Inhibition of ALAS1 activity exerts anti-tumour effects on colorectal cancer *in vitro*

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Abstract

Background/Aims: Colorectal cancer (CRC) is the third most common malignant tumour worldwide and the second leading cause of cancer-related deaths. Commonly, 5'-aminolevulinic acid synthase1 (ALAS1) is the rate-limiting enzyme for haem biosynthesis. Recent studies have shown that ALAS1 is involved in a number of cellular functions and has significant effects on non-small cell lung cancer (NSCLC). However, current concepts of disease pathogenesis fail to fully explain the role of ALAS1 expression and biological functions in CRC.

Materials and Methods: A total of 67 paired tumour tissues and adjacent colorectal tissues were used to detect ALAS1 levels and further analyse the correlation between ALAS1 expression levels and clinical features. Using HCT116 cell lines, we studied the impact of ALAS1 on biological function by knocking down or inhibiting ALAS1.

Results: We found an increase in the levels of ALAS1 in cancer tissues compared to adjacent colorectal tissues. The increase in ALAS1 expression was closely related to the invasion depth, N staging and tumour size of CRC patients. The proliferation and metastasis of CRC cells could be inhibited by suppressing ALAS1.

Conclusions: The abnormal expression of ALAS1 is closely related to the proliferation and metastasis of CRC cells, suggesting that ALAS1 may be a novel therapeutic target for the treatment of CRC.

Keywords: Cell proliferation, colorectal neoplasms, neoplasm metastasis

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy, as well as the second leading cause of death worldwide.[1] The main treatment options for CRC include surgery, chemotherapy and radiotherapy.[2-4] Due to the late detection of tumours and the development of drug resistance, the curative effect of traditional therapy is greatly restricted. Therefore, further understanding of the mechanisms underlying the progression and metastasis of CRC will be crucial to the development of new therapies.

ALAS1 is the rate-limiting enzyme for haem biosynthesis,[5] which is involved in numerous cellular functions and has a significant effect on non-small cell lung cancer (NSCLC). Haem degradation products are highly expressed in tumour tissues and play an important role in

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<tr><td></td><td>**DOI:**
10.4103/sjg.SJG_477_19</td></tr>
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How to cite this article: Zhao Y, Zhang X, Liu Y, Ma Y, Kong P, Bai T, *et al*. Inhibition of ALAS1 activity exerts anti-tumour effects on colorectal cancer *in vitro*. Saudi J Gastroenterol 2020;26:144-52.

tumour development.[6-8] Studies have indicated that small interfering RNAs specific for ALAS1 are highly effective in preventing and treating biochemically induced attacks in a mouse model of acute intermittent porphyria.[9] The ALAS1 protein level was significantly enhanced in NSCLC cells. Upon inhibition of the activity of ALAS1, the proliferation, colony formation and migration ability of NSCLC cells were significantly reduced.[10,11] However, the role of ALAS1 has not been studied in the occurrence and development of CRC.

In this study, we found that the expression of ALAS1 was increased in CRC tissues. Therefore, we hypothesized that ALAS1 plays a pivotal role in the development and metastasis of CRC. To assess the impact of ALAS1 on the clinical progress and prognosis of CRC patients, we detected the expression of ALAS1 in 67 CRC tissues and analysed the relationship between ALAS1 expression and clinicopathological parameters. In addition, we conducted a cell function assay to assess the impact of ALAS1 on the proliferation and migration of CRC cells and its potential mechanisms.

MATERIALS AND METHODS

Reagents
Succinylacetone was purchased from MedChemExpress (Cat# HY-W010184). Antibodies were used to determine protein expression including anti-ALAS1 (Proteintech, Rosemont, IL, USA Cat# NBP1-91656), anti-MMP2 (Wanleibio, Shenyang, China Cat# WL03224), anti-MMP9 (Wanleibio, Shenyang, China, Cat# WL03206), anti-PCNA (Cell Signaling Technology, USA, Cat# 13110), anti-c-Myc (Abcam, Cambridge, MA, USA, Cat# ab32072), and anti-β-actin (Cell Signaling Technology, USA, Cat# 4967).

The small interfering RNA (siRNA) oligonucleotides [siRNA-NC: 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) and 5'-ACGUGACACGUUCGGAGAATT-3' (anti-sense); siRNA-ALAS1#1: 5'-GGUGCAGUAAU GACUACCUTT-3' (sense) and 5'-AGG UAG UCAUUACUGCACCTT-3' (anti-sense); siRNA-ALAS1#2: 5'-GCAGACAUAAC AUCUACGUTT-3' (sense) and 5'-ACGUAGAUG UUAUGUCUGCTT-3' (anti-sense)] were constructed and provided by GenePharma (Shanghai, China).

