

REPORT



Long non-coding RNA ZFAS1 sponges miR-484 to promote cell proliferation and invasion in colorectal cancer

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ABSTRACT

The incidence and mortality rate of colorectal cancer (CRC) have been significantly increasing. However, mechanisms involved in CRC progression are still unclear. LncRNA ZFAS1 has been verified as oncogenic molecular in a series of tumors, including CRC. However, the underlying mechanism of ZFAS1 in CRC carcinogenesis remains unclear. In the present study, our data showed that ZFAS1 expression was significantly upregulated in CRC tissues and cell lines. Correlation analysis showed that high ZFAS1 expression was significantly associated with *Helicobacter pylori* infection, lymph nodes metastasis, advanced TNM stage and poor overall survival of CRC patients. Loss-of-function experiments revealed that ZFAS1 inhibition could markedly suppress CRC cells proliferation and invasion both in vitro and in vivo. Bioinformatics analysis and luciferase reporter assay revealed that ZFAS1 directly interacted with miR-484. Rescue experiments showed that miR-484 inhibitor reversed the tumor suppressing roles of ZFAS1 knockdown on CRC cells. Therefore, our study suggested that ZFAS1 could act as an oncogene in CRC tumorigenesis, and discovered the functional regulatory pathway of ZFAS1 sponging miR-484.

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide. Approximately 1.2 million patients worldwide have been diagnosed with CRC [1,2]. Although aggressive therapeutic strategies including surgery, radiotherapy, and chemotherapy have greatly improved, CRC morbidity and mortality remain high [3]. Therefore, a better understanding of the molecular mechanisms involved in CRC will provide novel diagnostic biomarkers and effective therapeutics for the treatment of CRC patients.

Long non-coding RNAs (lncRNAs) are RNA molecules whose transcripts are more than 200 nt in length [4]. They do not encode proteins, but play an important role in regulating gene expression through both epigenetic and posttranscriptional mechanisms [5,6]. Aberrant expression levels of lncRNAs have been correlated with various malignant biological processes, including carcinogenesis, cell proliferation, migration, invasion and apoptosis [7,8]. Increasing studies showed that lncRNAs play critical roles in tumor progression. For example, Lin et al showed that increased expression of the lncRNA ANRIL promoted lung cancer cell metastasis and correlates with poor prognosis [9]. Li et al suggested that overexpression of lncRNA HOTTIP increased chemoresistance of osteosarcoma cell by activating the Wnt/ β -catenin pathway [10]. Wang et al indicated that lncRNA XIST exerted oncogenic functions in human glioma by targeting miR-137 [11]. Thus, emerging evidences are proving the more and more significant role of lncRNA in tumor progression.

LncRNA zinc finger antisense 1 (ZFAS1) is an antisense transcript from 5' end of *Znfx1* gene, being firstly identified

to play a functional role in patients with acute myocardial infarction [12]. Moreover, the dysregulated ZFAS1 expression in cancer tissues has been discovered. For example, Gao et al found that lncRNA ZFAS1 could exhibit an oncogenic role in glioma progression by regulating EMT and Notch signaling pathway [13]. Xia et al showed that lncRNA ZFAS1 interacted with miR-150-5p to regulate Sp1 expression and ovarian cancer cell malignancy [14]. Recent studies showed that ZFAS1 play important roles in CRC. For example, Wang et al showed that upregulation of lncRNA ZFAS1 predicted poor prognosis and promoted invasion and metastasis in colorectal cancer [15]. Thorenor et al indicated that ZFAS1 interacted with CDK1 and is involved in p53-dependent cell cycle control and apoptosis in colorectal cancer [16]. However, the underlying mechanism of ZFAS1 on CRC progression remains unclear.

In the present study, our data elucidated the regulatory function of ZFAS1 in the development of CRC via regulating miR-484, suggesting an effective therapeutic strategy for CRC treatment.

Materials and methods

Tissue specimens and ethics statement

The CRC tissues and adjacent non-tumor tissues were collected from 49 patients who underwent surgical resection at Huaihe Hospital of Henan University (China) between 2012 and 2013. All patients did not receive any local or systemic treatment before surgery. All the CRC tissues were confirmed by two experienced pathologists. All patients signed the informed

consent and the experimental protocol was approved by the Ethics Committee of Huaihe Hospital of Henan University.

Cell lines and cell transfection

Human CRC cell lines (HCT116, SW480, SW620, HT-29 and LOVO) and the human colonic epithelial cells HCoEpiC were obtained from the American Type Culture Collection. All cells were cultured in RPMI-1640 medium (Gibco) supplemented with 10% FBS, 100 U/mL streptomycin and 100 U/mL penicillin (Gibco). The culture condition was at 37 °C in a humidified atmosphere containing 5% CO₂.

