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Research article

ALYREF promotes malignant behaviors and inhibits ferroptosis in colon cancer cells by stabilizing PCSK9 mRNA ☆

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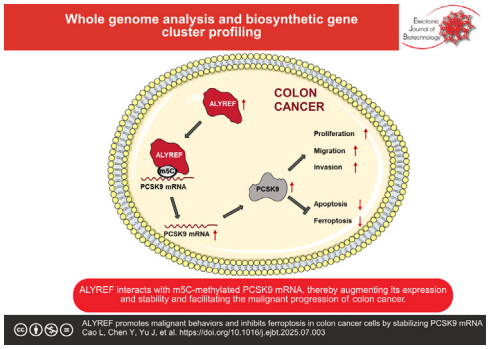
GRAPHICAL ABSTRACT

ALYREF promotes malignant behaviors and inhibits ferroptosis in colon cancer cells by stabilizing PCSK9 mRNA



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ABSTRACT

Background: Colon cancer is a prevalent malignancy causing significant global morbidity and mortality. The RNA methyltransferase Aly/REF export factor (ALYREF), which binds 5-methylcytosine (m5C)-modified messenger RNA, represents a potential diagnostic and therapeutic target in cancer. However, its specific role and mechanism in colon cancer progression remain unexplored.
Results: ALYREF expression was significantly elevated in colon cancer tissues and cell lines compared to normal controls. Depletion of ALYREF suppressed colon cancer cell proliferation, migration, and invasion, while simultaneously promoting apoptosis and ferroptosis. Analysis revealed proprotein convertase subtilisin/kexin type 9 (PCSK9) is highly expressed in colon cancer and positively regulated by ALYREF. Mechanistically, ALYREF directly bound to and stabilized PCSK9 messenger RNA in a manner dependent on m5C modification. Crucially, the anti-tumor effects resulting from ALYREF knockdown were reversed by overexpressing PCSK9. Consistent with cellular findings, silencing ALYREF significantly inhibited tumor growth *in vivo* using xenograft models.

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Conclusions: This study demonstrates that ALYREF drives colon cancer malignancy by stabilizing PCSK9 messenger RNA via m5C methylation, thereby enhancing PCSK9 expression. These findings establish the ALYREF/PCSK9 axis as a critical mechanism in colon cancer progression, highlighting its potential as a novel therapeutic target for intervention.

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1. Introduction

Colon cancer represents a prevalent malignant neoplasm within the digestive tract, primarily arising from the mucosal epithelium of the colon [1]. As one of the most prevalent global malignancies, it exhibits remarkably high morbidity and mortality rates, posing a severe threat to human health and imposing a heavy burden on society and families [2,3]. The prognosis of colon cancer hinges upon diverse elements, encompassing tumor stage, pathological classification, and therapeutic approach [4,5,6]. Early-stage patients typically have a relatively high 5-year survival rate after prompt treatment, whereas advanced-stage patients face a poorer outcome [7,8,9]. Thus, in-depth investigations into the various aspects of colon cancer are of paramount importance in enhancing patient prognoses and alleviating the disease's deleterious impacts [10].

5-methylcytosine (m5C) is an important form of RNA modification catalyzed by specific methyltransferases. Its chemical modification site significantly affects RNA structure and function [11,12]. With the ceaseless progress of technology and intensive research, m5C is anticipated to act as a crucial target for cancer diagnosis and treatment [13]. A study has demonstrated that NOP2/Sun RNA methyltransferase 6 (NSUN6)-mediated m5C modification of METTL3 and promoted colon adenocarcinoma (COAD) progression [14]. Researchers found that prognostic features obtained using m5C-related regulators were valuable in COAD. Moreover, biological processes and pathways associated with m5C-related RNA modifications promoted the malignant development of COAD [15]. RNA methyltransferase Aly/REF export factor (ALYREF) is an RNA-binding protein associated with m5C modification [16]. A recent study has demonstrated that its aberrant expression can contribute to multiple malignant phenotypes, such as sustaining proliferation, generating malignant heterogeneity, causing metastasis, and inducing drug resistance and cell death through various regulatory mechanisms like pre-mRNA processing, mRNA stabilization, and nucleo-cytoplasmic shuttling [17]. Few studies have shown that ALYREF is of vital importance in different cancers and diseases [18,19,20]. Nevertheless, whether ALYREF plays a role in colon cancer progression is unclear.

Proprotein convertases (PCs), a family consisting of nine serine proteases, are involved in the regulation of homeostasis of protein substrates in the cell [21]. They give rise to the activation, deactivation or functional alterations of numerous hormones, neuropeptides, growth factors and receptors [22,23,24]. As a member of the PC family, proprotein convertase subtilisin/kexin type 9 (PCSK9), a protein related to cholesterol, may be of great significance in cardiovascular disease, liver disease, infectious diseases, neurocognitive disorders and cancer [25,26,27,28]. In addition, it has been reported that PCSK9 is related to several diseases, such as cardiovascular disease and hypercholesterolemia [29,30]. A previous study has demonstrated that PCSK9 depletion inhibits the growth of APC/KRAS-mutated colorectal cancer cells *in vitro* and *in vivo*, whereas PCSK9 overexpression induces tumorigenesis [31]. Additionally, PCSK9 plays a critical role in the progression and metastasis of colon cancer by regulating epithelial-mesenchymal transition (EMT) and PI3K/AKT signaling in tumor cells [32]. Nevertheless, the regulatory relationship between ALYREF and PCSK9 and their roles in colon cancer progression remain unclear.

Hence, the present work is dedicated to revealing the underlying molecular mechanism of ALYREF in the development of colon cancer and providing novel clues for the treatment of colon cancer.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines

NCM460 cells were procured from Suncell (Wuhan, China), which were cultured in RPMI-1640 medium (BIOSUN, Shanghai, China) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (P/S) (Invitrogen, Carlsbad, CA, USA). HT29, SW620, HCT116, and SW480 cell lines were acquired from Pricella (Wuhan, China). HT29 and HCT116 cells were cultured in McCoy's 5A medium (Pricella) with 10% FBS (Gibco) and 1% P/S (Invitrogen). SW620 and SW480 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% FBS (Gibco) and 1% P/S (Invitrogen).

2.1.2. Clinical samples

A total of 63 pairs of colon cancer tissues and matched normal tissues were gathered from patients who underwent resection at Nantong First People's Hospital. Written informed consent from all subjects has been obtained, and the protocol has gained approval from the Ethics Committee of Nantong First People's Hospital.

2.2. Methods

2.2.1. Bioinformatics and database analysis

Differential expression of ALYREF in normal tissue and colon Adenocarcinoma (COAD) was first examined using the GEPIA database (https://gepia.cancer-pku.cn/detail.php). UALCAN Clinical Proteomic Tumor Analysis Consortium (CPTAC) (https://ualcan.path.uab.edu/analysis-prot.html) database was utilized to show ALYREF protein expression in colon cancer. In addition, the Linkedomics website was utilized to screen genes associated with ALYREF in the CPTAC-Colon Adenocarcinoma cohort.