Clinical samples
The human normal colorectal mucosa cell line foetal human cells (FHC) and human CRC cell line HCT116 were purchased from the Cell Bank Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The human normal colorectal mucosa cell line FHC

and human CRC cell line HCT116 were cultured in DMEM/F12 medium (Gibco, USA) and McCoy's 5A medium (Gibco, USA), respectively. In addition, 1% foetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin and 100 μg/mL streptomycin (Huayao, China) were added to the medium. Cells were cultured in an incubator containing 5% CO_2 at 37°C. Cells were inoculated in 6-well plates and transfected with Lipofectamine RNAiMAX (Invitrogen, CA, USA). The succinylacetone (SA) treatment concentration was 0.5 mM. A total of 67 patients with primary CRC enrolled in this study were treated at The Second Surgical Department, The Fourth Hospital of Hebei Medical University from December 2013 to May 2014. Prior to the study, written informed consent was obtained from all participants. This study was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University. All experiments conformed to current national laws.

Cell cycle analysis
Cell cycle distribution was assessed by propidium iodide staining and flow cytometry. The cell cycle detection kit instructions were followed closely. Each experiment was repeated three times, and similar results were obtained.

Cell proliferation assay
A total of 3×10^3 cells were inoculated in a 96-well plate. After inoculation for 24 h/48 h/72 h/96 h, 10 μL of Cell Counting Kit-8 reagent (CCK-8; Abmole, Shanghai, China) was added to each well and incubated for 2 h at 37°C. The absorbance of each well was determined at 450 nm.

Colony assays
For the colony formation assay, HCT116 cells were counted and seeded at a density of 1×10^3 cells per well on 6-well plates and cultured with medium containing 10% FBS. Cells were treated with or without 0.5 mM SA, and the cells were transfected as described previously. The medium was replaced every four days. After 10–12 days, cells were fixed in 4% paraformaldehyde and stained with 0.5% crystal violet.

Transwell migration assay and Matrigel invasion assay
Transwell chambers (BD Bioscience) were used to detect cell migration. Transwell chambers covered with Matrigel were used to detect cell invasion. A total of 2×10^5 cells were added to a chamber. After incubation for 24 h, cells were fixed with 4% paraformaldehyde for 20 min, washed twice and stained with crystal violet for 15 min at 25°C. Transwell chambers were washed twice with 1× phosphate buffer saline (PBS). Cells that had not migrated or invaded were scraped from the top of the chamber. The number of migrated and invaded cells were calculated at

least in five random fields of view by optical microscopy (Olympus Corp, Tokyo, Japan).

Wound healing assay

The cells were seeded on a 6-well plate. After cell confluence reached 80–90%, a wound was scratched with a 200 μL sterile pipette tip. Cells were washed with serum-free PBS and then cultured in FBS-free medium for 36 h.

Immunofluorescence staining

Tissue or cells were placed on glass slides, fixed with 4% paraformaldehyde for 30 min and rinsed twice with PBS. Subsequently, the cells were incubated with anti-ALAS1 (Proteintech, Rosemont, IL, USA, Cat# NBP1-91656) at 4°C for 12 h and then with the secondary antibody at 25°C for 2 h. After washing three times in PBS, the nuclei were labelled with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (1 μg/mL, Sigma) for 5 min. ALAS1 and DAPI fluorescence images were captured using a multichannel fluorescence microscope.

Western blot

Cells were collected and lysate was added that contained 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 1% NP-40, and 0.5% sodium deoxycholate. Total protein concentration was detected by the Lowry method. 15–25 3 μg protein was taken for experiment. Proteins were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoresed and transferred to a transmembrane. After blocking in skim milk, the membranes were incubated with primary antibodies at 4°C overnight. The secondary antibodies were incubated at 37°C for 1 h. A chemiluminescence imaging analyser was used for image analysis.

Total RNA extraction and reverse transcription

CRC tissue RNA was extracted using TRIzol Reagent (Invitrogen, Karlsruhe Germany). The RNA concentration and purity were measured by a NanoDrop 1000. cDNA was synthesized by Invitrogen M-MLV following the manufacturer's protocol.