For RNA interference assay, cells were seeded at 70–80% confluency before transfection and transfected with siRNAs using Lipofectamine 2000 (Invitrogen). All the siRNAs (20nM) were synthesized by Genepharma (Shanghai, China) and the sequences were as follows: si-ZFAS1-1, 5'-CUGGCUGAAC-CAGUCCACAAGGUU-3'; si-ZFAS1-2, 5'-CCCTGTGCTT-TCATGAAAGTGAAGA-3'; si-NC, 5'-CCAAAACCAGGCU-UUGAUUGA-3'.

Total RNA extraction and quantitative real-time PCR

Tissues and cells samples were dissolved in TRIzol reagent (Invitrogen) and total RNAs was extracted according to the manufacturer's instruction. The cDNA was synthesized with the Reverse Transcription System (Promega). RT-PCR was performed using the SYBR Green Master Mixture (Roche) reagent in ABI 7500 Real-time PCR instrument. U6 was acted as an internal control. Relative expression levels were calculated and normalized using the 2^{-ΔΔC_t} method. All the primers were listed as follows: ZFAS1 forward, 5'-AAGCCACGTGCAGACATCTA-3', reverse, 5'-CTACTTCCAACACCCGCATT-3'; miR-484, forward, 5'-CTCAACTGGTGTCTTCAGTTGAGG-GTGGAGTCGGCC-3', reverse 5'-ACACTCCAGGGCCGCGCTGGGAGGCGG-3'; U6, forward, 5'-CGCTAGCACAT-ATCGGCTA-3', reverse, 5'-TTCTGCGACGAATTTGTCAT-3'.

Cell proliferation assay

Cell Counting Kit-8 (CCK-8, Dojindo) was employed to determine cell proliferation. Briefly, 1 × 10⁴ cells were seeded into 96-well plates and 10 μl CCK-8 solution was added to each well. Then, the cells and plates were incubated at 37°C for 90 min. At the indicated time points (24, 48, 72 and 96 h), the absorbance at 450 nm was measured using a spectrophotometer.

Colony formation assay

For the colony formation assay, cells were seeded into six-well plates at density of 1 × 10³ per well. After being cultured for 14 days, cells were washed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min. then, cells were stained with crystal violet for 10 min and washed with PBS to remove the staining solution. Colonies were examined and counted under microscope. The assays were repeatedly performed in triplicate.

Transwell invasion assay

The cell invasion assay was performed using 24-well insert transwell chambers (8 μm, Corning). Cells (2 × 10⁴) suspended in 200 μL serum-free medium were added to the upper chamber, and culture medium containing 20% FBS was placed in the bottom chamber. The cells were then incubated for 48 h at 37°C; the cells on the upper surface were scraped and washed away, whereas the cells on the lower surface were fixed with 20% methanol and stained with 0.1% crystal violet. The number of invaded cells was counted in 5 randomly selected fields under a microscope.

Xenograft nude mouse model

Male BALB/c nude mice (6 weeks) were maintained in clean conditions and used for xenograft assay. cells (4 × 10⁵/100ml) were transfected with sh-ZFAS1 or control (sh-NC), and then subcutaneously injected into back of nude mice. After that, tumor size was measured every week, and tumor weight was measured after sacrifice of mice. The xenograft mice assay was approved by the Committee on Animal Welfare of Huaihe Hospital of Henan University.

Luciferase reporter assay

For luciferase assay, the 3'-UTR of ZFAS1 cDNA was amplified using PCR, then, cDNA were cloned into the downstream of the firefly luciferase gene pGL3 (Invitrogen). HEK293T cells were seeded into 96-well plates at density of 1 × 10⁴ cells/well. Afterwards, pGL3-ZFAS1-Wt (wild type) and pGL3-ZFAS1-Mut (mutant) was transfected into HEK293T cells with miR-484 mimics using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h of transfection, luciferase activities were measured using the dual-luciferase reporter gene assay kit (Promega) according to the manufacturer's instructions.

RNA immunoprecipitation

Cells were lysed using a complete RNA lysis buffer containing protease inhibitor and RNase inhibitor using an EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore) following the manufacturers' instructions. 100uL of whole cell lysate was incubated with the RIP immunoprecipitation buffer containing magnetic beads coated with Ago2 antibody and was designated as the test group, while the control group consisted of normal mouse IgG. After being incubated for 2 h at 4°C, the immunoprecipitated RNA was isolated. The purified RNA was further used in the qRT-PCR analysis of ZFAS1 and miR-484.

Statistical analysis

All data were represented as mean ± standard deviation (SD) and statistical analyses were performed using SPSS 19.0. The differences were evaluated by the Student t-test and one way ANOVA. Pearson correlation analysis was used to estimate the relationship between the expression level of ZFAS1 and miR-484. Kaplan–Meier methods with the log-rank test were performed to calculate overall survival. p<0.05 was considered statistically significant.