2.2.2. Quantitative real-time polymerase chain reaction (qRT-RCR)

Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was utilized for the isolation of the total RNA from colon cancer tissues. Then, the cDNA was obtained using Transcriptor First Strand cDNA Synthesis Kit (Roche, Vilvoord, Brussel, Belgium). Subsequently, the FastStart Universal SYBR Green Master (Roche, Germany) was utilized to detect the mRNA expression level. The sequences of each primer are shown in Table 1, and all primers were synthesized by Songon (Shanghai, China).

Table 1
Primers sequences used for PCR.

Name		Primers for PCR (5′-3′)
ALYREF	Forward	GCAGGCCAAAACAACTTCCC
	Reverse	AGTTCCTGAATATCGGCGTCT
PCSK9	Forward	CCTGGAGCGGATTACCCCT
	Reverse	CTGTATGCTGGTGTCTAGGAGA
β-actin	Forward	CTTCGCGGGCGACGAT
	Reverse	CCACATAGGAATCCTTCTGACC

2.2.3. Western blot analysis

First, the RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) was utilized for the extraction of proteins. Then, the protein samples were isolated through sodium dodecyl sulfate–polyacrylamide gel electrophoresis, after which, the protein bands were transferred onto the PVDF membrane (The β-actin was used as an internal reference). Next, 5% skimmed milk was incubated with the membrane at 37°C for 2 h. After incubation with primary antibodies (ALY Polyclonal antibody (1:600, Proteintech, Wuhan, China), PCSK9 Polyclonal antibody (1:1500, Proteintech), Beta Actin Monoclonal antibody (ab8226, 1:5000, Proteintech)) at 37°C for 1.5 h, the membrane was incubated with the horseradish peroxidase (HRP)-conjugated secondary antibodies (Goat Anti-Mouse IgG H&L (ab205719, 1:5000, Abcam), Goat Anti-Rabbit IgG H&L (ab6721, 1:5000, Abcam)). Finally, the chemiluminescence intensity analysis was carried out using the Clarity™ Western ECL Substrate Kit (Bio-Rad, Shanghai, China).

2.2.4. Cell transfection

Short hairpin RNAs (shRNAs) targeting ALYREF (sh-ALYREF#1: 5′-AACCGACCGGCGCCCTACA-3′ and sh-ALYREF#2: 5′-GTTGACGCA CAGCGGAGGC-3′) were synthesized by GenePharma (Shanghai, China). A scrambled shRNA (5′-CAACAAGATGAAGAGCACCA-3′) with no significant homology to any known mammalian gene was used as a negative control. For PCSK9 overexpression, the PCSK9 coding sequence was cloned into the pcDNA3.1 (+) vector (Invitrogen), with the empty vector (oe-NC) as a control. When SW620 and HCT116 cells were grown to 60–70% confluency, shRNAs and overexpression plasmids were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the protocol provided by the manufacturer.

2.2.5. 5-Ethynyl-2′-deoxyuridine (EdU) assay

Cell proliferation was estimated using the E-Click EdU Cell Proliferation Imaging Assay Kit (Red, Elab Fluor®594) (Elabscience, Wuhan, China). The reagents needed for this assay were prepared in advance. At first, cells were incubated with EdU working solution at 37°C for 2 h. Then, cell fixation and permeabilization were performed using 4% paraformaldehyde (Beyotime, Shanghai, China) and 0.3% Triton X-100 (Beyotime). Fluorescent labeling was performed by adding 500 μL of Click reaction solution. After incubation in the dark for 30 min, the cells were incubated with 500 μL of DAPI working solution for 5 min. At last, the proper filter was selected to analyze the results under a fluorescence microscope.

2.2.6. Colony formation assay

SW620 and HCT116 cells were completely resuspended in the complete medium after digestion with PerfCell™ Trypsin-EDTA (0.25%) (Yeasen, Shanghai, China). Then, cells were accurately counted and inoculated into 6-well plates (500 cells/well). After 14 d (media changed every 3 d), cells were fixed with 4% paraformaldehyde, washed with PBS, stained with crystal violet (Beyotime) for 15 min, and photographed.

2.2.7. Transwell assay

Transwell assay was conducted using Millicell® Standing Cell Culture Inserts (Millipore, Billerica, MA, USA) to evaluate cell migration and invasion. For migration, cells were suspended with 200 μL serum-free medium and inoculated into the upper chamber with a density of 1×10^5 cells/well, while 600 μL of complete McCoy's 5A medium was introduced to the bottom chamber. After 48 h of incubation at 37°C, cells were fixed with 4% paraformaldehyde (Beyotime), stained with 0.1% crystal violet (Beyotime), and counted under a microscope. As for the invasion assay, prior to the start of the experiment, the Matrigel matrix (Corning, Bedford, MA, USA) was diluted in a ratio of 1:8 with the serum-free cell culture medium. Subsequently, 60 μL of the Martrigel was added to the upper chamber and incubated for 3 h in the incubator at 37°C. The remaining steps were identical to those for the migration assay.

2.2.8. Cell apoptosis measurement

The Annexin V-FITC Apoptosis Detection Kit (Beyotime) was utilized for cell apoptosis assessment. First, 5×10^4 cells were collected into a centrifuge tube and suspended using 195 μL of binding buffer. Secondly, 5 μL of Annexin V-FITC and 10 μL of Propidium Iodide (PI) were successively supplemented to the cell suspension for 20 min of incubation away from the light. Then, cell apoptosis was evaluated by flow cytometry.

2.2.9. Ferroptosis assessment

Reactive oxygen species (ROS) level was detected by flow cytometry using the Reactive Oxygen Species Assay Kit (Beyotime); in short, the cells were suspended in DCFH-DA at a concentration of 1×10^7 cells/mL and incubated for 20 min at 37°C in a cell culture incubator. Then, the samples were tested using flow cytometry. Meanwhile, the Malondialdehyde (MDA) levels were determined using the MDA Content Detection Kit (Solarbio, Beijing, China). Cells were broken by ultrasonic waves (200 W, 3 s, 10 s intervals, 30 repetitions) at a rate of 1 mL of extraction solution per 5 million cells. Then, the MDA detection working solution was added to the samples, and the final detection was performed using a Microplate Reader. The MDA contents were calculated according to the instructions. Besides, the contents of glutathione (GSH) and Fe^{2+} were separately examined using Reduced Glutathione (GSH) Colorimetric Assay Kit (Elabscience) and Cell Ferrous Iron Colorimetric Assay Kit (Elabscience). These assays were carried out according to the steps in the manual. The absorbance values at 405 nm and 593 nm were respectively measured by a Microplate reader. Finally, the contents of GSH and Fe^{2+} were calculated.

