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Original Article

VPS9D1-AS1, a novel long-non-coding RNA, acts as a tumor promoter by regulating the miR-324-5p/ITGA2 axis in colon adenocarcinoma

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Abstract: Background: Colon adenocarcinoma (COAD) is among the most common malignancies worldwide. Elucidating the function and mechanism of action of the lncRNA VPS9D1-AS1 in COAD will be of great value for identifying potential therapeutic targets. Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) analysis was used to measure the expression levels of lncRNA VPS9D1-AS1 in COAD tissues and cell lines. After knocking down the expression of VPS9D1-AS1 in two COAD cell lines, namely SW1116 and LoVo, their proliferation rate was measured by the 5-ethynyl-2'-deoxyuridine (Edu) incorporation and cell counting kit-8 (CCK-8) viability assays, migration and invasion abilities were assessed by wound healing and Transwell assays, and apoptosis rate was measured withflow cytometry. Additionally, the dual luciferase reporter assay system was used to investigate the targeting of miR-324-5p to VPS9D1-AS1 and *ITGA2* 3'-UTR. The inhibitory effects of the miR-324-5p/ITGA2 axis on the function of VPS9D1-AS1 were also examined. *In vivo* tumorigenesis assay was performed in nude mice injected with VPS9D1-AS1 shRNA or control shRNA lentivirus-transfected LoVo cells. Results: VPS9D1-AS1 was found to be upregulated in COAD tissues and cell lines. VPS9D1-AS1 knockdown inhibited the COAD cell proliferation, migration and invasion and increased the apoptosis rate. In addition, we have demonstrated that miR-324-5p targets VPS9D1-AS1 and *ITGA2* 3'-UTR, and miR-324-5p silencing or forced *ITGA2* expression attenuated the effect of VPS9D1-AS1 knockdown. Conclusion: This study identified a novel competing endogenous RNA (ceRNA) pathway that potentially associates with the oncogenic functions of VPS9D1-AS1, miR-324-5p, and ITGA2 in COAD, which could contribute to the identification of new therapeutic approaches targeting COAD.

Keywords: VPS9D1-AS1, ceRNA, miR-324-5p, ITGA2, colon adenocarcinoma

Introduction

Colorectal cancer is the third most commonly diagnosed malignant tumor and second leading cause of cancer death worldwide, with about 1.9 million new cases diagnosed and 935,172 colorectal cancer-related deaths in 2020. In recent years, with the advances in diagnostic and surgical techniques and improvement of postoperative adjuvant treatment, the comprehensive treatment of colorectal cancer has made great progress [1, 2]. However, the mortality rate increased about 6.2% in the last two years, compared the estimated 880,792 deaths in 2018, to 935, 172 colorectal cancer-related deaths in 2020 [3]. Studies have shown that the proportion of proximal colon adenocarcinoma (COAD) increases with age [4]. The occurrence and development of COAD is a complex process involving the accumulation of multiple independent genetic alterations that lead the transformation of normal colonic cells to form an adenoma and ultimately adenocarcinoma through a regulatory mechanism that mainly involves the activation of proto-oncogenes and inactivation of tumor suppressor genes [5]. Despite extensive studies, the pathogenesis of COAD is not fully understood. Therefore, in-depth studies of the mechanism of the development of COAD and elucidation of the key molecules and their roles in the metastatic process are critical not only for early diagnosis and postoperative recurrence monitoring, but also to develop new drugs to prevent

the development of COAD and increase the survival rate of the patients with COAD.

Long non-coding RNAs (lncRNAs) are a group of non-protein coding RNA transcripts longer than 200 nucleotides [6]. Due to their non-protein coding property, lncRNAs were once regarded as evolutionarily accumulated genetic "junk" without functional relevance in the process of transcription [7]. In recent years, increasing evidence has shown that lncRNAs have a variety of important regulatory functions in various cellular processes. They can regulate gene expression at the epigenetic, transcriptional and post transcriptional levels, and participate in pathological and physiological processes of the body through their functions as signaling, bait, guide and scaffold lncRNA molecules [8-10]. Numerous lncRNAs have been found to be abnormally expressed in tumors and have gained drawn attention in cancer research due to their important role in tumor development. LncRNAs are involved in tumor cell growth, apoptosis, migration and invasion, and may act as an oncogene or tumor suppressor gene during tumorigenesis [11]. The VPS9D1 antisense RNA 1 (VPS9D1-AS1) gene located on chromosome 16q24.3 is 1,753 bp in length [12]. VPS9D1-AS1 was found to be upregulated in non-small-cell-lung cancer (NSCLC) [13, 14] and prostate cancers [15]. In particular, in NSCLC patients the increased level of VPS9D1-AS1 was significantly correlated with the adverse clinicopathological features and shorter overall survival time [14], suggesting that it could serve as a prognostic biomarker. However, the functions and mechanism of VPS9D1-AS1 in COAD remain unknown.

MicroRNAs (miRNAs) are small non-coding RNAs of 20 to 25 nucleotides in length, which inhibit the expression of their target genes by specifically binding to their messenger RNA sequences [16, 17]. As important members of the non-coding RNA family, the mechanism by which lncRNAs exert their effects on malignant tumors, and their interaction with miRNAs to play a regulatory role in tumorigenesis have attracted increasing attention among cancer researchers. Salmena L *et al.* found that lncRNAs regulate the expression of target genes by acting as an endogenous sponge for miRNAs, which bind to lncRNAs and affect their stability [18]. Increasing attention has been paid to the importance of ceRNA regulatory networks in the development of various cancers, sincluding cervical [19], gastric [20], colorectal [21], and breast cancers [22], *etc*., which have been gradually verified by multiple biological methods.