Results

LncRNA ZFAS1 was upregulated in CRC tissues

To explore the clinical relevance of lncRNA ZFAS1 in human CRC. We detected ZFAS1 expression in CRC tissues using qRT-PCR. The results showed that ZFAS1 expression was significantly upregulated in CRC tissues compared to adjacent non-tumor tissues (Figure 1A and 1B; $p < 0.05$). According to the median value of ZFAS1 expression in CRC tissues, patients were divided into two groups: the high ZFAS1 expression group ($n = 25$) and low ZFAS1 expression group ($n = 24$). Results showed that high ZFAS1 expression was positively associated with Helicobacter pylori infection, lymph nodes metastasis and advanced TNM stage (Table 1; $p < 0.05$). As shown in Figure 1C, high ZFAS1 expression in CRC tissues is significantly associated with worse overall survival ($p < 0.05$, log-rank test). In addition, ROC (Receiver Operating Characteristic) curve showed that comparing with CRC patients and controls, ZFAS1 had an AUC (Area under the Curve) value of 0.837 (95% CI: 0.713-0.921). Besides, sensitivity, specificity and p value were respectively 0.752, 0.788 and 0.006 (Figure 1D). Taken together, those results indicated that ZFAS1 could act as an oncogene in CRC progression.

Table 1. Correlation between ZFAS1 expression and clinicopathological features of CRC.

Parameters	Group	Total	lncRNA ZFAS1 expression		P value
			High	Low	
Gender	Male	30	17	13	0.320
	Female	19	8	11	
Age (years)	<60	23	11	12	0.674
	≥60	26	14	12	
Tumor size (cm)	<5 cm	25	15	10	0.453
	≥5 cm	24	10	14	
Histological grade	Well and moderately	36	16	20	0.199
	Poorly	13	9	4	
Helicobacter pylori	Negative	20	6	14	0.015
	Positive	29	19	10	
Lymph nodes metastasis	Negative	38	16	22	0.020
	Positive	11	9	2	
TNM stage	I-II	19	6	13	0.030
	III-IV	30	19	11	

ZFAS1 knockdown suppressed CRC cells proliferation and invasion in vitro

It had been testified that ZFAS1 was upregulated in CRC tissue, and the high ZFAS1 expression was closely correlated with advanced clinical features and poor overall survival. Thus, we