2.2.10. RNA immunoprecipitation (RIP) assay

The RIP assay was performed using BeyoRIP™ RIP Assay Kit (Protein A/G Agarose) (Beyotime) to prove the correlation between ALYREF protein and PCSK9 mRNA. Concisely, Protein A/G Agarose was washed and pre-conjugated to the antibodies. The anti-IgG was used as a negative control in an amount consistent with the amount of ALYREF-specific antibody. Then, cells were lysed and incubated with Protein A/G Agarose at 4°C for 4 h. The supernatant was then discarded after four washes, and the precipitate was then incubated with 100 μL of Elution Buffer at 55°C for 30 min. The RNA was purified using RNA isolation kit (Beyotime). Finally, qRT-PCR was used to quantify RNA.

2.2.11. Detection of RNA stability

To analyze the steadiness of PCSK9 mRNA, SW620 and HCT116 cells received treatment using 5 μg/mL actinomycin D (Yeasen) and were cultured at 37°C. The RNA was isolated at the specified time points (0, 2, 4, 6, and 8 h) and quantified by qRT-PCR analysis.

2.2.12. Xenograft tumor models

To confirm the impacts of ALYREF on colon tumors *in vivo*, the animal tumor models were established using female BALB/c nude mice purchased from Aniphe Biolaboratory Inc. (Jiangsu, China). The mice were kept in ventilated cages and were provided a standard specific-pathogen-free (SPF) environment at 24°C, having unrestricted access to food and water. Briefly, the mice were sorted into two groups (5 mice/group), based on the types of cells injected. Approximately 5×10^6 HCT116 cells treated with sh-NC and sh-ALYREF were subcutaneously injected into the right lateral abdomen of the nude mice. Tumor volumes were recorded every 5 d. After 30 d, the mice were sacrificed and the tumor tissues were removed for further analysis. All animal experiments gained approval from the Animal Ethics Committee of Nantong First People's Hospital, and all procedures were conducted in accordance with the National Institutes of Health guide for the Care and Use of Laboratory Animals (animal ethics welfare number: P20230218-040).

2.2.13. Immunohistochemistry (IHC)

Tumor tissues were fixed in 4% paraformaldehyde, dehydrated, paraffin-embedded, and sectioned (4 μm). Sections were then deparaffinized, rehydrated and antigen retrieved. Subsequently, the 5% BSA (Yeasen) was used for blocking. 30 min later, the slides were incubated with ALYREF antibody (1:200, Proteintech) and PCSK9 antibody (1:500, Proteintech) at room temperature for 2 h. Then, the HRP-conjugated Goat Anti-Rabbit IgG (H + L) (1:1000, ab205718, Abcam) was added to the slides for signal amplification. DAB staining was performed using the DAB Horseradish Peroxidase Color Development Kit (Beyotime), and slides were counterstained with hematoxylin, dehydrated, and mounted for microscopy.

2.2.14. Statistical analysis

All data were analyzed with GraphPad Prism 8.0.1 software and presented as mean ± standard deviation (SD). The difference between the two groups was evaluated via Student's *t*-test, and the distinction among multiple groups was gauged by means of the one-way analysis of variance (ANOVA) test. A two-tailed Pearson correlation test was used to examine the correlation between PCSK9 expression and ALYREF. $p < 0.05$ was regarded as a statistically significant difference.\

3. Results

3.1. ALYREF was elevated in colon cancer

The GEPIA database analyzed ALYREF expression in 349 normal tissues and 275 COAD tissues, showing higher ALYREF levels in COAD (Fig. 1A). Similarly, UALCAN database results indicated elevated ALYREF protein expression in colon tumor tissues versus normal tissues (Fig. 1B). qRT-PCR analysis of 63 pairs of clinical samples confirmed increased ALYREF mRNA in tumor tissues (Fig. 1C), and western blot validated higher ALYREF protein expression in tumors (Fig. 1D). In cell lines, ALYREF was upregulated in colon cancer cells (SW620, HCT116, HT29, SW480) compared to normal NCM460 cells (Fig. 1E). Collectively, ALYREF mRNA and protein exhibited high-level expression in colon cancer tissues and cells.

3.2. Knockdown of ALYREF suppressed the malignant behaviors of colon cancer cells

The roles of ALYREF in SW620 and HCT116 cells were ascertained by loss-function assays. ALYREF protein levels in SW620 and HCT116 cells were decreased (Fig. 2A). Furthermore, EdU and colony formation assays illustrated that cell proliferation was restrained by sh-ALYREF (Fig. 2B-C). Interestingly, the transwell assay demonstrated that the migration (Fig. 2D) and invasion (Fig. 2E) abilities of colon cancer cells were similarly inhibited by