Integrin subunit alpha 2 (ITGA2), a heterodimeric transmembrane glycoprotein in the family of integrin alpha subunits, frequently forms the heterodimer α2β1 with the integrin beta 1 (ITGB1) glycoprotein [23]. In normal tissues, ITGA2 has been found to be mainly expressed in skin glands, lungs and saliva. The expression of ITGA2 was found to be upregulated in various types of tumors, such as glioblastoma [23], ovarian cancer [24], and breast cancer [25], suggesting that ITGA2 could promote tumor progression.

This study investigated the role of the lncRNA VPS9D1-AS1/miR-324-5p/ITGA2 axis in the development of COAD and found the lncRNA VPS9D1 acts as a sponge for miRNA miR-324-5p to upregulate the expression of *ITGA2*. The findings may help with the understanding of the pathogenesis of COAD and possibly COAD diagnostics and therapeutics.

Materials and methods

Tissues specimens

A total of 23 COAD tissue samples paired with matched normal colon tissue adjacent to the tumor (normal mucosa 10 cm from the edge of the tumor) were collected from COAD patients who underwent surgical resection cat the Clinical Research Center, Kunming Maternal and Child Health Hospital from October 2017 to October 2018. All specimens were confirmed to be COAD by two pathologists. The research was conducted in accordance with the Declaration of Helsinki. All the patients signed informed consent and none of them had a history of other cancers, or undergone radiotherapy, chemotherapy, or immunotherapy before surgery. The Ethics Committee of the Clinical Research Center at Kunming Maternal and Child Health Hospital reviewed and approved this study (NO. 2018005). Tissue specimens were resected, frozen and stored in liquid nitrogen at -80°C until used for RNA extraction.

Cell lines and culture conditions

The COAD cell lines (SW620, SW1116, CW-2 and LoVo) and the normal colonic epithelial cell line (NCM460) were supplied by the Cell Bank of the Chinese Academy of Sciences Type Culture Collection (Shanghai, China). Cells were cultured in Dulbecco's Modified Medium (DMEM) (Gibco/Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin/streptomycin and 10% fetal bovine-serum (FBS) (Gibco/Thermo Fisher Scientific Inc.) in an incubator with a humidified atmosphere of 5% CO_2, at 37°C.

Cell transfection

The cells were passaged when the cell confluence reached about 80%. Log phase SW1116 and LoVo cells were detached by digestion with 0.25% trypsin (Solarbio, Beijing, China), washed, resuspended in culture medium at a cell density of 10^4 cells/mL, and seeded in a 6-well plate (200 μL/well). When the cell confluence reached 70%, the culture medium was replaced with serum-free culture medium. Then, si-VPS9D1-AS1, miR-324-5p inhibitor, miR-324-5p mimics and/or controls (Ctrl) (RiboBio, Guangzhou, China) were transfected into COAD cells for 6 h using the Lipofectamine 2000 transfection kit (Invitrogen/Thermo Fisher Scientific Inc.) following the manufacturer's instructions. Afterwards, the culture medium was replaced with DMEM containing serum, and the cells were cultured for 48 h. Then, log phase cells were collected for subsequent studies.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from each group using Trizol reagent (Invitrogen/Thermo Fisher Scientific Inc.), and RNA concentration and quality of each sample was determined. For analysis of miR-324-5p expression, qRT-PCR analysis was performed in using the Mir-X miRNA qRT-PCR-TB-Green® Kit (TaKaRa Bio, Shiga, Japan), and total RNA of each sample was reverse transcribed into cDNA using the Mir-X miRNA First-Strand-Synthesis Kit (TaKaRa Bio) first. To analyze the expression of VPS9D1-AS1 and *ITGA2* mRNA, the total RNA of each sample was reverse transcribed into cDNA using the PrimeScript-RT-Reagent Kit with gDNA Eraser (TaKaRa Bio). The analysis of the expression of VPS9D1-AS1 and ITGA2 mRNA was performed by PCR amplification using the TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa Bio). The miR-324-5p PCR amplification was performed in the following conditions: 95°C 5 min, 95°C 10 s, 60°C 30 s for 40 cycles, while the amplifications of VPS9D1-AS1 and ITGA2 PCR were performed in the following conditions: 95°C 15 min, 95°C 10 s, 55°C 20 s and 72°C 30 s for 40 cycles. The small-nuclear U6 RNA and β-actin were used as internal controls for miR-324-5p and VPS9D1-AS1/*ITGA2* mRNA levels, respectively, and the $2^{-\Delta\Delta Cq}$ method was used to calculate the relative gene expression. The following primers were used: forward primer of miR-324-5p 5'-ACACTCCAGCTGGGCGCATCCCCTAGGGCAT-3', reverse primer of miR-324-5p 5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGACACCAAT-3'; forward primer of VPS9D1-AS1 5'-ATGGGTAACCAGGGGTCAAG-3', reverse primer of VPS9D1-AS1 5'-GCCTTTTCCGTTGCCTCATTT-3'; forward primer of *ITGA2* 5'-GTGCAAACAGACAAGGCTGG-3', reverse primer of *ITGA2* 5'-CAGCTGCCGTTAGCTGTACT-3'.

Cell-Counting Kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) assays

Log phase COAD cells were collected and then seeded in the 96-well culture plates at a cell density of 6×10^4 cells/ml. Cell growth rate was determined at 0, 24, 48, and 72 h after transfection. After culturing for the indicated time, 20 μL of CCK-8 solution (Solarbio) was added into each well and cell culture was continued for 2 h, at 37°C. The absorbance value of each well was measured by colorimetry at the wavelength of 450 nm on a microplate reader. The cell growth curve was plotted with the different time points on the horizontal axis and light absorption values on the vertical axis.