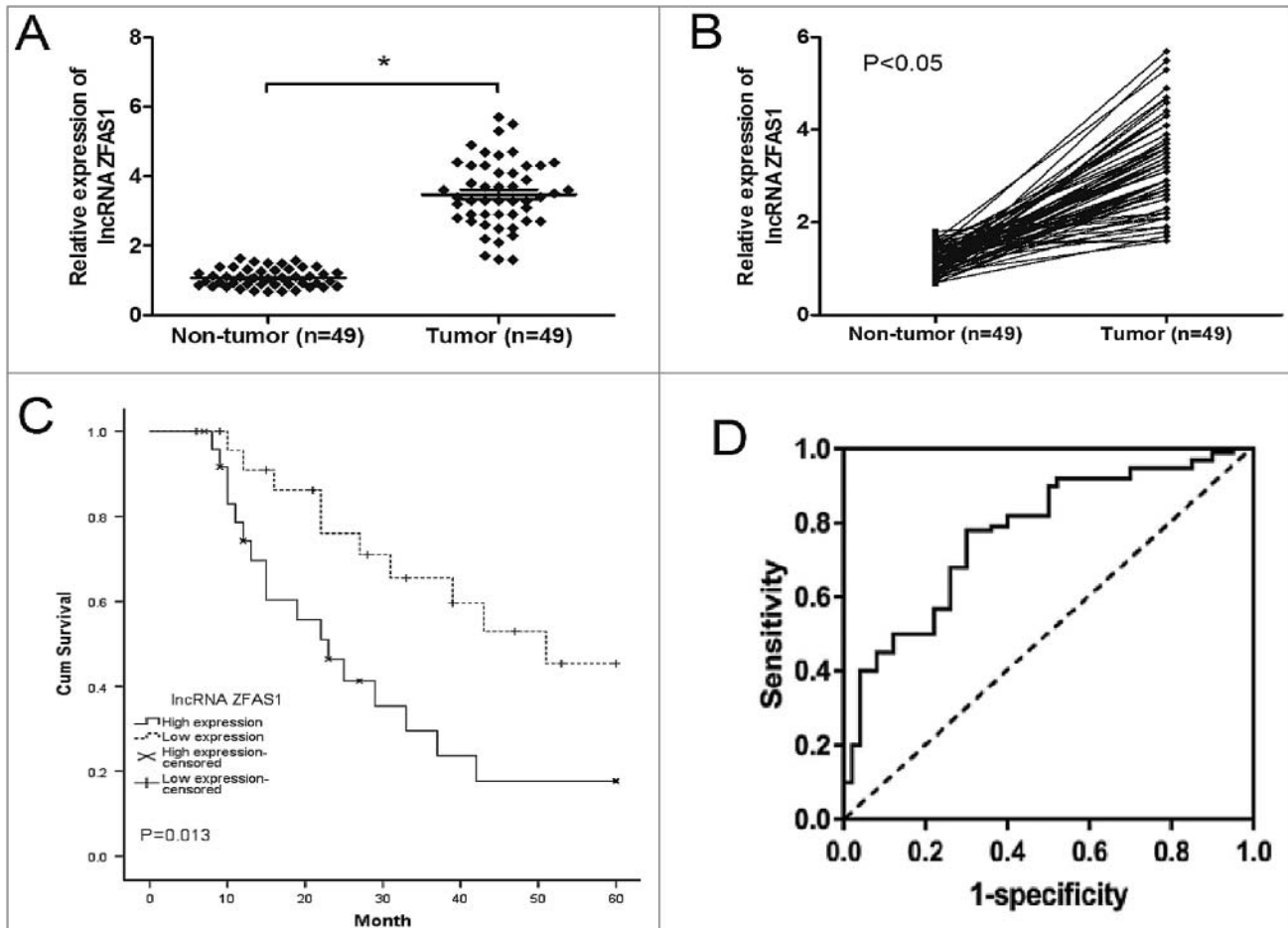


Figure 1. LncRNA ZFAS1 was upregulated in CRC tissues and associated with poor prognosis. (A,B) Relative expression of ZFAS1 measured by qRT-PCR in CRC tissues and adjacent non-tumor tissues. (C) Kaplan-Meier analysis and log-rank test were performed to assess the prognosis of CRC patients with high- and low- ZFAS1 expression. (D) ROC curve showed the AUC value of ZFAS1 (0.837, 95% CI: 0.713-0.921). * $p < 0.05$

performed loss-of-functional experiments to explore the role of ZFAS1 on CRC cells in vitro. QRT-PCR showed that ZFAS1 expression in CRC cells (HCT116, DLD1, SW480, SW620, HT-29 and LOVO) was significantly increased compared to HCoEpiC cells (Figure 2A; $p < 0.05$). The synthesized small interfering RNAs were respectively transfected into SW480 and HT-29 cells. The ZFAS1 expression was significantly decreased in si-ZFAS1 transfecting cells (Figure 2B; $p < 0.05$). CCK-8 assay showed that ZFAS1 knockdown inhibited cell viability in SW480 and HT-29 cells compared to si-NC group (Figure 2C; $p < 0.05$). Furthermore, colony formation assay showed that ZFAS1 inhibition effectively suppressed the clone formation ability of SW480 and HT-29 cells (Figure 2D; $p < 0.05$). Additionally, the invasion ability of SW480 and HT-29 cells transfected with si-ZFAS1 was inhibited compared with si-NC group (Figure 2E; $p < 0.05$). Taken together, those results suggested that ZFAS1 suppression could inhibit CRC cell viability and invasion.

ZFAS1 knockdown suppressed tumor growth in vivo

To confirm the biological function of ZFAS1 in CRC in vivo, SW480 cells stably transfected with sh-ZFAS1 or sh-NC were subcutaneously injected into the right flank of the nude mice. Tumor volume was measured every weeks for 7 weeks. As shown in Figure 3A, ZFAS1 knockdown suppressed tumor growth in vivo compared with sh-NC group ($p < 0.05$). Xenograft tumors were resected and weighed after 7 weeks of injection. Tumor weight in sh-ZFAS1 group was lower than that in sh-NC group (Figure 3B and 3C; $p < 0.05$). Thus, these data demonstrated that ZFAS1 knockdown could suppress tumor growth in vivo.

miR-484 was directly regulated by ZFAS1

Recently, increasing studies demonstrated that lncRNA might act as a competing endogenous RNA (ceRNA) or a molecular sponge in regulating the biological functions of miRNAs [17]. To investigate the underlying mechanism of ZFAS1 on CRC tumorigenesis, the bioinformatics tool DIANA was used to explore the targets of ZFAS1. Results showed that miR-484 shared complementary binding sites with ZFAS1 3'-UTR (Figure 4A). Luciferase reporter assay revealed that miR-484 mimics could evidently suppress the luciferase activity of the ZFAS1-Wt, but not of ZFAS1-Mut (Figure 4B; $p < 0.05$). In addition, we explored the expression of miR-484 in CRC tissues and cell lines, results showed that miR-484 was significantly downregulated in CRC tissues and cell lines (Figure 4C and 4D; $p < 0.05$). Pearson's correlation analysis revealed an inverse correlation between miR-484 and ZFAS1 in CRC samples (Figure 4E; $p < 0.05$). Furthermore, we found that miR-484 expression was upregulated after ZFAS1 inhibited in CRC cells (Figure 4F; $p < 0.05$). RNA immunoprecipitation experiments showed that both miR-484 and ZFAS1 were in the Ago2-pulled down pellet (Figure 4G; $p < 0.05$). Thus, those data indicated that ZFAS1 might acted as a molecular sponge for miR-484.