Quantitative real-time PCR

Divergent primers were designed with Primer 5. The sequences of ALAS1 were 5'-CACACACCCCAGATG ATGAA-3' (forward primer) and 5'-CCTGCAGAAGTT GCACTCAG-3' (reverse primer). The primer sequences for GAPDH as control were 5'-ATCTTCCAGGAGCG AGATCCC-3' (forward primer) and 5'-TGAGT CCTTCCAAGATACCAA-3' (reverse primer). The expression levels of ALAS1 in tissue specimens were calculated by ΔCt. A larger ΔCt number indicates a lower

expression level of ALAS1. All experiments were repeated three times.

Statistical analysis

Experimental data are presented as the mean ± standard deviation (SD). The two groups were compared using a *t*-test. One-way analysis of variance (ANOVA) was applied for comparisons among multiple groups. $P < 0.05$ was considered statistically significant. Data were analysed by SPSS 21.0.

RESULTS

ALAS1 is upregulated in CRC tissues

The expression of ALAS1 was detected by qRT-PCR and western blot (WB). The tumour tissues and normal colon tissues of 67 patients with CRC were collected. Compared with normal intestinal tissue, the expression of ALAS1 was significantly upregulated in tumour tissues. [Figure 1a and b; $P < 0.001$]. In addition, the expression of ALAS1 in CRC tissues was verified by immunofluorescence, and the result was consistent with western blot data [Figure 1c; $P < 0.01$]. The analysis demonstrated that the expression of ALAS1 in tumour tissues was significantly higher than that in the matched adjacent normal colonic tissue (ANT).

Relationship between ALAS1 expression and clinicopathological features of CRC patients

The correlation between ALAS1 expression and the clinicopathological features of CRC patients was analysed [Table 1]. ALAS1 expression was closely related to the invasion depth, N staging and tumour size of CRC patients. Using the Gehan-Breslow-Wilcoxon test, we found that the overall survival in patients with high expression of ALAS1 was significantly lower than that in patients with low ALAS1 expression [Figure 1d; $P = 0.04$].

ALAS1 is downregulated in ALAS1-siRNA cell lines

We verified that ALAS1 was highly expressed in colorectal tumour tissues and then studied the biological behaviour of tumour cells after reducing its expression. First, western blot and immunofluorescence were used to verify the expression of ALAS1 in the cell lines FHC representing human normal colorectal mucosal epithelium and HCT116 representing CRC. The results showed that the expression of ALAS1 in HCT116 cells was significantly higher than that in FHC cells [Figure 2a and b; $P < 0.01$]. Next, siRNA was transfected into HCT116 cells with the aim of knocking out ALAS1. After transfection for 48 h, western blot analysis showed that the expression of ALAS1 in HCT116 cells was significantly reduced [Figure 2c; $P < 0.01$]. The results indicated that ALAS1 was effectively knocked down in HCT116 cells. ALAS1 knockdown was more efficient in cells transfected with siRNA-ALAS1#2.
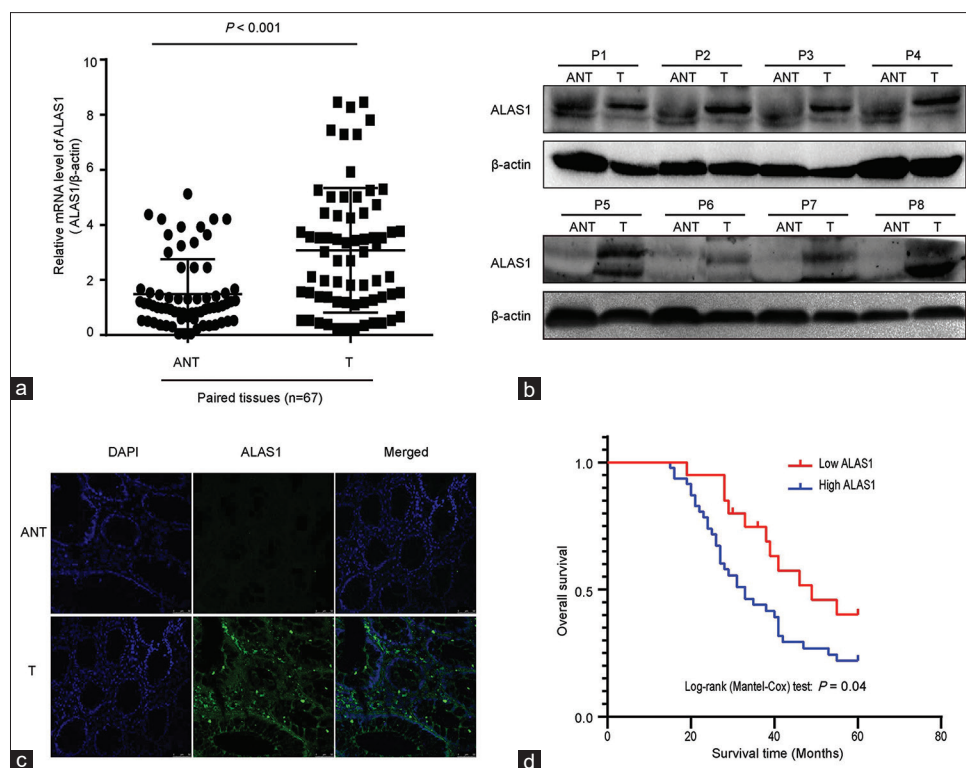
Figure 1: ALAS1 is upregulated in colorectal cancer (CRC) tissues. (a) The mRNA expression of ALAS1 in CRC ($n = 67$). (b) Western blot of eight paired CRC patients. (c) The expression of ALAS1 in the tissue detected by immunofluorescence. (Scale bar, 50 μm). (d) Patients with high expression of ALAS1 showed reduced survival times compared with patients with low expression of ALAS1. **$P < 0.01$; ****$P < 0.0001$