After harvesting and resuspending cells in DMEM medium (10% FBS) at a concentration of 5×10^5 cells/mL for the EdU assay, the obtained transfected SW1116 and LoVo cells were cultured in 12-well plates (q100 μL in each well) and 12 h later, the cells were incubated with EdU at 10 μm for 2 h at 37°C for incorporation into DNA. After fixation for 15 min with 4% paraformaldehyde, the cells were stained for 15 min using the ClickiT EdU Assay kit (Invitrogen/

Thermo Fisher Scientific Inc.) to detect the levels of incorporated EdU in the cells.

Flow cytometry for cell apoptosis

After harvesting and washing twice with cold PBS, the obtained transfected SW1116 and LoVo cells were resuspended in a binding buffer, followed by the sequential addition of 5 μL of fluorescein isothiocyanate (FITC)-Annexin V (BD Biosciences, San Jose, CA, USA) and 5 μL propidium iodide (PI; BD Biosciences) and incubation for 20 min in the dark. The cell apoptosis analysis was performed by flow cytometry using a FACScan flow cytometer (BD Biosciences).

Dual-luciferase reporter assay

The wild-type (WT) or mutant (MUT) predicted binding sequences of miR-324-5p in VPS9D1-AS1 and *ITGA2* 3'-UTR were separately amplified and subcloned into the psiCHECK-2 luciferase reporter vector, After seeding COAD Cells in 96-well plates, cells were co-transfection with either miR-324-5p mimics or control mimics and the WT/MUT vector using the Lipofectamine 2000 reagent following the manufacturer's instructions. The cells were collected and normalized 48 h after transfection, and the luciferase activity was measured using ta dual-luciferase reporter assay kit (Promega Corporation, Madison, WI, USA).

Wound healing assay

The cell migration ability was assessed using a wound healing assay. Cells from each group were seeded in six well plates at a density of 5×10^5 cells/well and allowed to grow until he cell confluence reached about 90%. Then, to measure the rate at which cells repopulate artificially created scratches, linear scratches were made on the confluent monolayer of cells with the use of a 200 μL pipette tip. After 48 hours of incubation in serum-free media, the wound healing process was observed under an inverted microscope to determine the migration ability of the cells.

Invasion assay

To perform the invasion assay, 3×10^5 cells were seeded in the upper chamber of a 24-well Millicell chambers (8-μm-pore-size cell culture inserts; Millipore, Billerica, MA, USA) with Matrigel (BD Biosciences, San Jose, CA, USA). Serum-free was added to the upper chamber, and medium containing 10% FBS was added to the lower-chamber. After 24 h of incubation, chambers were removed and the cells were fixed in 4% paraformaldehyde and then stained with 1% crystal-violet. The invaded cells were imaged and counted under an inverted microscope.

In vivo tumorigenicity model

All animal experiments were approved by the Animal Care and Use Committee of the Clinical Research Center of Kunming Maternal and Child Health Hospital (NO. 2019001). BALB/c nude mice (4-5-week old, 18-20 g) were obtained from the Vital River Laboratories (Beijing, China) and maintained under specific pathogen free (SPF) conditions. The LoVo cells were transfected with theVPS9D1-AS1 knockdown lentivirus or control lentivirus for 48 h. Afterwards, the transfected cells were collected and administered to the mice by injection into their flanks. After 28 d, the experiments were terminated, tumors were exercised, weighed, and photographed.

Statistical analysis

The results of at least three independent experiments are expressed as the mean ± standard deviation and were analyzed using the SPSS software (IBM Corporation, Armonk, NY, USA). The Students two-tailed t-test was used to compare differences between two treatment groups, and two-way analysis of variance (ANOVA) test was used to compare differences when more than two groups are involved. $P<0.05$ ($^*P<0.05$ or $^\#P<0.05$) and $P<0.01$ were considered to indicate statistically significant differences.

Results

VPS9D1-AS1 is upregulated in COAD

The qRT-PCR expression analysis of VPS9D1-AS1in COAD tissue samples and their paired adjacent normal colon tissue samples revealed that VPS9D1-AS1 expression was significantly higher in COAD tissues than that in the paired adjacent normal tissues (**Figure 1A**). These results were confirmed using the UALCAN software [26] to analyze and visualize the expres-