miR-484 reversed the effect of ZFAS1 on CRC progression

To explore whether ZFAS1 exerted biological functions through miR-484, we performed rescue experiments by inhibiting miR-484 expression in ZFAS1 knockdown SW480 cells (Figure 5A; $p < 0.05$). MTT and colony formation assay showed that miR-484 inhibitor could reverse the inhibition of ZFAS1 on proliferation capacity of SW480 cells (Figure 5B and 5C; $p < 0.05$). Transwell invasion assay showed that miR-484 inhibitor increased the invasion ability of SW480 cells reduced by ZFAS1 knockdown (Figure 5D; $p < 0.05$). Overall, miR-484 inhibitor could reverse the tumor suppressing of ZFAS1 knockdown on CRC cells, indicating the antagonism regulation of ZFAS1 and miR-484.

Discussion

Despite the great therapeutic advances made in CRC, including surgical resection and adjuvant therapy, the long-term prognosis of CRC patients with distant metastases remains unfavorable [18]. Increasing functional studies have indicated that lncRNAs, which may act as oncogenes or tumor suppressors, are involved in the carcinogenesis and progression of several tumors, including CRC [19]. For example, Liu et al showed that enhanced expression of lncRNA Sox2ot promoted cell proliferation and motility in colorectal cancer [20]. Wang et al revealed that lncRNA AB073614 regulated proliferation and metastasis of colorectal cancer cells via the PI3K/AKT signaling pathway [21]. Liu et al suggested that lncRNA SPRY4-IT1 promoted proliferation and invasion by acting as a ceRNA of miR-101-3p in colorectal cancer cells [22].

In the present study, we focused on the lncRNA ZFAS1 and found that its expression was significantly upregulated in CRC tissues and correlated with Helicobacter pylori infection, lymph nodes metastasis, advanced TNM stage, and poor overall survival. Furthermore, function experiments showed that ZFAS1 inhibition markedly suppressed the proliferation and invasion ability of CRC cells. In addition, our results showed that ZFAS1 knockdown suppressed tumor growth in vivo. Thus, our data strongly suggested that ZFAS1 could function as an oncogene in CRC tumorigenesis.

lncRNA is type of non-protein coding RNA and modulate pathophysiology process involving multi-levels regulation [5]. One of the major regulating approach is to bind target miRNAs to absorb the miRNAs abundance and further modulate functional genes expression [17]. For example, Zhang et al lncRNA UCA1 promoted cell progression by acting as a competing endogenous RNA of ATF2 in prostate cancer [23]. Wang et al indicated that lncRNA TUG1 promoted migration and invasion by acting as a ceRNA of miR-335-5p in osteosarcoma cells [24].

Recent studies showed that miR-484 play important roles in tumor progression. For example, Hu et al showed that miR-484 suppressed proliferation and epithelial-mesenchymal transition by targeting ZEB1 and SMAD2 in cervical cancer cells [25]. While, Li et al showed that miR-484 promoted non-small-cell lung cancer progression through inhibiting Apaf-1 associated with the suppression of