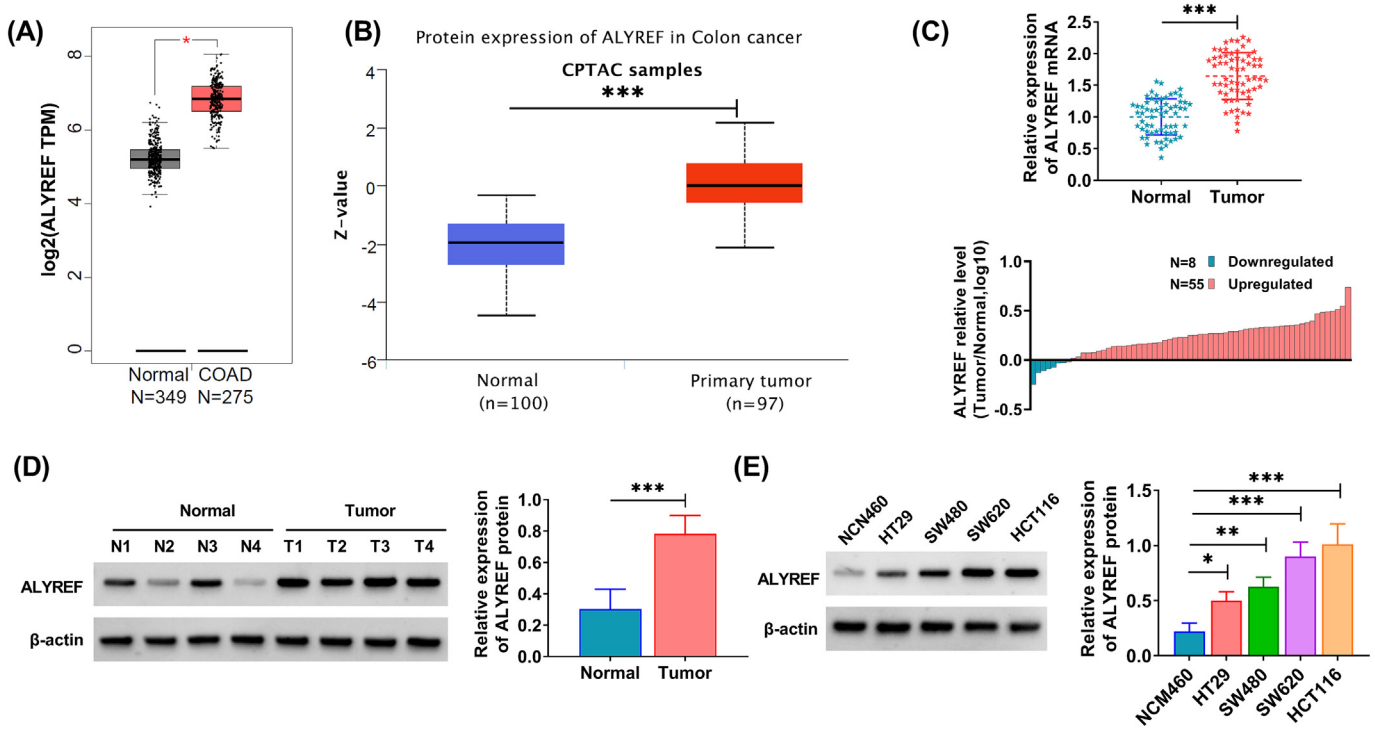

Fig. 1. Expression patterns of ALYREF in colon tissues and cells. (A) The GEPIA database showed ALYREF expression in 349 normal samples and 275 COAD samples. (B) UALCA database showed ALYREF expression in colon cancer based on CPTAC samples. (C) qRT-PCR assay was applied to measure the expression level of ALYREF mRNA in 63 pairs of colon tumor tissues and adjacent normal tissues. (D) ALYREF protein level was determined using Western blot in 4 tumor tissues and 4 normal tissues. (E) Western blot analysis of ALYREF protein level in normal colon cells (NCM460) and human colon cancer cell lines (HT29, SW480, SW620 and HCT116). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

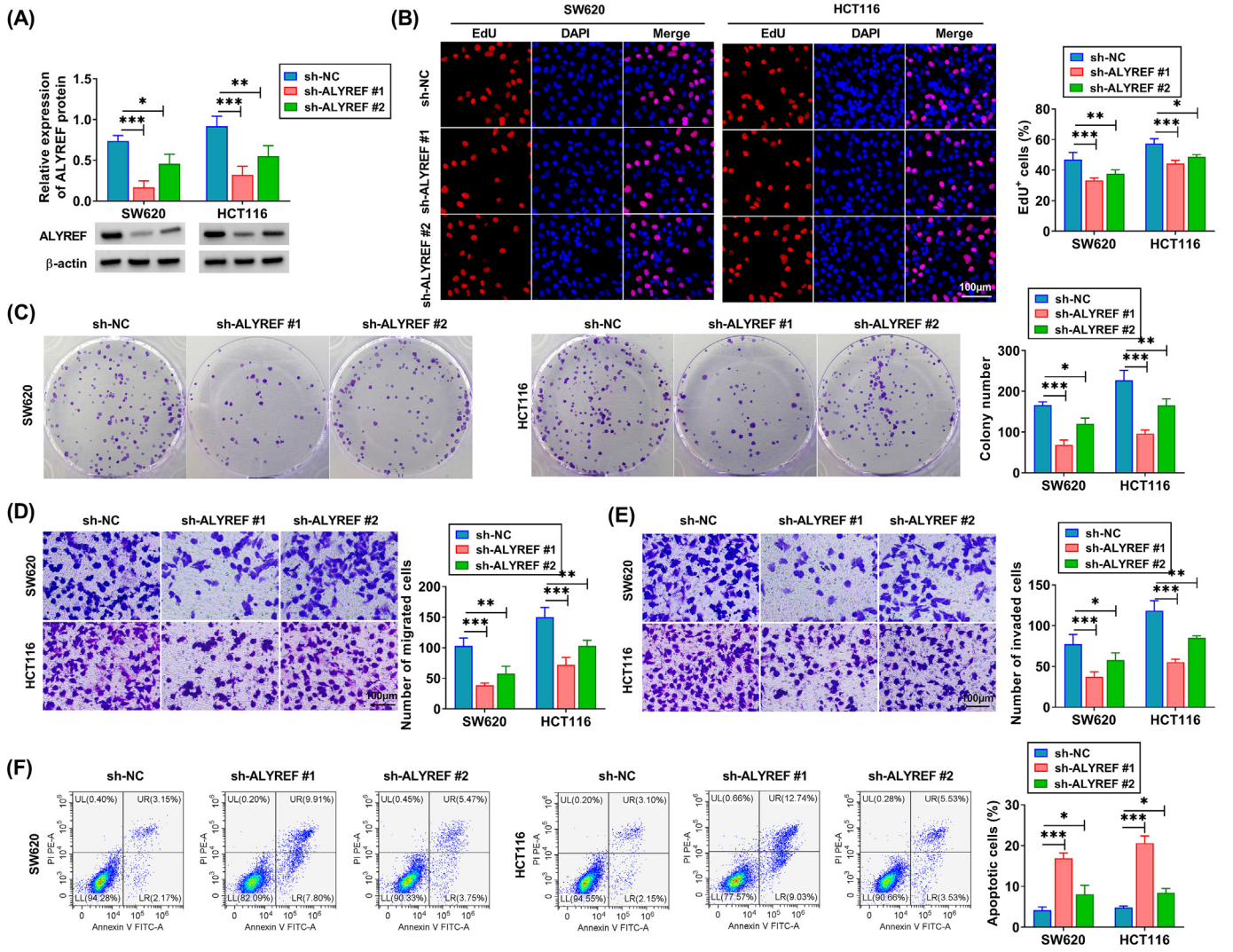

Fig. 2. Malignant invasion of colon cancer cells can be suppressed by knockdown of ALYREF. SW620 and HCT116 cells were transfected with sh-NC and sh-ALYREF. (A) Western blot analysis of ALYREF expression in colon cancer cells. (B-C) Cell proliferation detected by EdU assay and colony formation assay in inhibited ALYREF SW620 and HCT116 cells. (D-E) Transwell assay was used to measure cell migration and invasion capacity of transfected colon cancer cells. (F) Apoptotic cells were measured by flow cytometry after SW620 and HCT116 cells were treated with sh-NC and ah-ALYREF. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

sh-ALYREF, while cell apoptosis was promoted (Fig. 2F). In conclusion, ALYREF depletion restrained the proliferation, migration, and invasion of colon cancer cells and triggered their apoptosis.

3.3. Silencing ALYREF promoted ferroptosis of colon cancer cells

It is reported that inhibiting ferroptosis is an important cause of malignant progression of various tumors, including colon cancer [33]. Ferroptosis, a controlled cell death dependent on iron, is caused by fatty acid peroxidation damage resulting from polyunsaturated fatty acids [34]. Next, in colon cancer, the relationship between ALYREF and ferroptosis was analyzed. The ROS level was measured using a commercial kit. Fig. 3A illustrated that the ROS levels were elevated in colon cancer cells after transfection with sh-ALYREF#1/#2. MDA and Fe^{2+} levels were elevated, whereas GSH levels were decreased upon ALYREF knockdown (Fig. 3B-D). Furthermore, knocking down ALYREF significantly downregulated the protein level of glutathione peroxidase 4 (GPX4) and upregulated the protein level of acyl-CoA synthetase long-chain family member 4 (ACSL4) (Fig. 3E), suggesting that ALYREF knockdown affected ferroptosis-related pathways. The results above illustrated that the knockdown of ALYREF effectively facilitated ferroptosis in colon cancer cells.