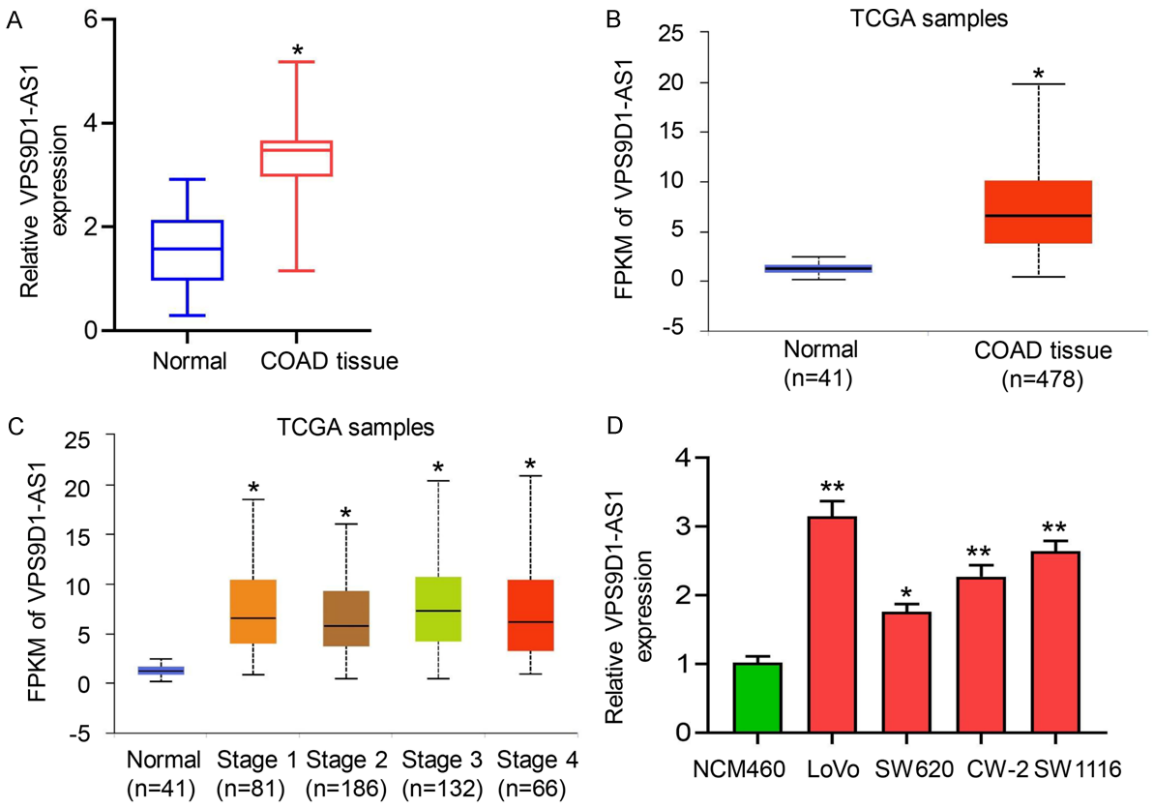

Figure 1. VPS9D1-AS1 was upregulated in COAD tissue-samples and cell-lines. A. Expression of VPS9D1-AS1 in COAD tissue-samples, and their paired adjacent normal colon tissue-samples, was measured by qRT-PCR-analysis. $^{*}P<0.05$, *vs*. normal colon tissues. B, C. Expression of VPS9D1-AS1 was analyzed in samples from TCGA database using UALCAN. $^{*}P<0.05$, *vs*. normal colon-tissues. D. Expression of VPS9D1-AS1 was measured in the four COAD cell lines (SW620, SW1116, CW-2 and LoVo) compared to the normal colonic-epithelial cell-line NCM460. $^{*}P<0.05$, $^{**}P<0.01$, *vs*. NCM460.

sion data of VPS9D1-AS1 in TCGA database, which generated results that were consistent with the expression analysis of the tissue samples (**Figure 1B**, **1C**). In addition, the results obtained by qRT-PCR analysis showed that VPS9D1-AS1 was often upregulated in all four COAD cell lines (SW620, SW1116, CW-2 and LoVo) analyzed compared to its expressions in the normal colonic epithelial cell line NCM460 (**Figure 1D**).

VPS9D1-AS1 promotes proliferation, migration and invasion in COAD cells

The function of VPS9D1-AS1 in COAD cells was investigated by studying the effect of suppressing its expression with specific small interfering RNA (siRNA) in SW1116 and LoVo cells (**Figure 2A**). The results of the cell proliferation analysis by the CCK-8 and Edu assays indicated that VPS9D1-AS1 knockdown reduced the proliferation rate of COAD cells (**Figure 2B**, **2C**). Additionally, the results of both wound healing and Transwell-assays revealed that suppression of VPS9D1-AS1 markedly inhibited migration and invasion of COAD cells compared with control siRNA (**Figure 2D**, **2E**). Moreover, According to the cell apoptosis analysis by flow cytometry, VPS9D1-AS1 knockdown led to an increase in apoptotic cell death (**Figure 2F**).

VPS9D1-AS1 acts as a sponge for miR-324-5p

To investigate the mechanisms of action of VPS9D1-AS1 in COAD cells, searches for predicted miRNA targets of VPS9D1-AS1 were performed in the databases on the miRDB and StarBase 3.0 on-line bioinformatics tools. The miRNA miR324-5p was predicted to be a target of VPS9D1-AS1 and was selected for further studies (**Figure 3A**). A dual-luciferase reporter assay system, including luciferase vectors containing WT or MUT binding sites for miR-324-5p in VPS9D1-AS1, was constructed to confirm