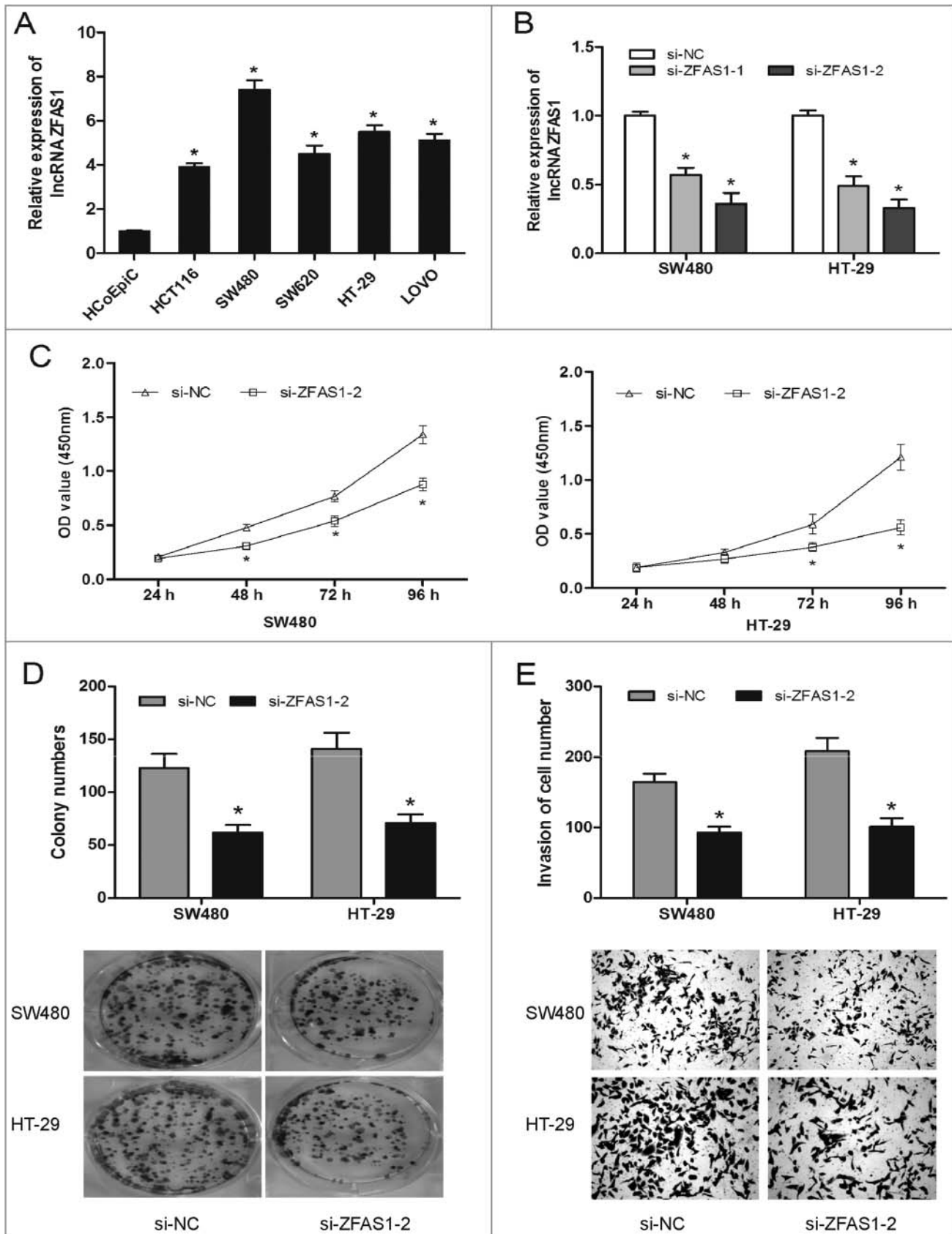


Figure 2. LncRNA ZFAS1 inhibition suppressed CRC cells proliferation and invasion. (A) Relative expression of ZFAS1 measured by qRT-PCR in CRC cells and human colonic epithelial cells HCoEpiC. (B) ZFAS1 expression was examined by qRT-PCR assay in cells transfected with si-ZFAS1 or si-NC. (C) CCK-8 assay showed that ZFAS1 depletion inhibited the proliferation of SW480 and HT-29 cells. (D) colony formation assay revealed that ZFAS1 inhibition suppressed the clone formation of SW480 and HT-29 cells. (E) Transwell invasion assay suggested that ZFAS1 suppression inhibited the invasion ability of SW480 and HT-29 cells. * $p < 0.05$

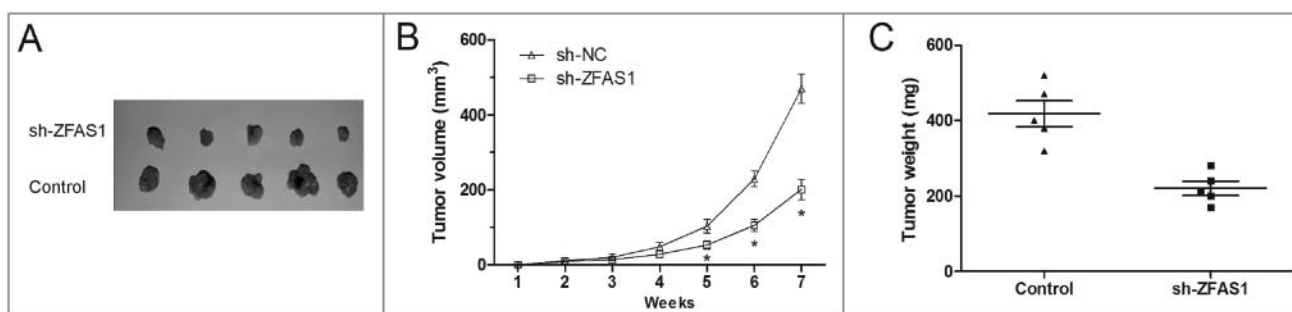


Figure 3. LncRNA ZFAS1 knockdown suppressed tumor growth in vivo. (A) SW480 cells transfected with sh-ZFAS1 were subcutaneously injected into back of nude mice. Tumor volume was measured every week. (B,C) The subcutaneous tumors were taken photos (B) and weighted (C) in sh-ZFAS1 group or sh-NC group. * $p < 0.05$

apoptosis [26]. However, the expression and roles of miR-484 on CRC remains unclear.