Table 1: The correlation between protein expression of ALAS1 and clinicopathological features in colorectal cancer (CRC)

Characteristics	Number of cases	Relative level of ALAS1 (Mean±S.E.)	P
Gender			0.349
Female	31	0.546±0.308	
Male	36	0.641±0.471	
Age			0.725
>55	44	0.584±0.371	
≤55	23	0.621±0.469	
Invasion depth			**0.035**
T1+T2	12	0.434±0.251	
T3+T4	55	0.694±0.399	
N classification			**0.007**
N0	28	0.453±0.256	
N1-N2	39	0.699±0.459	
Tumour Location			0.446
Colon	40	0.565±0.388	
Rectal	27	0.644±0.429	
Tumour size			**0.037**
>5 cm	42	0.639±0.318	
≤5 cm	25	0.477±0.272	
Histological differentiation			0.491
High or middle	51	0.581±0.401	
Low	16	0.662±0.428	

P values in bold were statistically significant

Inhibition of ALAS1 activity significantly affected cell proliferation and colony formation in CRC

First, the effect of ALAS1 on the proliferation of HCT116 cells was tested by CCK-8. The proliferative activity of HCT116 cells was significantly inhibited by knocking down ALAS1 [Figure 3a; $P < 0.01$]. Compared with the control group, the number of colonies formed in the ALAS1 group was significantly reduced [Figure 3c; $P < 0.01$], as indicated by the colony-forming experiment. To further investigate the effect of ALAS1 on the proliferation of HCT116 cells, the cell cycle was measured by flow cytometry. The results showed that the cell cycle was blocked at the G0/G1 phase and the distribution of the S phase was relatively reduced after knocking down ALAS1 [Figure 3e; $P < 0.01$].

SA,[12,13] an effective inhibitor of haem synthesis, inhibits ALAS1 activity.[9] To further verify the above results, cells were treated with SA. Compared with the control group, the proliferative activity of HCT116 cells treated with SA was significantly inhibited [Figure 3b, d and f; $P < 0.01$], which was in accordance with the ALAS1 knockdown results. These findings indicate that ALAS1 plays an important role in cell proliferation in CRC.

Inhibition of ALAS1 activity significantly affected the invasion and migration in CRC