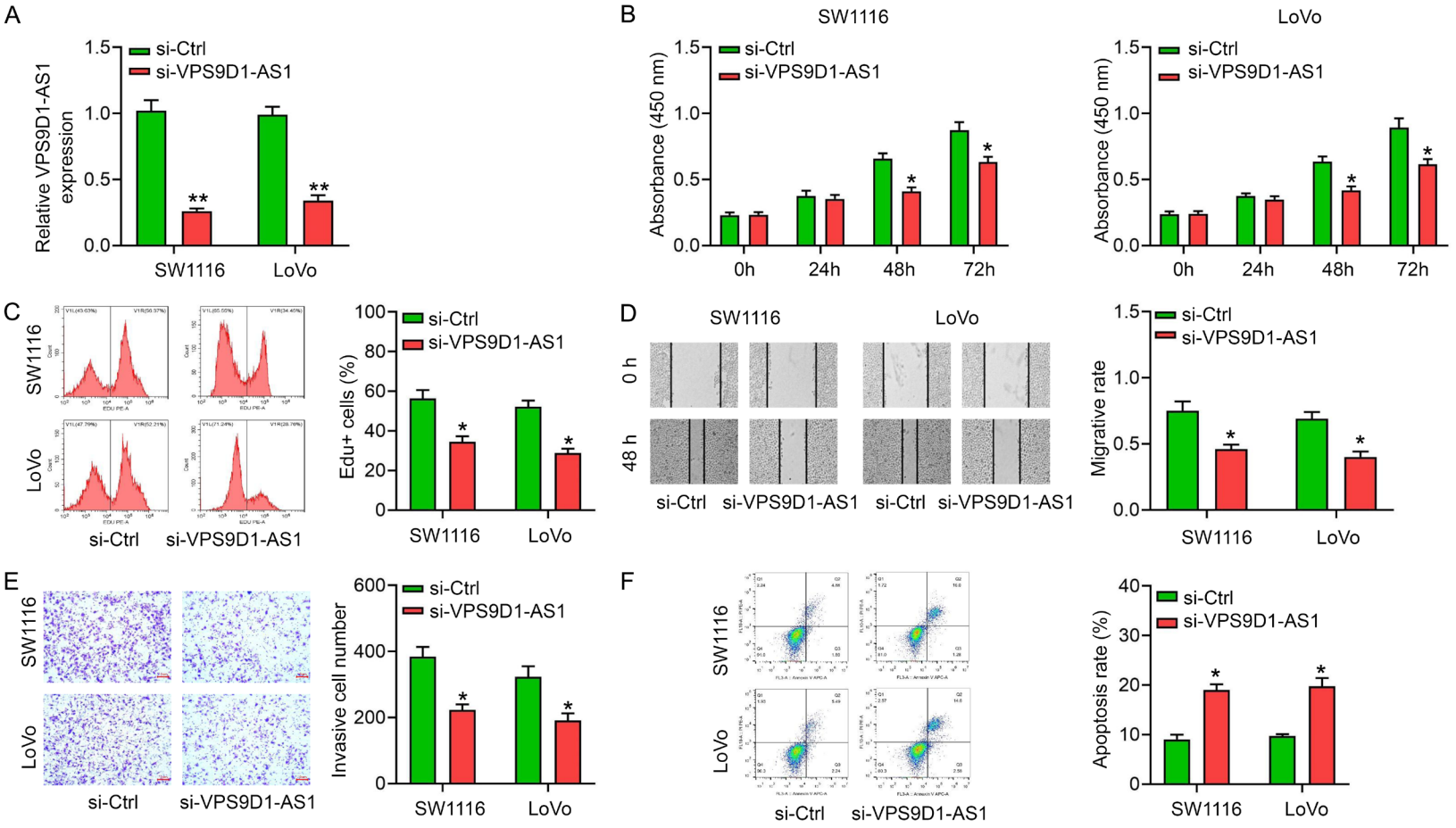

Figure 2. VPS9D1-AS1 promotes proliferation, migration and invasion in COAD cells. A. Suppression of the expression of VPS9D1-AS1 by siRNA in SW1116 and LoVo cells was measured by qRT-PCR-analysis. $^{*}P<0.05$, *vs*. Ctrl siRNA. B, C. Proliferation of COAD cells were measured by Edu and CCK-8 assays. $^{*}P<0.05$, *vs*. Ctrl siRNA. D, E. Migration and invasion abilities were evaluated using the wound-healing and Transwell-assays. $^{*}P<0.05$, *vs*. Ctrl siRNA. F. Analysis of cell apoptosis was performed by flow cytometry. $^{*}P<0.05$, *vs*. Ctrl siRNA.