In the present study, qRT-PCR showed that miR-484 expression was significantly decreased and negatively correlated with ZFAS1 expression in CRC tissues. Bioinformatics prediction and luciferase reporter assay validated that miR-484 was the target of ZFAS1 sharing complementary binding sites at 3'-UTR. Besides, miR-484 reversed the functions of ZFAS1 on

CRC cells proliferation and invasion, suggesting the competing endogenous RNA (ceRNA) mechanism in CRC progression.

In conclusion, our work revealed the overexpression of ZFAS1 and investigated the potential interaction with miR-484 in CRC tumorigenesis, indicating the ceRNA mechanism or miRNAs sponge in CRC. Thus, it could be argued the ZFAS1-miR-484 axis may become a new epigenetic therapeutic target for the treatment of CRC.

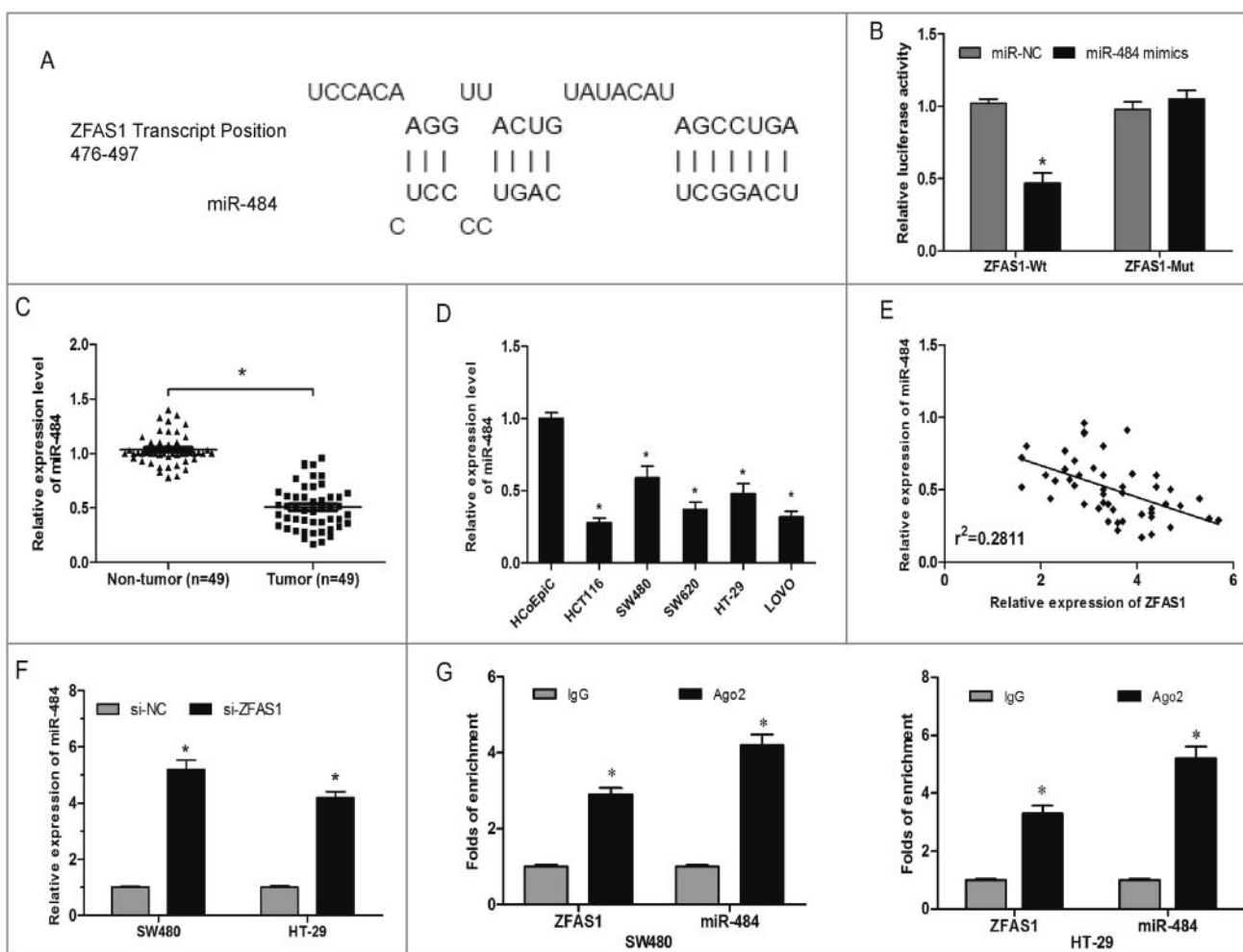


Figure 4. LncRNA ZFAS1 directly interacted with miR-507 in CRC. (A) The predicted complementary sequence between miR-484 and ZFAS1 3'-UTR. (B) HEK293T cells were transfected with ZFAS1 3'-UTR Wt or Mut reporter plasmids along with miR-484 mimics, and then luciferase activity