3.4. PCSK9 was positively related with ALYREF in colon cancer

Accordingly, the Linkedomics website was utilized to screen ALYREF-related genes in the CPTAC-COAD cohort (Fig. 4A). Venn diagram intersection of 2069 positively correlated genes (Linkedomics), 100 upregulated genes (GEPIA), 12,467 ALYREF-binding genes (ENCORI), and 772 ferroptosis-related genes (GeneCards) identified PCSK9 (Fig. 4B). Moreover, the GEPIA database showed that PCSK9 mRNA was upregulated in 275 COAD samples (Fig. 4C). Additionally, the Linkedomics website indicated a positive correlation existed between PCSK9 and ALYREF mRNA in CPTAC-COAD (Fig. 4D). PCSK9 mRNA expression was downregulated in 13 samples and upregulated in the remaining 50 samples (Fig. 4E). Fig. 4F demonstrated that PCSK9 mRNA level followed the same trend as ALYREF. Next, western blot analysis demonstrated that the PCSK9 protein was highly expressed in tumor tissues and colon cancer cells, whereas its expression was scarce in normal

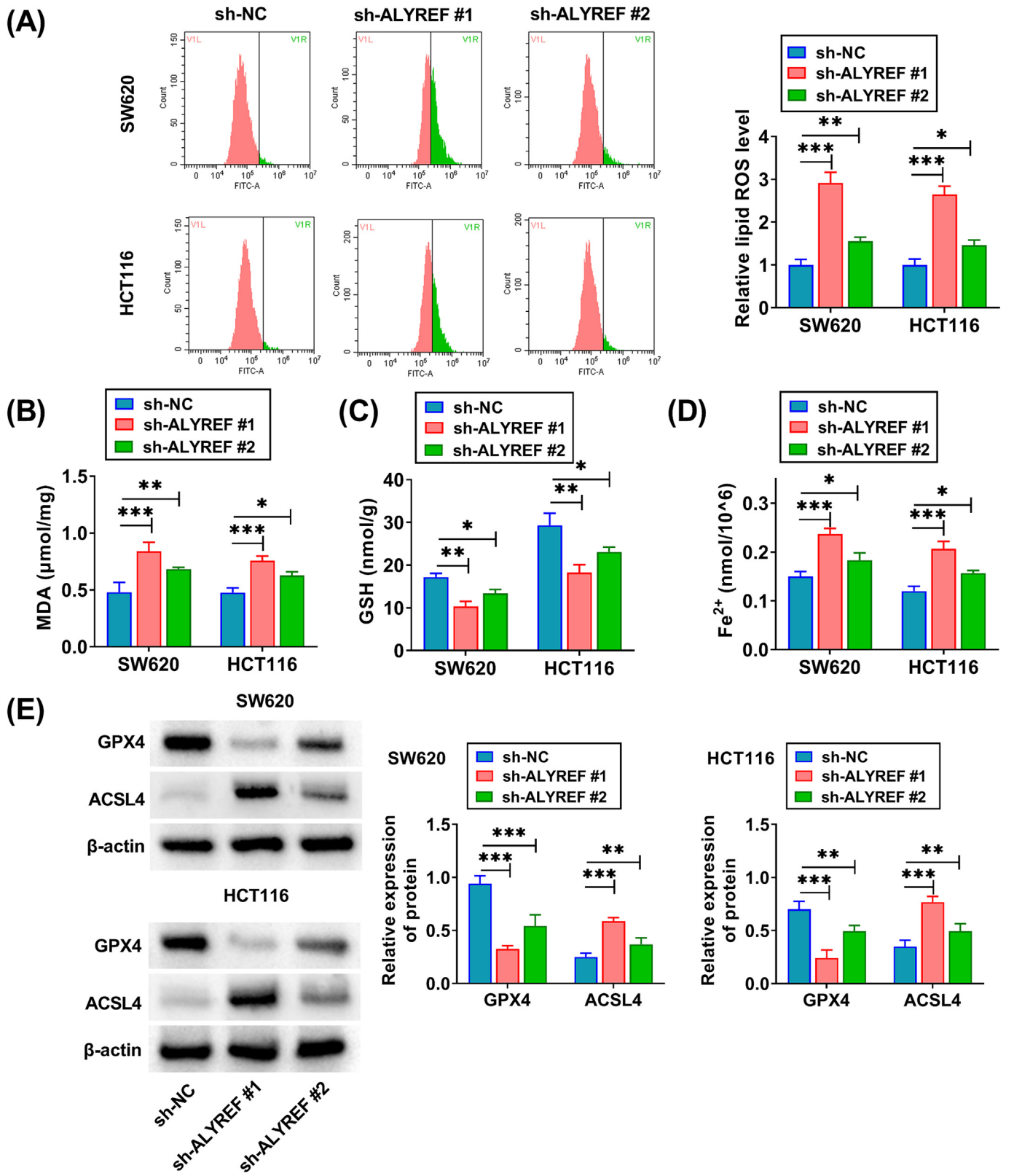

Fig. 3. Downregulation of ALYREF promotes ferroptosis. (A) The ROS levels in colon cancer cells were detected by flow cytometry. (B-D) The levels of MDA, GSH and Fe^{2+} were measured using corresponding kits. (E) Western blot analysis of GPX4 and ACSL4 expression levels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

tissues and colon cells (Fig. 4G–H). Consequently, a significant positive correlation between PCSK9 expression and ALYREF expression in colon cancer was noted. Moreover, PCSK9 exhibited a high expression level in colon cancer tissues.

3.5. ALYREF bound to PCSK9 to enhance the stability of PCSK9 mRNA

The interaction between ALYREF and PCSK9 in colon cancer was further confirmed. Knockdown of ALYREF hindered PCSK9 mRNA