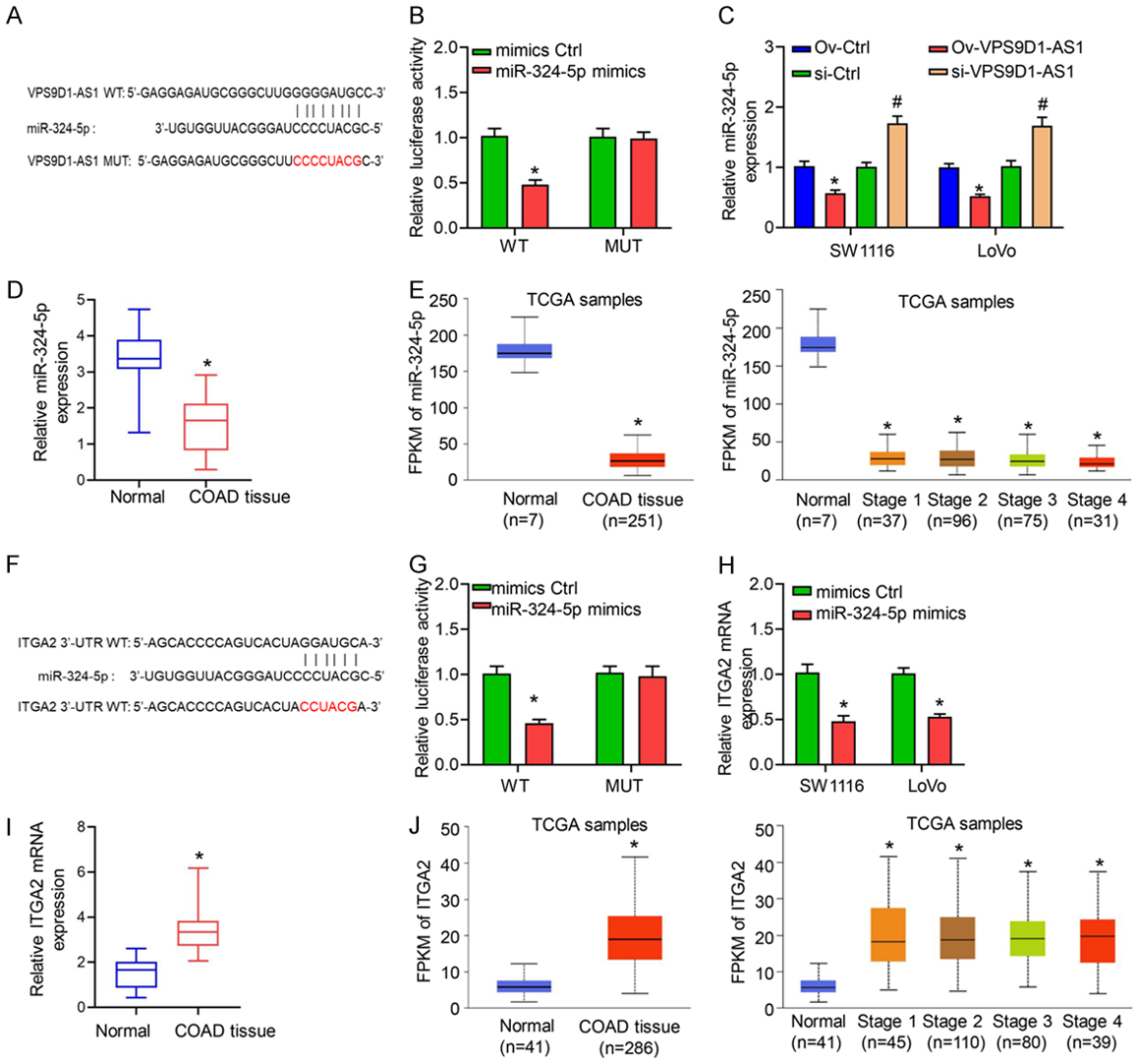

Figure 3. MiR-324-5p targets VPS9D1-AS1 and ITGA2 3'-UTR. A, F. MiR324-5p acts as a potential target of VPS9D1-AS1 and ITGA2 3'-UTR were analyzed using the StarBase 3.0 and miRDB, the online bioinformatics tools. B, G. MiR-324-5p targets VPS9D1-AS1 and ITGA2 3'-UTR as determined by dual-luciferase-reporter assay. C. VPS9D1-AS1 expression was measured in miR324-5p-overexpressing and miR136-5p-knockdown COAD cells. $^{*}P<0.05$, *vs*. Ov-Ctrl; $^{\#}P<0.05$, *vs*. si-Ctrl. D, I. Expression of miR324-5p and ITGA2 in COAD tissue samples, and their paired adjacent normal colon tissue samples, was measured by qRT-PCR analysis. $^{*}P<0.05$, *vs*. normal colon tissue-samples. E, J. Expression of miR324-5p and *ITGA2* was analyzed by UALCAN by using samples obtained from TCGA database. $^{*}P<0.05$, *vs*. normal colon tissue samples. H. *ITGA2* mRNA expression was measured in miR-324-5p-overexpressing COAD cells. $^{*}P<0.05$, *vs*. mimics Ctrl.

such prediction. Overexpression of miR324-5p significantly inhibited the luciferase activity in COAD cells transfected with WT VPS9D1-AS1, but not in those transfected with MUT VPS9D1-AS1 (**Figure 3B**). In addition, overexpression of VPS9D1-AS1 significantly inhibited the expression of the miR-324-5p, while VPS9D1-AS1 knockdown promoted the miR-324-5p expressions in COAD cells (**Figure 3C**). Moreover, miR324-5p expression was found to be decreased in COAD tissues by qRT-PCR analysis (**Figure 3D**), which was verified by TCGA database analysis using UALCAN (**Figure 3E**).

MiR-324-5p targets ITGA2 and regulates its expression

Bioinformatics analysis was conducted to search for the predicted target binding site of *ITGA2* and miR324-5p binding site (**Figure 3F**).

The luciferase-reporter-assay conducted above revealed that miR324-5p can directly target the 3'-UTR of *ITGA2* in LoVo cells (**Figure 3G**). In addition, it was also found that the overexpression of miR-324-5p significantly inhibited the expression of *ITGA2* mRNA (**Figure 3H**). Also, *ITGA2* mRNA was upregulated in COAD tissues by qRT-PCR analysis (**Figure 3I**) and UALCAN-TCGA database analysis (**Figure 3J**).

Impairment of the miR324-5p/ITGA2 axis attenuated the function of VPS9D1-AS1

Functional experiments involving the silencing of miR324-5p or forced expression of ITGA2 in the presence of VPS9D1-AS1 siRNA in LoVo cells indicated that anti-miR-324-5p or ITGA2 abolished the inhibitory effect of VPS9D1-AS1 siRNA on COAD progression. Specifically, after decreasing miR-324-5p or enhancing ITGA2 in VPS9D1-AS1-knockdown-LoVo cells, the results showed that they could abolish the suppressive effect of VPS9D1-AS1 siRNA on cell apoptosis (**Figure 4E**) and proliferation (**Figure 4A**, **4B**), migration (**Figure 4C**) and invasion (**Figure 4D**).

Knockdown of VPS9D1-AS1 inhibits in vivo growth of tumor

The regulatory effect of VPS9D1-AS1 on *in vivo* progression of COAD was investigated in a COAD xenograft mouse model developed by injecting mice with VPS9D1-AS1 shRNA- or control shRNA-lentivirus transfected LoVo cells. Mice injected with VPS9D1-AS1shRNA-lentivirus transfected cells had reduced tumor weight compared with those injected with control cells (**Figure 5A**). In addition, the xenograft expressed a lower VPS9D1-AS1 level after the injection with VPS9D1-AS1 shRNA (**Figure 5B**).