assays were performed. (C) Relative expression of miR-484 was measured by qRT-PCR in CRC tissues. (D) Relative expression of miR-484 was measured by qRT-PCR in CRC cells. (E) Pearson's correlation analysis between miR-484 and ZFAS1 expression in CRC tissues. (F) miR-484 expression in ZFAS1 knockdown CRC cells was determined by qRT-PCR. (G) Association of ZFAS1 and miR-484 with Ago2 in CRC cells. * $p < 0.05$

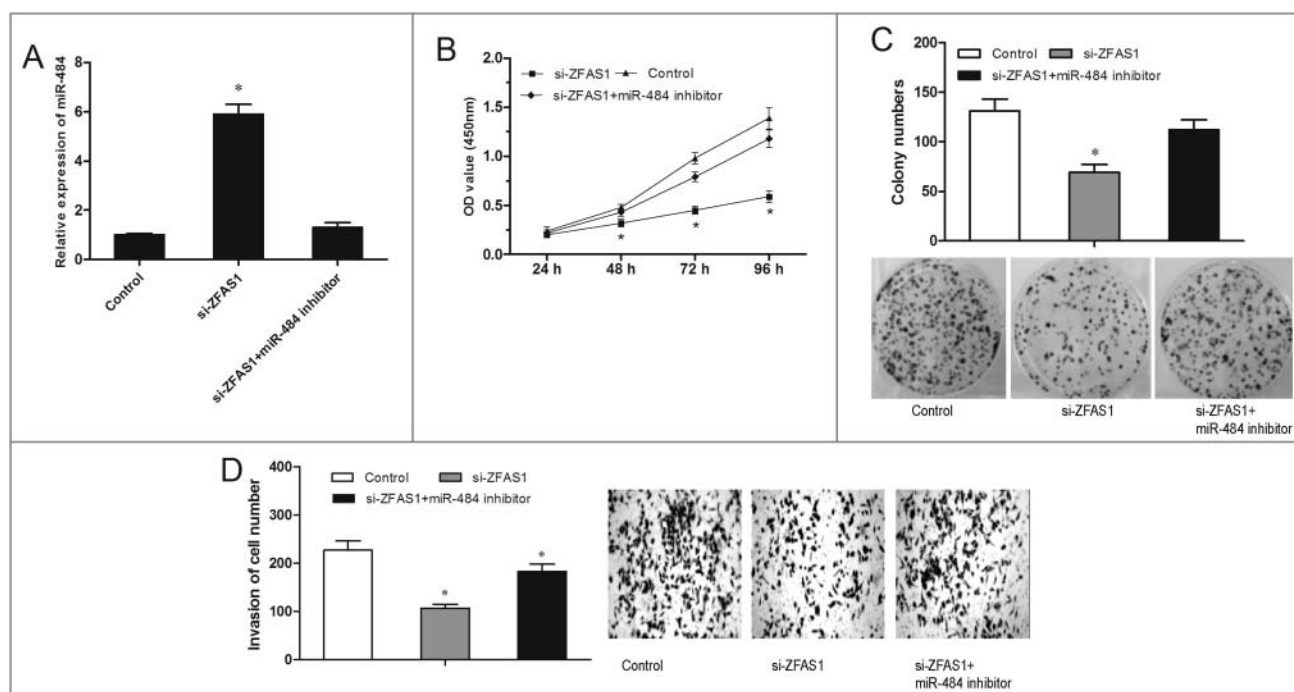


Figure 5. miR-484 reversed the effect of ZFAS1 on CRC tumorigenicity. (A) Relative expressions of miR-484 in CRC cells transfected with si-ZFAS1 and miR-484 inhibitor. (B) CCK-8 assay showed that proliferation of CRC cells transfected with si-ZFAS1, si-ZFAS1+miR-484 inhibitor. (C) Colony formation assay showed the clone number of CRC cells transfected with si-ZFAS1, si-ZFAS1+miR-484 inhibitor. (D) Transwell invasion assay showed the invasion of CRC cells transfected with si-ZFAS1, si-ZFAS1+miR-484 inhibitor. * $p < 0.05$

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this Article.

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