes, *E-cadherin* and *β-catenin,* in human CRC cells. Since ARID1A expression loss is not rare in colorectal cancer tumors, correlation of ARID1A and E-cadherin expression in CRC tissue samples remains a goal for future studies.

Materials and methods

Cell culture

HEK293T cell line and six CRC cell lines (HCT116, HT 29, SW48, SW742, SW480 and LS180) were purchased from the National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). HCT116, HT 29, SW48, SW742 and SW480 cells were cultured in RPMI 1640 medium (#11875093, Thermo Fisher Scientific, Inc., Bartlesville, OK, USA) and HEK293T and LS180 cells were cultured in DMEM medium (#12430062, Thermo Fisher Scientific, Inc., Bartlesville, OK, USA) containing 10% fetal bovine serum (Gibco-BRL), 1% penicillin(#16000044, Thermo Fisher Scientific, Inc., Bartlesville, OK, USA) and 1% streptomycin (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), in a humidified CO2 (5%) incubator at 37 °C, and regularly checked and confirmed to be mycoplasma-free.

Plasmids and lentivirus production

Overexpression plasmid of pcDNA 6- ARID1A was obtained from addgene (#39311, Addgene plasmid) for the overexpression assay. Empty vector was used as a negative control. The short hairpin RNA (shRNA) lentiviral plasmid (pLKO.1) which contains a puromycin resistance gene was obtained from Addgene (#10878, pLKO.1 - TRC Cloning Vector, Addgene). The shRNA oligonucleotide against *ARID1A* gene (oligo ID: TRCN0000059090) with the sequence of 5'- CCTCTCTTATACACAGCAGAT -3' was cloned into the pLKO.1 vector. A pLKO.1-puro Empty Vector (#RHS4080, Open Biosystems) was also used to produce empty lentivirus as a control. The empty lentivirus and lentivirus encoding *ARID1A* shRNA were produced using HEK293T cells transfected with pLKO.1 plasmids and the second generation packaging system, pMD2.G (#12259, Addgene plasmid) and pSPAX2 (#12260, Addgene plasmid). Briefly, about 80% confluent HEK293T cells were co-transfected with 0.5 µg of the envelope protein–coding plasmid (pMD2.G), and 1.5 µg of the packaging construct (pSPAX2) together with 2 µg of pLKO.1 vector encoding *ARID1A* shRNA or negative control pLKO.1 Empty Vector using a standard polyethylenimine (PEI) mediated method [18]. After 48 h, the virus-containing media was collected, centrifuged (1250 rpm for 5 min), and stored in small aliquots at − 80 °C.

Generation of the HCT116 and HT29 cells stably expressing shRNA against ARID1A

HCT116 and HT29 cells were seeded in 6-well plates. After 24 h, the lentiviral supernatant containing either a lentivirus expressing *ARID1A* shRNA or an empty lentivirus as a control was added at MOI (multiplicity of infection) of 5–50 together with 8 µg/ml polybrene (#sc-134220, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). 24 h later, the transduction medium was replaced with fresh complete medium and puromycin (#sc-108071, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) selection (1 µg/ml)

was started 72 h post transfection. Approximately, in every 2–3 days, the culture media was aspirated and replaced with freshly prepared selective media. After 3 weeks under puromycin selection the remaining resistant clones were assessed for knock down efficacy. *ARID1A* Knockdown efficacy was assessed by immunoblotting and qPCR.

ARID1A gene transfection into SW48 cells

SW48 cells were transfected with 2 µg pcDNA6- *ARID1A* or 2 µg pcDNA6 (as a negative control). Cell transfection was performed using Lipofectamine 2000 (#11668030, Invitrogen; Thermo Fisher Scientific, Inc. USA) according to the manufacturer's instructions. The efficiency of the transfection was examined 48 h post-transfection using real-time quantitative PCR and Western blotting.

RNA extraction and real-time PCR assay

RNA of cultured cells was extracted using the Tripure RNA isolation kit (#11667165001, Roche Applied Science, Germany) based on the manufacturer's guidelines. cDNA synthesis was conducted by RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Fisher Scientific, USA). mRNA analysis of *ARID1A* was determined by real-time quantitative PCR (qPCR) using SYBR Green master mix (#4309155, Thermo Fisher Scientific, USA) on an ABI 7500 Sequence Detection System (Applied Biosystems, USA). *GAPDH* was used as an internal housekeeping gene. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The primers used in this study are listed in Table 1.