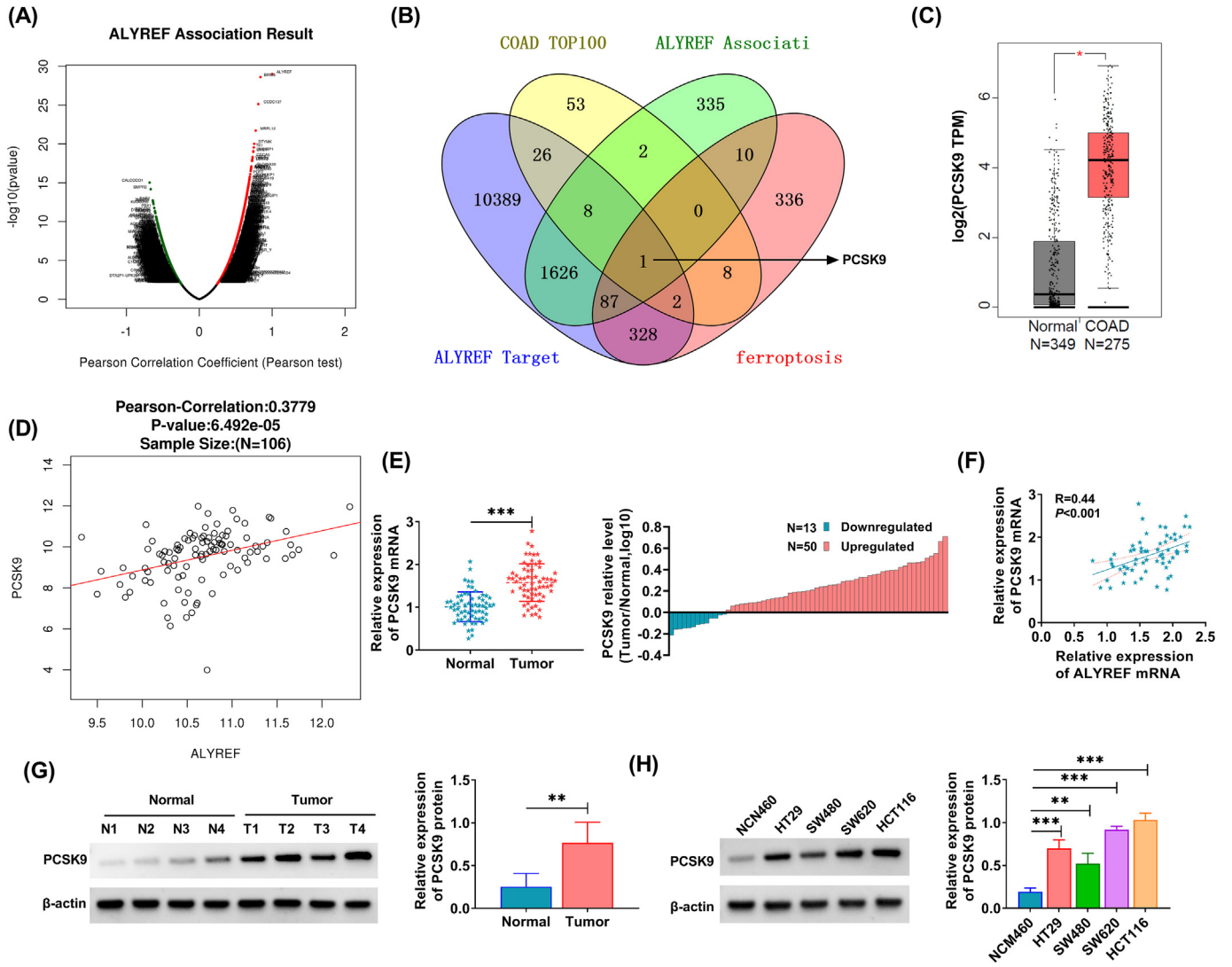

Fig. 4. PCSK9 is positively related to ALYREF in colon cancer. (A) The volcano figure of genes significantly positively or negatively related to ALYREF in colon cancer. (B) Intersecting genes from the Linkedomics, GEPIA, ENCORI and GeneCard databases were shown in a Venn diagram. (C) GEPIA displayed the PCSK9 expression level in 349 normal samples and 275 COAD samples. (D) Linkedomics website showed the correlation between PCSK9 and ALYREF mRNA in CPTAC – Colon Adenocarcinoma (Pearson correlation: 0.3779, N = 106). (E) PCSK9 mRNA levels in colon cancer samples and control samples were determined using qRT-PCR assays. (F) Co-expression relationship between ALYREF mRNA and PCSK9 mRNA in colon cancer. (G) PCSK9 protein expression levels in 4 normal tissues and 4 tumor tissues were detected by Western blot. (H) Western blot analysis of PCSK9 protein expression in normal colon cells (NCM460) and colon cancer cells (HT29, SW480, SW620 and HCT116). ** $p < 0.01$, *** $p < 0.001$.

and protein expression (Fig. 5A–B). Subsequently, the RIP assay was utilized to determine the binding activity between ALYREF protein and PCSK9 mRNA. It was found that the ALYREF protein had a binding ability to PCSK9 mRNA (Fig. 5C). Actinomycin D treatment showed reduced PCSK9 mRNA stability upon ALYREF depletion (Fig. 5D–E). These findings indicate that ALYREF promotes PCSK9 expression by stabilizing its mRNA.

3.6. Overexpression of PCSK9 undermined the potency of ALYREF knockdown on the aggressiveness of colon cancer

As per the foregoing, whether ALYREF fulfilled its function in colon cancer by targeting PCSK9 was further investigated. The transfection efficiency of PCSK9 overexpression was validated by Western blot (Fig. 6A–B). The suppression of cell proliferation, migration, and invasion caused by ALYREF knockdown was reversed due to PCSK9 upregulation (Fig. 6C–F). In the same way, the effect of decreased apoptosis resulting from ALYREF knockdown was also alleviated by PCSK9 overexpression (Fig. 6G). Moreover, the impacts of sh-ALYREF on ROS, MDA, GSH, and Fe^{2+} levels in colon cancer cells were reversed by PCSK9 overexpression (Fig. 6H–K). Overall, ALYREF facilitated cell proliferation, migration, and invasion, and suppressed apoptosis and ferroptosis of colon cancer cells by modulating PCSK9.

3.7. Downregulation of ALYREF restrained tumor growth in vivo

The tumor models were constructed to elucidate the functional role of ALYREF in tumor growth of colon cancer *in vivo*. The neoplasm in the sh-NC group was evidently larger than that in the sh-ALYREF group (Fig. 7A). The tumor growth curves showed a significant reduction in the sh-ALYREF group (Fig. 7B). Additionally, the suppression of ALYREF led to a significant reduction in tumor weight (Fig. 7C). Besides, western blot and IHC confirmed decreased ALYREF and PCSK9 expression in sh-ALYREF tumors (Fig. 7D–E). Collectively, ALYREF promoted tumor growth by regulating PCSK9 expression *in vivo*.