Discussion

Recent studies have shown that although lncRNAs do not encode proteins, their abnormal expression can lead to a variety of human diseases, including cancers. LncRNAs can regulate downstream genes at the epigenetic, transcriptional and post-transcriptional levels in different tumors, thereby affecting tumor development [7, 8]. Indeed, it has been reported that aberrantly expressed lncRNAs greatly contribute to the emergence and development of various tumors, thus examining their expression levels is helpful for tumor diagnosis, the treatment and prognosis analysis [11]. To date, an increasing number of lncRNAs that abnormally expressed in COAD tissues have been identified and play a significantrole in the regulation of COAD development and progression. Differential expression of lncRNAs is closely correlated with the prognosis of patients with COAD, which makes them potentially useful as effective prognostic biomarkers, *e.g.*, ZEB1-AS1 [27], SNHG6 and CTD-2354A18.1 [28], *etc*. Recently, a marked upregulation of ZEB1-AS1 expression in COAD tissues was reported by Ni *et al.* [27], suggesting a relationship between the high ZEB1-AS1 level and undesirable prognosis of patients with COAD. In addition, Xue *et al.* [28] reported the identification of two lncRNAs (SNHG6 and CTD-2354A18.1), which served as independent prognostic factors for predicting the clinical outcome of patients with COAD.

In the mentioned research above, lncRNA VPS9D1-AS1 was found to be highly expressed in a variety of tumors [13-15]. In addition, differential expression of VPS9D1-AS1 was detected between the COAD tissues and their paired adjacent normal tissues. VPS9D1-AS1 was sharply increased in COAD tissues compared with normal tissues. These results were further supported by the findings that VPS9D1-AS1 was also frequently upregulated in COAD cell lines compared with the normal colonic epithelial NCM460 cells, suggesting that VPS9D1-AS1 is associated with COAD development and may be involved in the carcinogenesis of COAD. VPS9D1-AS1 has been found highly expressed in clinical COAD samples, and showed COAD-promoting properties. In addition, a series of experiments have been conducted and confirmed the effects of VPS9D1-AS1 on the proliferation, migration, invasion and cell apoptosis of COAD-cells. The results of the proliferation assays suggested that interfering with the expression of VPS9D1-AS1 will reduce the growth rate of SW1116 and LoVo cells. Furthermore, in subsequent experiments, interfering with VPS9D1-AS1 expression was found to significantly reduce the invasion and migration ability of SW1116 and LoVo cells, indicating that VPS9D1-AS1 may have a role in enhancing the invasion and migration ability of COAD cells. The VPS9D1-AS1 knockdown effect was evaluated by flow cytometry analysis, which revealed that its knockdown promoted cell