Western blot analysis

Cultured cells were lysed using ice-cold lysis buffer in the presence of a protease inhibitor cocktail (Roche Applied Science) and the protein concentration was quantified using a Pierce BCA protein assay kit (#23225, Thermo Fisher Scientific, USA). Proteins with an equal amount were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were then blocked using 5% skim milk/PBS for 1 h. After blocking, membranes were incubated with the following primary antibodies overnight at 4 °C: ARID1A (#sc-373784; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), β-catenin (#sc-7963; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), E-cadherin (#sc-71009; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and GAPDH (#ab181602; Abcam, UK) as an internal control. Each membrane was subsequently washed with TBST three times and incubated with a corresponding HRP-conjugated secondary antibody at room temperature for 1 h. Protein detection was performed using a Pierce™ enhanced chemiluminescence (ECL) western blotting substrate (#32209, Thermo Fisher Scientific, Inc., Bartlesville, OK, USA). Band intensity data was obtained using Image lab software (version 5.2, Bio-Rad).

Wound healing (scratch) migration assay

In order to determine the cell migration, the HCT116 cells were cultured in 6-well plates to confluence. Subsequently, the monolayer cells were scratched using 200 µl pipette tip from the top to the bottom of the culture plates. After the debris was removed with PBS, the cells were further maintained at 37 °C in a humidified incubator with 5% CO_2. Wounded area was visualized after 0, 24 and 48 h using an Olympus CX31 light microscope (Olympus Corporation, Tokyo, Japan).and calculated by NIH ImageJ software (https://imagej.nih.gov/ij/). Cell motility was estimated through the quantification of the % of recovery using the equation: R (%) = [1 − (wound area at Tt/wound area at T0)] × 100, where T0 is the wounded area at 0 h and Tt is the wounded area after t.

Statistical analysis

All the experiments were repeated at in triplicate and all data were expressed as mean ± SD. The statistical package IBM SPSS Statistics (IBM Corp. Released 2017. IBM SPSS Statistics for Windows, Version 25.0. Armonk, NY: IBM Corp.) was used for all analyses. graphs were performed using Prism version 6.00 (GraphPad Software, Inc., San Diego, CA, USA). The one-way ANOVA, accompanied by Tukey's multiple comparison tests were performed to analyze data. Differences with a P value ≤ 0.05 were statistically significant.

Table 1 Sequences of primers used for real-time RT-PCR

Gene	Forward (5'-3')	Reverse (5'-3')
ARID1A	CAGTACCTGCCTCGCACATA	GCCAGGAGACCAGACTTGAG
E-cadherin	ATTTTTCCCTCGACACCCGAT	TCCCAGGCGTAGACCAAGA
β-catenin	AAAATGGCAGTGCGTTTAG	TTTGAAGGCAGTCTGTCGTA
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG

Results

The efficiency of overexpression and shRNA-mediated knockdown of ARID1A in CRC cell lines

We examined the expression of *ARID1A* in a panel of CRC cell lines using qPCR (Fig. 1a). Among the six CRC cell lines, the highest expression level of *ARID1A* was in the HCT116 and HT29 cells, whereas SW48 cells lacked *ARID1A* expression (Fig. 1a). Thus, these cells were selected for subsequent knockdown and overexpression analyses, respectively. We stably silenced *ARID1A* in HCT116 and HT29 cells using *ARID1A* shRNA lentivirus and overexpressed *ARID1A* in SW48 cells by plasmid transfection. Successful knockdown and overexpression of *ARID1A* in these cells were confirmed using Western blot and qPCR analysis (Fig. 1b and c).

ARID1A silencing suppresses the expression of E-cadherin protein and mRNA, whereas the ARID1A overexpression exerts an opposite effect

Western blot analysis and RT-qPCR indicated that the HCT116 and HT29 cells with stable knockdown of *ARID1A* by *ARID1A* shRNA virus exhibited a lower expression of E-cadherin protein and mRNA as compared to cells transduced with control virus carrying pLKO.1. On the other hand, the SW48 cells transfected with pCDNA6-*ARID1A* exhibited a higher expression of E-cadherin protein and mRNA as compared to the control group of cells lacking *ARID1A* expression (Fig. 2). The above results suggest that ARID1A may modulate E-cadherin expression.

β-catenin remains stable regardless of ARID1A silencing or overexpression

To identify the role of ARID1A in the expression of β-catenin in CRC cells, protein and mRNA levels of β-catenin in CRC cells were analyzed after overexpression or stable knockdown of ARID1A. As shown in Fig. 2, the expression of β-catenin remained largely stable regardless of ARID1A silencing or overexpression in CRC cells.