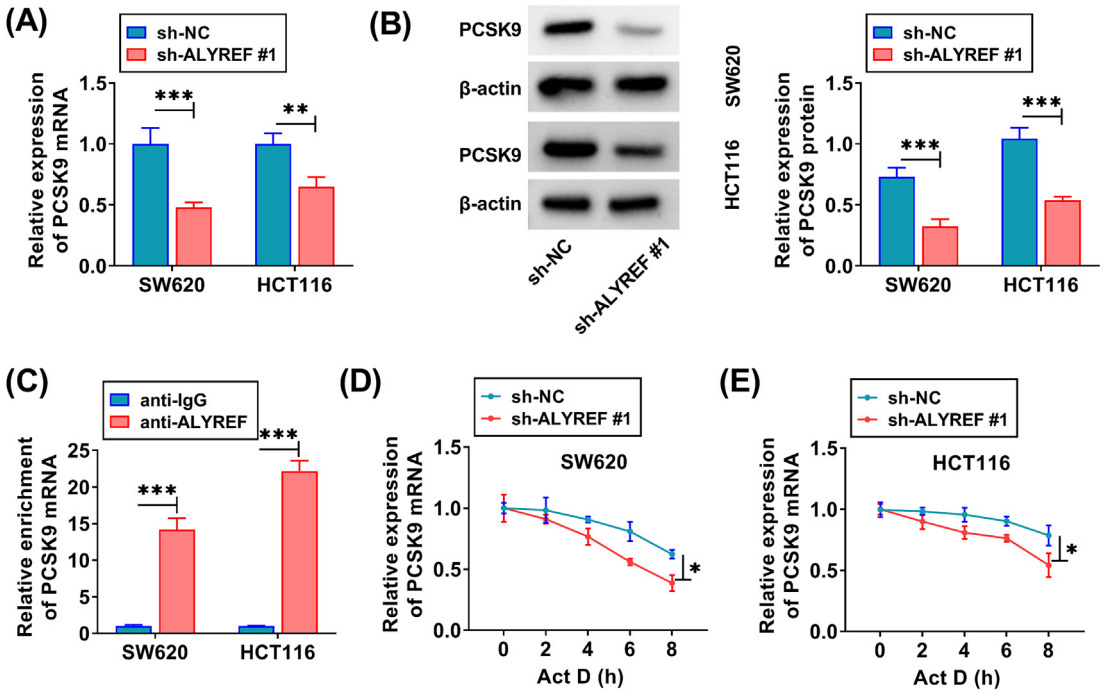

Fig. 5. ALYREF methylates PCSK9 to promote the stability of PCSK9. (A-B) qRT-PCR assay and western blot analysis were conducted to determine the expression of PCSK9 mRNA and protein in transfected SW620 and HCT116 cells. (C) The binding between ALYREF protein and PCSK9 mRNA was verified in SW620 and HCT116 cells using RIP assay. (D-E) The role of ALYREF in PCSK9 mRNA stability was assessed by using actinomycin D and qRT-PCR. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

4. Discussion

Over 1.9 million new instances of colorectal cancer were identified in 2020, thus rendering it the second leading cause of cancer deaths [2]. Globally, colorectal cancer accounts for 11% of all cancer diagnoses [35]. Due to the high morbidity and lethality of colon cancer, exploring the molecular mechanisms underlying disease pathogenesis and identifying novel therapeutic and diagnostic targets hold substantial significance for the prevention and treatment of the disease.

ALYREF is highly expressed in major cancer types, and its upregulation correlates with poor prognosis in various malignancies [36,37,38]. In our current work, it was confirmed that ALYRERF showed high expression in colon cancer and it manifested oncogenic features during the advancement of colon cancer. Ferroptosis, a form of iron-dependent cell death, plays a critical role in tumor biology and therapy [39,40,41]. Notably, ferroptosis is not only triggered by classical cancer therapies but also intersects with multiple cancer signaling pathways [42,43,44,45]. The main features of ferroptosis are disruption of iron homeostasis and accumulation of lipid ROS, which can additionally be measured by MDA detection [46,47]. GSH, as a potent reducing agent, effectively scavenges ROS in collaboration with GPX4, thus preventing the onset of ferroptosis [48]. By evaluating the changes in ROS, MDA, GSH and Fe^{2+} levels in different groups of colon cancer cells, it could be concluded that ALYREF knockdown effectively promoted the ferroptosis process of colon cancer cells.

The m5C modification is required for the invasion and metastasis of cancer cells [49]. A prior study has shown that the m6A and m5C modifications of GPX4 might be a potential target for cancer immunotherapy by activating the cGAS-STING signaling pathway in COAD [50]. ALYREF acts as a reading protein for m5C modification and recognizes and binds mRNAs containing m5C modifications, thereby facilitating nuclear export and translation of these mRNAs [16]. Therefore, whether ALYREF can promote the expression of related genes through m5C modification and thus promote the progression of colon cancer is the question we next explore. Bioinformatics databases were utilized to screen for genes with potential binding sites for ALYREF in colon cancer, resulting in the screening of PCSK9. The roles of PCSK9 in cancers have been reported in several studies [31,51,52]. A recent study has expounded the function of PCSK9 in colon cancer, which validates that PCSK9 enhances the advancement and metastasis of colon cancer cells via modulating EMT and PI3K/AKT signaling pathways within tumor cells and the phenotypic polarization of macrophages [32]. Consistent with previous reports, PCSK9 was overexpressed in colon cancer relative to normal tissues and cells. However, unlike the canonical transcriptional regulation (EMT/PI3K/AKT axis) or indirect cellular interaction mechanisms (macrophage polarization) previously implicated in PCSK9-mediated oncogenesis, our study uncovered a novel epitranscriptomic mechanism: ALYREF, acting as an m5C reader protein, directly binds to m5C-modified sites in PCSK9 mRNA, thereby stabilizing its transcript and upregulating its protein expression. Functional experiments demonstrated that downregulation of ALYREF retarded the proliferation, migration and invasion of colon cancer cells. The inhibitory effects were reversed by overexpression of PCSK9. Notably, our findings also intersect with ferroptosis regulation. As ferroptosis is characterized by iron dyshomeostasis and lipid ROS accumulation (key features measurable via MDA, GSH, and Fe^{2+} assays), we observed that ALYREF knockdown (by destabilizing PCSK9) promoted ferroptosis in colon cancer cells. Conversely, PCSK9 overexpression reversed these effects, positioning the ALYREF-PCSK9 axis as a novel bridge between epitranscriptomic control and ferroptotic susceptibility in colon cancer. *In vivo*, ALYREF silencing suppresses tumor growth, accompanied by reduced PCSK9 expression.

Overall, ALYREF promotes colon cancer malignancy by stabilizing PCSK9 mRNA via m5C modification. This study provides theoretical support for the understanding of colon cancer pathogenesis, as well as offers a novel concept for formulating efficacious molecular therapies for patients with colon cancer.