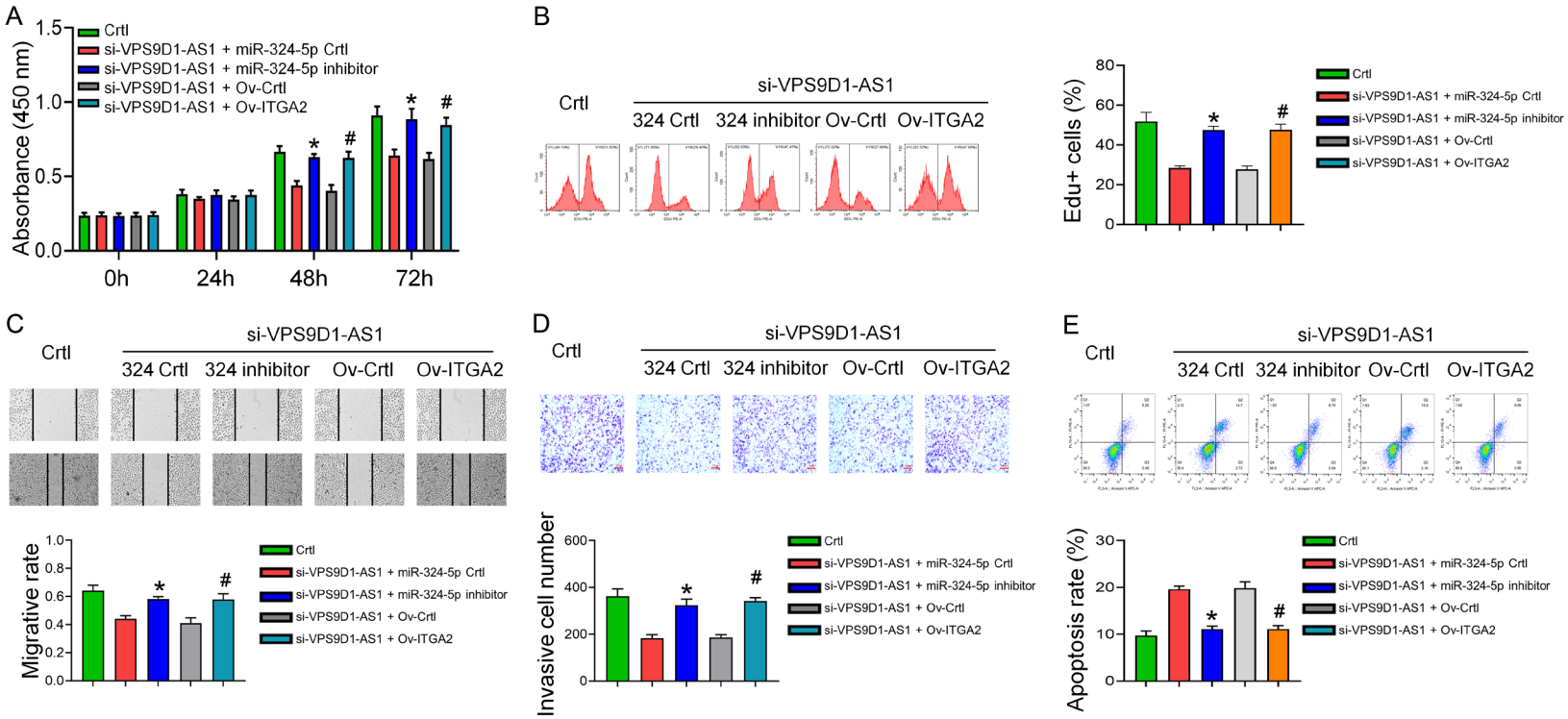

Figure 4. Silencing miR324-5p or inducing the expression of *ITGA2* in the presence of VPS9D1-AS1 siRNA in LoVo cells attenuated the function of VPS9D1-AS1 siRNA. A, B. Proliferation of LoVo cells were measured by CCK-8 and Edu assays. C, D. Migration and invasion abilities were evaluated by the wound healing and Transwell assays. E. Cell-Apoptosis analysis was performed by flow cytometry. *$P<0.05$, *vs*. VPS9D1-AS1 siRNA + miR-324-5p Ctrl; #$P<0.05$, *vs*. VPS9D1-AS1 siRNA + Ov-Ctrl.

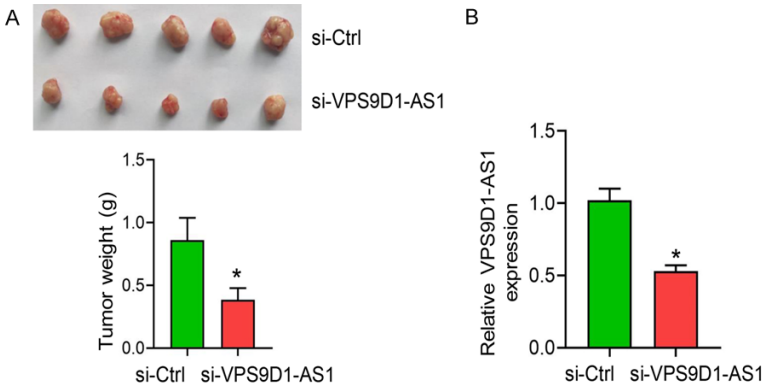

Figure 5. Knockdown of VPS9D1-AS1 inhibits tumor growth *in vivo*. A. Tumor weight was measured in mice injected with VPS9D1-AS1 shRNA and Ctrl shRNA. B. Expression of VPS9D1-AS1 was measured in isolated tumors by qRT-PCR analysis. *$P<0.05$, *vs*. Ctrl shRNA.

apoptosis, suggesting that VPS9D1-AS1 may increase the proliferation of COAD by inhibiting cell apoptosis. More importantly, the evaluation of the VPS9D1-AS1 knockdown effect in a COAD xenograft mouse model revealed that VPS9D1-AS1 knockdown inhibited tumor growth in *vivo*. Taken together, these findings indicate that VPS9D1-AS1 is an important regulator of gene expression and plays different functions in multiple biological processes in COAD cells, including tumor cell proliferation, metastasis, invasion and apoptosis. These effects of VPS9D1-AS1 may enable it to serve as a diagnostic and prognostic biomarker in COAD.

Understanding the molecular mechanisms underlying COAD cell proliferation and metastasis is essential to develop novel effective treatment approaches. In recent years, the role of ceRNA regulatory networks in tumorigenesis receiving increasing attention. Non-coding ceRNAs, such as lncRNAs, contain multiple binding sites for certain miRNAs [29-31]. Through competitive binding the inhibitory effects of miRNAs on target genes can be mitigated. According to the theory of ceRNA, a large body suggests that the interaction between lncRNAs and miRNAs is involved in the regulatory mechanism of COAD tumorigenesis. For instance, Liu J *et al.* [32] demonstrated a novel SNHG17/miR-375/CBX3 network that participates in COAD progression, and may provide promising therapeutic targets for COAD. Also, the identification of the functions of LINC00491 as a ceRNA, by sponging miR-145, leading to the to increased expression of *SERPINE1* in COAD cells, was reported by Wan J *et al.* [33]. Based on the above studies, we hypothesize that VPS9D1-AS1 is quite likely to have a part in promoting COAD evolution and progression by acting as a ceRNA. Through bioinformatics prediction tools and a dual-luciferase assay, miR-324-5p was determined to be capable of binding both VPS9D1-AS1 and the ITGA2 3'-UTR region. In addition, it was also confirmed by qRT-PCR analysis and UALCAN-TCGA database analysis that miR-324-5p and ITGA2 were lowly and highly expressed, respectively, in COAD cells. An increasing number of studies have found decreased expression of miR324-5p in glioma [34], colorectal cancer [35] and gallbladder carcinoma [36], indicating the suppressive effect of miR324-5p in the progression of different human cancers. The expression of *ITGA2* determined to be upregulated in several types of tumors, and was also shown to be closely associated with tumor invasion [23-25]. Overall, this study suggested that VPS9D1-AS1, miR-324-5p and ITGA2 may form a ceRNA regulatory network, which provides a molecular basis for the function of the VPS9D1-AS1/miR-324-5p/ITGA2 axis.

To conduct an in-depth investigation of the function and activity performed by VPS9D1-AS1, miR324-5p and ITGA2, the activity of miR-324-5p was silenced or the expression of *ITGA2* was induced in the presence of si-VPS9D1-AS1 in LoVo cells. The results of the functional rescue experiments revealed that AMO-miR-324-5p or ITGA2 abolished the inhibitory effects of VPS9D1-AS1 siRNA on the COAD progression processes, including cell-proliferation, migration and invasion. Overall, the results of this study led to the identification of a novel ceRNA network involving VPS9D1-AS1, miR324-5p, and *ITGA2* in the development of COAD. A comprehensive and detailed understanding of the VPS9D1-AS1/miR324-5p/ITGA2 ceRNA pathway may lead to the identification of the new treatment approaches targeting COAD.

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Disclosure of conflict of interest

None.

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