Knockdown of ARID1A promotes HCT116 cells migration

The migration ability of HCT116 cells was examined by wound healing assay. Microscopic examination after wounding revealed that HCT116 cells transduced with *ARID1A* shRNA lentivirus have obviously increased ability of wound closure at all time points (up to 48 h post-scratch) compared to HCT116 cells transduced with control lentivirus (Fig. 3).

Discussion

EMT, an absolute requirement for tumor metastasis and invasion, plays a key role in cancer progression, including CRC [16, 17, 19]. Due to its emerging role as a pivotal driver of tumorigenesis, inhibiting EMT is an attractive therapeutic strategy in combating metastasis in cancer patients [20]. The hallmark of EMT is the decreased expression of the E-cadherin protein [21, 22]. When E-cadherin is downregulated, epithelial cells lose their polarity and cell–cell adhesion, and EMT then occurs. Therefore, the downregulation mechanism of E-cadherin is a key topic of EMT research [15, 21]. In recent years, an increasing number of studies have found that ARID1A downregulation is associated with EMT and tumor invasion via the repression of E-cadherin in several different types of cancer including some types of gastrointestinal cancers [11–13, 23]. Although, decreased expression of ARID1A protein has been associated with enhanced invasion and metastasis of colorectal carcinoma in mice and humans [6, 10]; however, there is limited evidence for the ARID1A role in regulating E-cadherin expression in CRC. To address this issue, we sought to delineate the impact of *ARID1*A knockdown and overexpression on the expression of E-cadherin protein and mRNAs in CRC cells. Here, we demonstrated that the expression of E-cadherin was strongly correlated with that of ARID1A. shRNA-mediated knockdown of *ARID1A* significantly downregulated E-cadherin expression in HCT116 and HT29/219 cells. Conversely, in the SW48 cells transfected with *ARID1A* overexpression vector, *E-cadherin* expression was significantly upregulated. These results are in good agreement with the findings from the only available experimental study in CRC, in which Mathur.et al. indicated the *E-cadherin* downregulation in HCT116 isogenic lines with monoallelic ($ARID1A^{+/-}$) or biallelic ($ARID1A^{-/-}$) deletion of *ARID1A* [6]. Importantly, our results were also consistent with data from the previous studies, which suggest that *ARID1A* knockdown decreased the expression of E-cadherin, whereas *ARID1A* overexpression increased the expression of E-cadherin in human gastric, breast and hepatocellular cancer cells [12, 13, 24]. Moreover, *ARID1A* knockdown strongly downregulated *E-cadherin* expression in cells derived from nasopharyngeal, neuroblastoma and pancreatic carcinomas [23, 25, 26]. Interestingly, Yan.et al. indicated that the expression of ARID1A was strongly correlated with that of E-cadherin in the analyzed gastric cancer tissues. Expression of ARID1A and E-cadherin were synergistically downregulated in 23.5% of analyzed gastric cancer tissues [13]. Notably, controversial