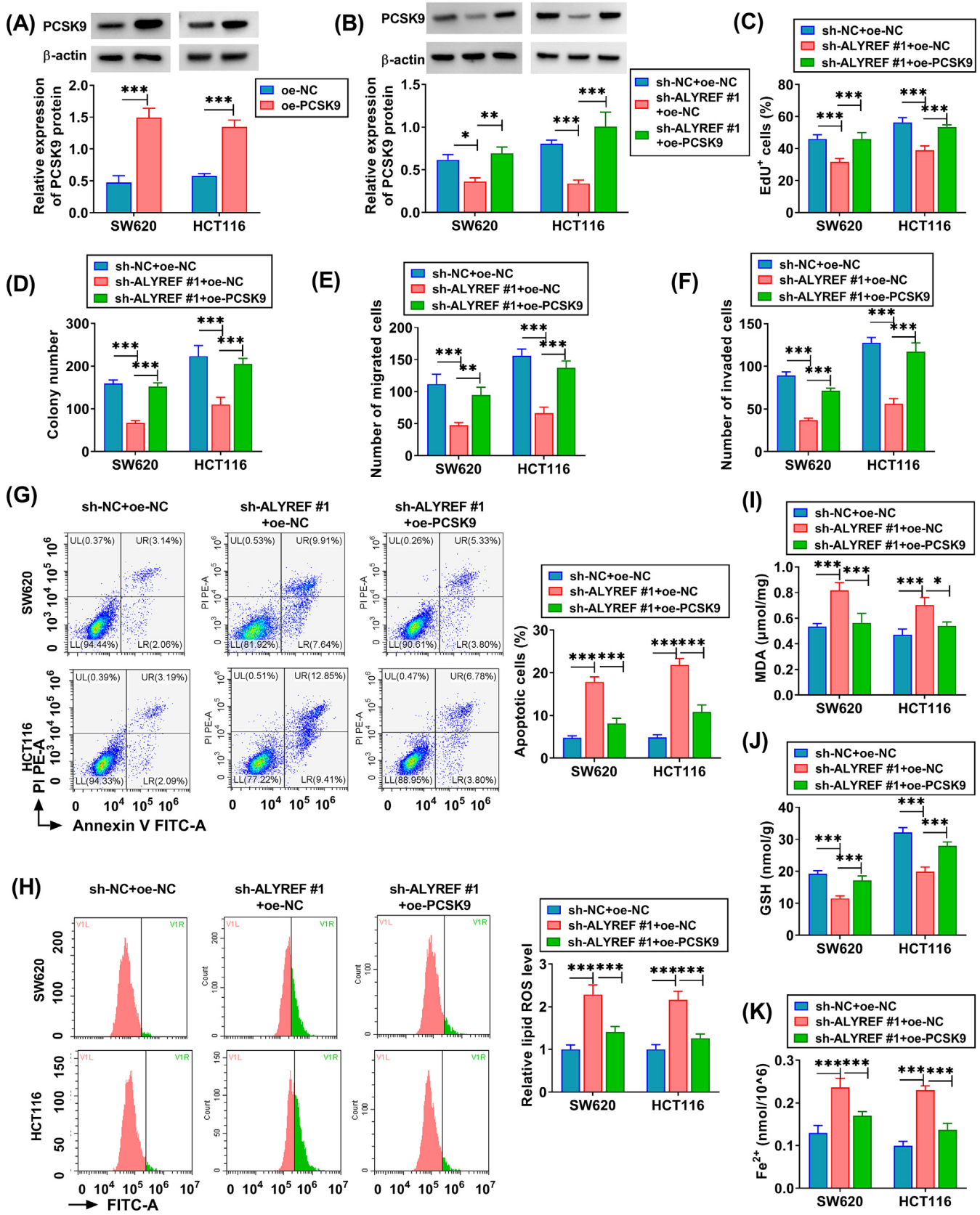

Fig. 6. Overexpression of PCSK9 mitigated the potency of ALYREF knockdown on aggressiveness of colon cancer cells. SW620 and HCT116 cells were transfected with sh-NC + oe-NC, sh-ALYREF #1 + oe-NC, and sh-ALYREF#1 + oe-PCSK9. (A-B) PCSK9 protein levels were measured using Western blot. (C-D) Cell proliferation ability was monitored by EdU assay and colony formation assay. (E-F) Transwell was performed to analyze the migration and invasion of SW620 and HCT116 cells. (G) Cell apoptosis was determined by flow cytometry. (I-K) Levels of MDA, ROS, GSH, and Fe^{2+} were measured using corresponding kits. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

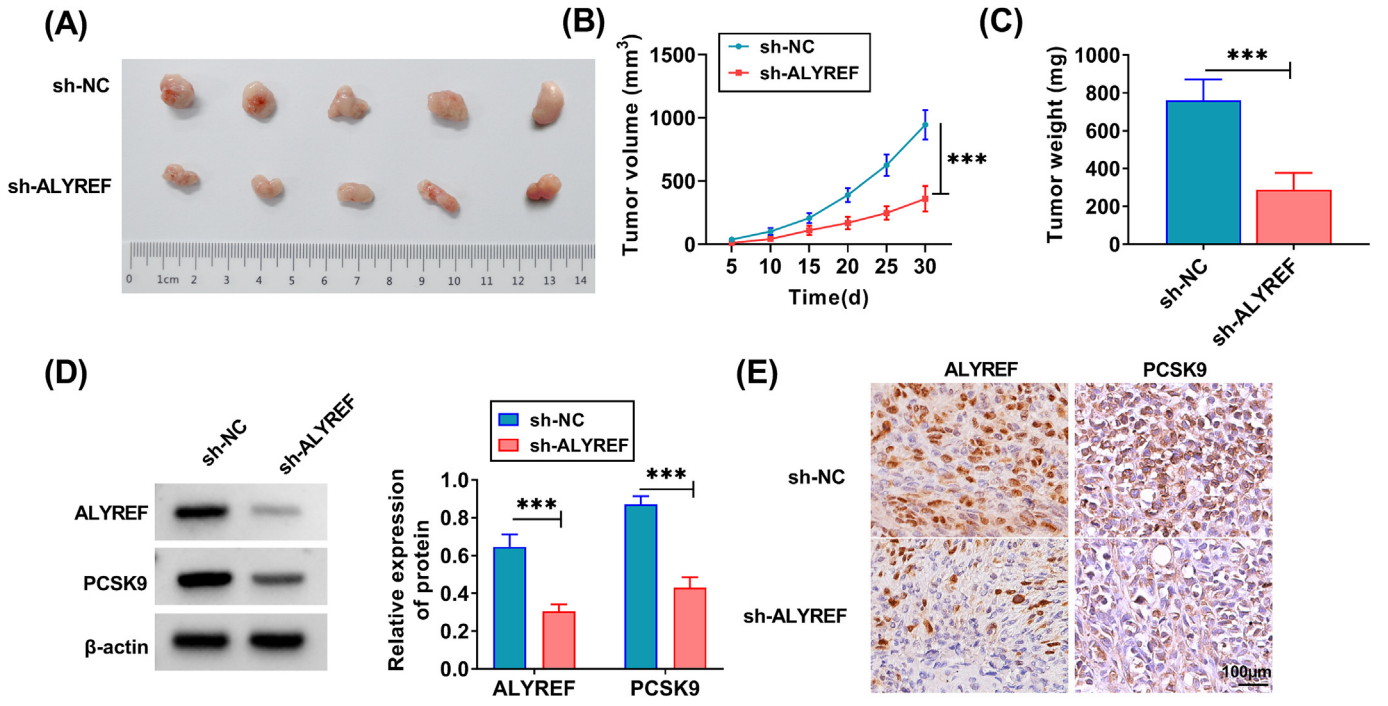

Fig. 7. Knockdown of ALYREF suppressed tumor growth of colon cancer *in vivo*. (A) The photographs, (B) tumor volume, and (C) tumor weight of xenograft tumors from control, knockdown of ALYREF groups. (D) The expression levels of ALYREF and PCSK9 in tumor tissues were examined by Western blot. (E) IHC images of tumor tissues obtained from xenograft mice. *** $p < 0.001$.

CRediT authorship contribution statement

Lili Cao: Writing – original draft, Validation, Supervision, Resources, Project administration. **Ying Chen:** Methodology, Data curation. **Jing Yu:** Software, Formal analysis. **Dian Yin:** Writing – review & editing, Visualization, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

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Data availability

Data will be made available on request.

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