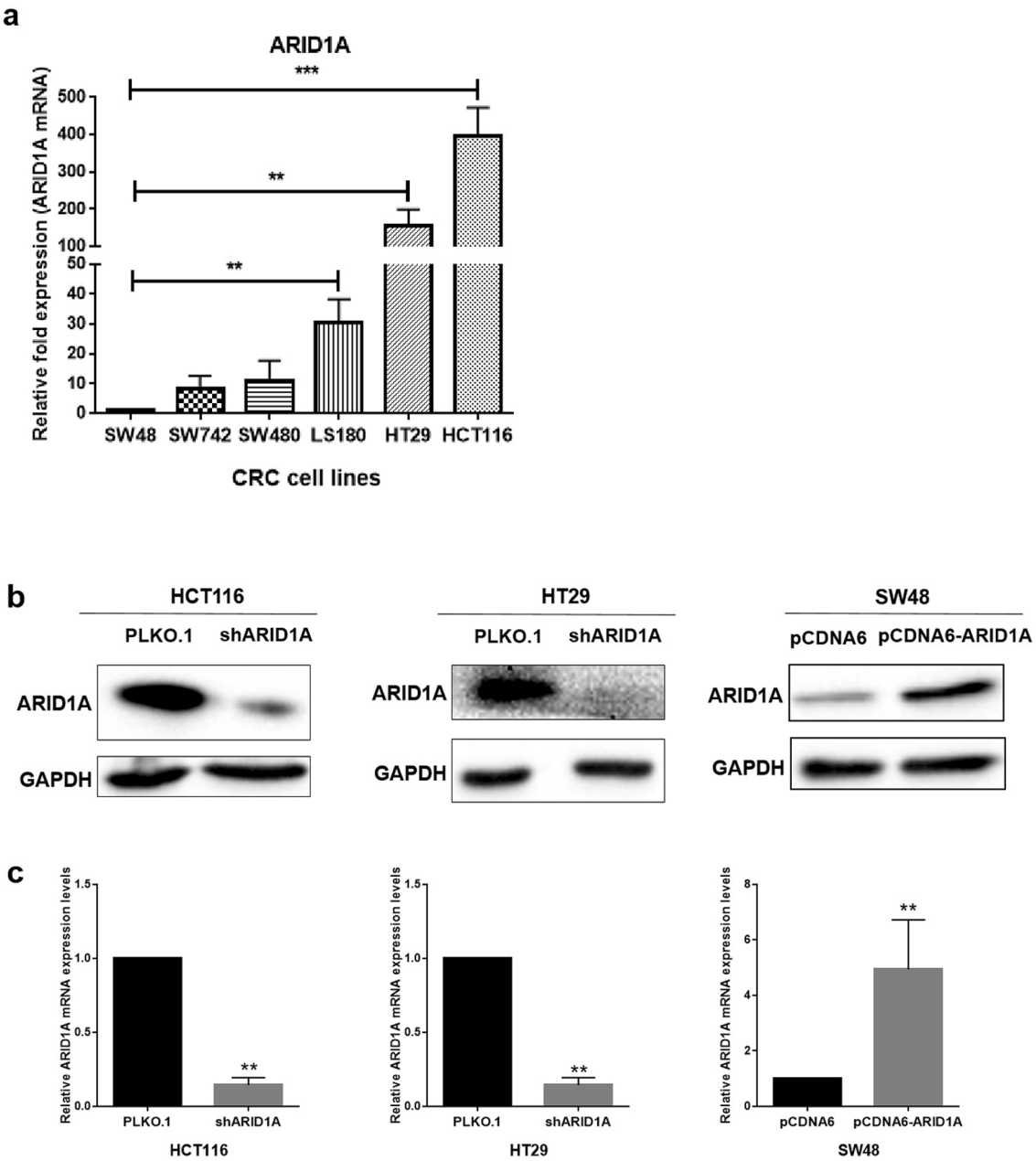

Fig. 1 *ARID1A* relative mRNA expression in several colorectal carcinoma cell lines and efficiency of overexpression and shRNA-mediated knockdown of *ARID1*A. **a** Endogenous expression of *ARID1A* was examined in HCT116, HT29, LS180, SW742 by qPCR. SW48 cell line was utilized as a reference, with an expression level set to 1.0, and expressions in all other cell lines were presented as an n-fold difference, compared to the SW48. Analysis of western blot (**b**) and qPCR (**c**) showed reduction in *ARID1A* expression in HCT116 and HT29 cells transduced with *ARID1A* shRNA virus, compared to control virus transduced cells, while pCDNA6-*ARID1A* plasmid transfection markedly enhanced *ARID1A* levels in SW48 cells. The *ARID1A* expression was normalized relative to *GAPDH*. For panel A and C, Mean ± standard deviation (SD) of three experiments is reported. (**$p < 0.01$)

report also exists. In a clinical study of esophageal squamous cell carcinoma, Ozawa et al. found that *ARID1A* expression status did not significantly correlate with *E-cadherin* expression levels of the patients [27]. The lack of rescue experiments to prove and justify the obtained results is one of the limitations of our study. Another limitation of this study was the lack of immunohistochemical analysis in clinical samples and animal experiments. Further investigations to provide more comprehensive mechanisms of the correlation between ARID1A and E-cadherin expression status in CRC tissues are under consideration for future study. However, additional studies would be helpful to explore the exact

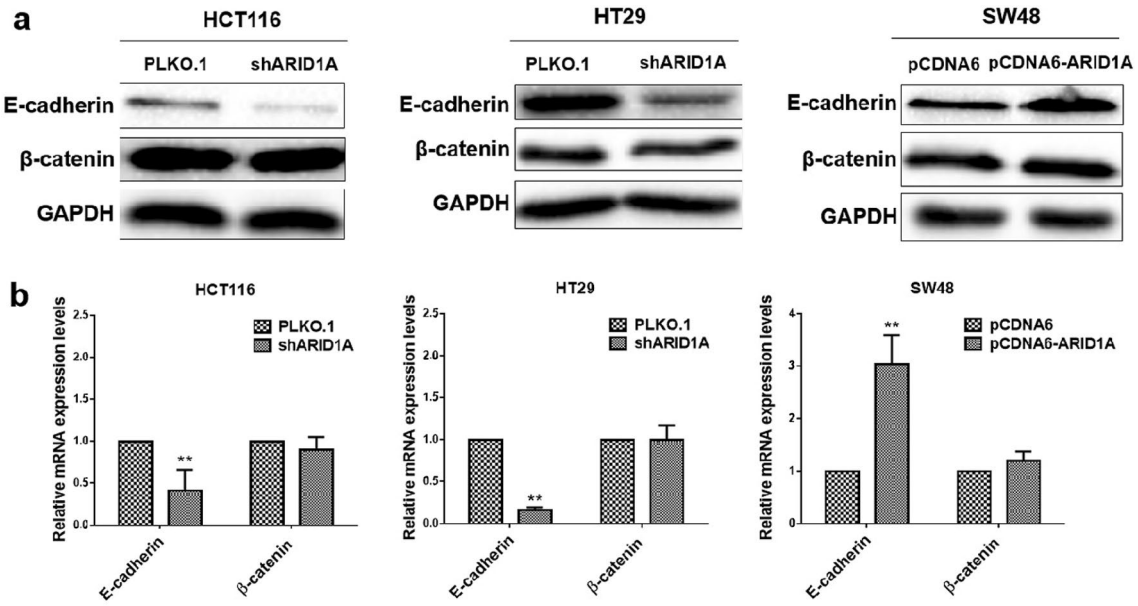

Fig. 2 Effects of ARID1A on the protein and mRNA expression of E-cadherin and β-catenin in HCT116, HT29 and SW48 cells. Western blot (**a**) and qPCR (**b**) demonstrates that *ARID1A* stable knockdown results in reduced expression of *E-cadherin* in both HCT116 and HT29 cells, while induction of *ARID1A* expression leads to upregulation of E-cadherin in SW48 cells. The β-catenin expression is not significantly affected following *ARID1A* knockdown or overexpression. Values are expressed as mean ± SD (n = 3). (**P < 0.01)

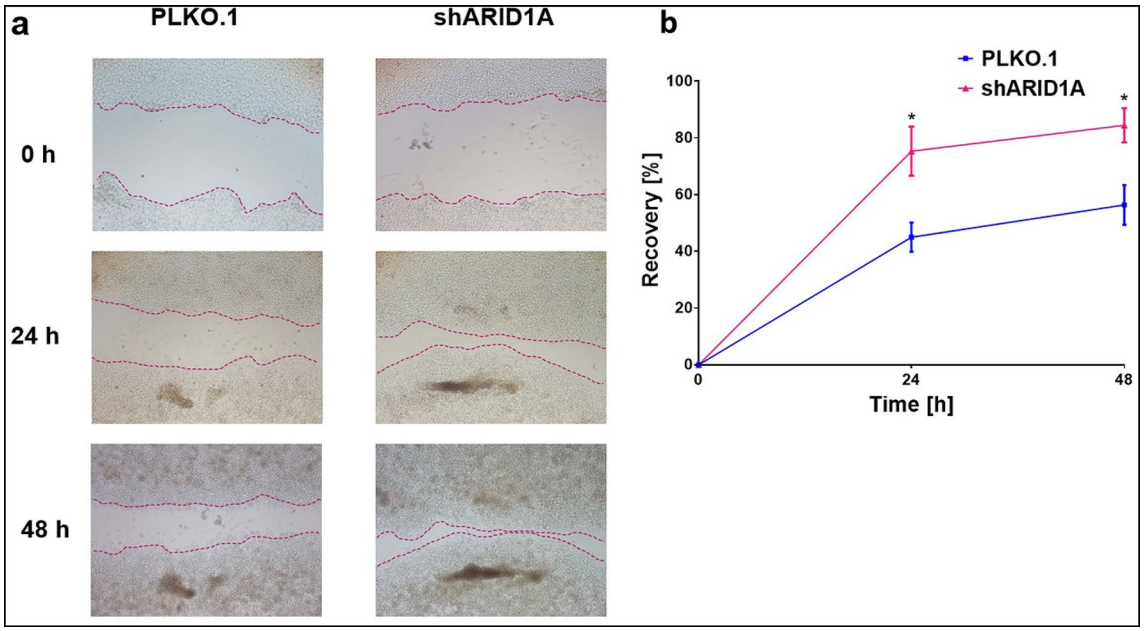

Fig. 3 Knockdown of ARID1A promotes HCT116 cells migration. **a** The Effects of ARID1A knockdown on migratory activity of HCT116 cells was examined by wound healing assay. Representative images of wound healing assay at indicated time points (0, 24 and 48 h after the scratch) are shown. Dashed line indicates boundary of the scratched wound. **b** Cell motility was estimated through the quantification of the % of recovery using the equation detailed in section of Materials and Methods. Data were presented as means ± SD from three replicates. (*p < 0.05)

mechanism by which ARID1A regulates E-cadherin, such as ChIP-sequencing of histone markers of repression and activation coupled with ATAC-seq [28] will be useful to examine the interplay of ARID1A and chromatin accessibility of E-cadherin in CRC cells or detecting of ARID1A effects on transcriptional repressors of E-cadherin such as

Slug, Zeb, Twist, Snail and some microRNAs especially the miR-200 family [15, 16].

Considering that E-cadherin downregulation marks the initiation of EMT and cancer cells invasion and metastasis, we postulated that *E-cadherin* downregulation by *ARID1A* silencing may partly account for enhanced migration activity of CRC cells. Therefore, using wound healing, we also noted that knockdown of *ARID1A* significantly enhanced the migration activity of the HCT116 cells, consistent with the data reported in pancreas, kidney, neuroblastoma, gastric, breast, and liver cancers, in which loss of ARID1A promoted cell migration and invasion [11, 13, 14, 24, 29, 30].

β-catenin, a major component of the canonical Wnt signaling pathway, has a crucial role in the negative regulation of E-cadherin expression and the EMT induction [19, 31–33]. Upon Wnt stimulation, β-catenin is stabilized, translocate to the nucleus where it triggers constitutive activation of Wnt target genes [32]. Aberrant activation of the Wnt pathway underlies many cancers including CRC [19]. In this study, *ARID1A* knockdown or overexpression did not alter the expression level of β-catenin. This finding is in agreement with a previous study in human gastric cells, in which the expression of β-catenin remained largely stable regardless of *ARID1A* silencing [13]. With respect to the ARID1A role in the regulation of β-catenin, the documented results to date are controversial. For instance, in the only available experimental study in CRC, Mathur.et al. found that $Arid1a^{fl/fl}$ mice drive colorectal tumorigenesis by a mechanism that is independent of Wnt signaling and distinct from established genetic models of colon cancer [6]. In the mentioned research, β-catenin localized exclusively outside of the nucleus in mice colon tumors, indicating that *ARID1A* inactivation does not cooperate with aberrant Wnt signaling in driving tumorigenesis [6]. In contrast, *ARID1A* silencing in human gastric and neuroblastoma cells induced disassociation of the E-cadherin/ β-catenin adhesion complex at the membrane and a redistribution of β-catenin from the plasma membrane to the nucleus, where β-catenin triggers the EMT process [13, 25]. Additional studies are needed to elucidate the effect of the manipulation of *ARID1A* expression on nuclear translocation of β-catenin and the activation of Wnt signaling in CRC cells.

Conclusion

Our findings, together with those of previous studies, further demonstrate that ARID1A regulates E-cadherin expression in cancer cells. Considering that downregulation or loss of E-cadherin expression can activate EMT, therefore, our data suggest that *ARID1A* downregulation may contribute to enhancing the migratory and invasive abilities of CRC cells. In addition, we present evidence for the role of E-cadherin as one potential therapeutic target for ARID1A-deficient tumors as it was affected by ARID1A re-expression. Although, low ARID1A expression is not rare in colorectal cancer tumors [9, 34–36], the correlation between ARID1A and E-cadherin expression status in CRC tissue samples remains a goal for future studies.

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Author contributions PM and ME contributed to the study conception and design. Material preparation, data collection and analysis were performed by ME, MZ, SYH, ZM and SMS. The first draft of the manuscript was written by ME and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability All data are included in this article and datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declared that they have no conflict of interest.

Ethical approval Ethics approval was obtained from the Ethics Committee of Shiraz University of Medical Sciences prior to this study (IR.SUMS.REC.1398.1390).

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