Transwell migration and Matrigel invasion assays were performed to investigate the effect of ALAS1 on the invasion and migration of HCT116 cells. Compared with those in the control group, the migration and invasion
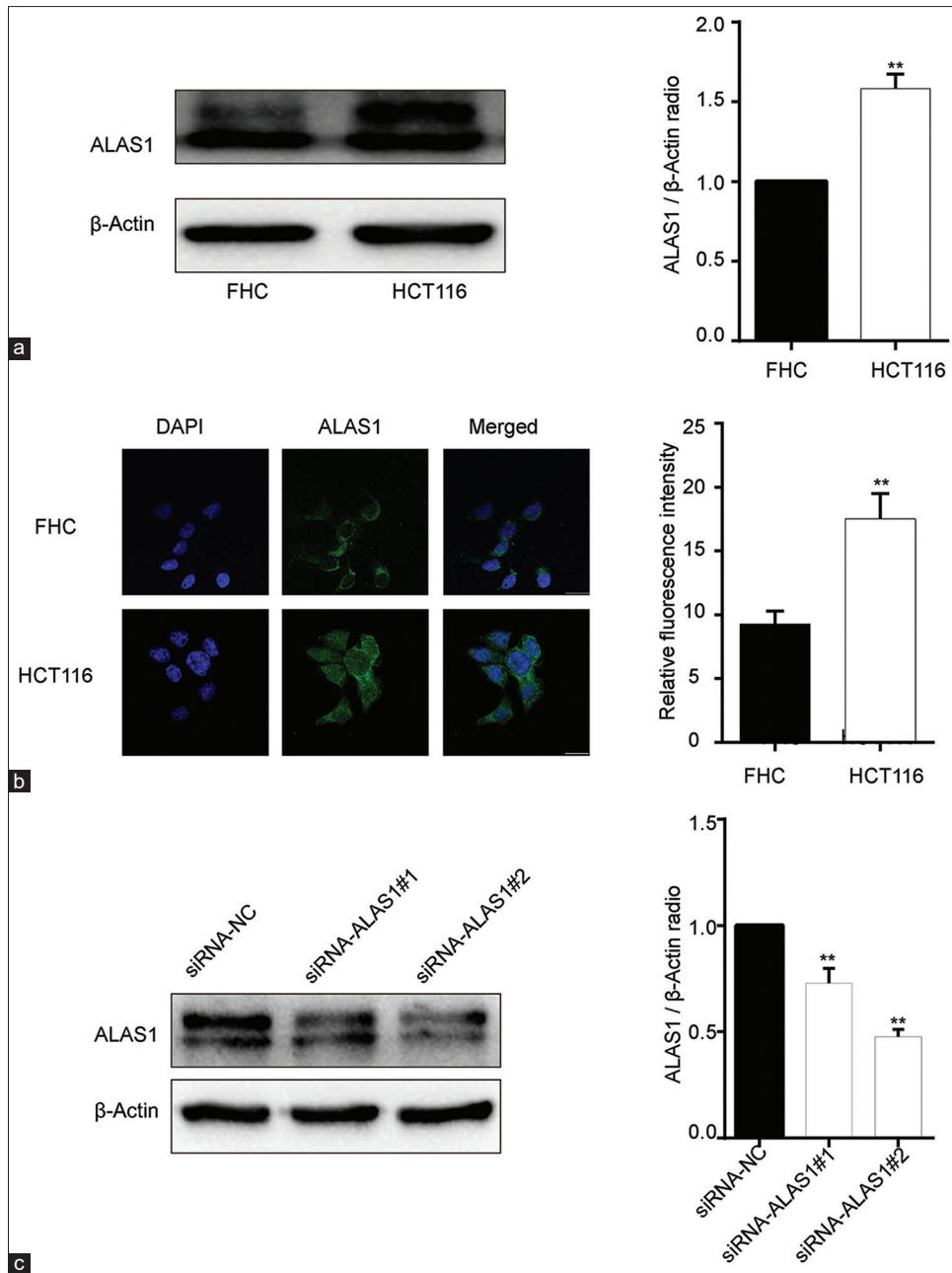
Figure 2: ALAS1 is downregulated in ALAS1-siRNA cell lines. (a) The protein expression of ALAS1 in human normal colorectal mucosa cell line foetal human cells (FHC) and human CRC cell lines HCT116 verified by western blot. (b) The protein expression of ALAS1 in human normal colorectal mucosa cell line FHC and human CRC cell lines HCT116 cells verified by immunofluorescence. (Scale bar, 25 μm) (c) The efficiency of si-RNA knockdown on ALAS1 in HCT116 cells. **$P < 0.01$

abilities of the experimental group were decreased [Figure 4a; $P < 0.01$]. The wound healing experiment[14] showed that the cells in the experimental group were slow and the cell migration area was significantly reduced [Figure 4c; $P < 0.01$].

After treatment with SA, the migration and invasion abilities of HCT116 cells in the experimental group were also remarkably inhibited [Figure 4b and d; $P < 0.01$]. The results show that suppression of ALAS1 activity

can inhibit the migration and invasion of HCT116 cells in CRC.

DISCUSSION

In this study, we found that the levels of ALAS1 were significantly elevated in most CRC tissues tested. Overall, the ALAS1 levels were related to cell proliferation and migration, suggesting that ALAS1 plays an important role in CRC.
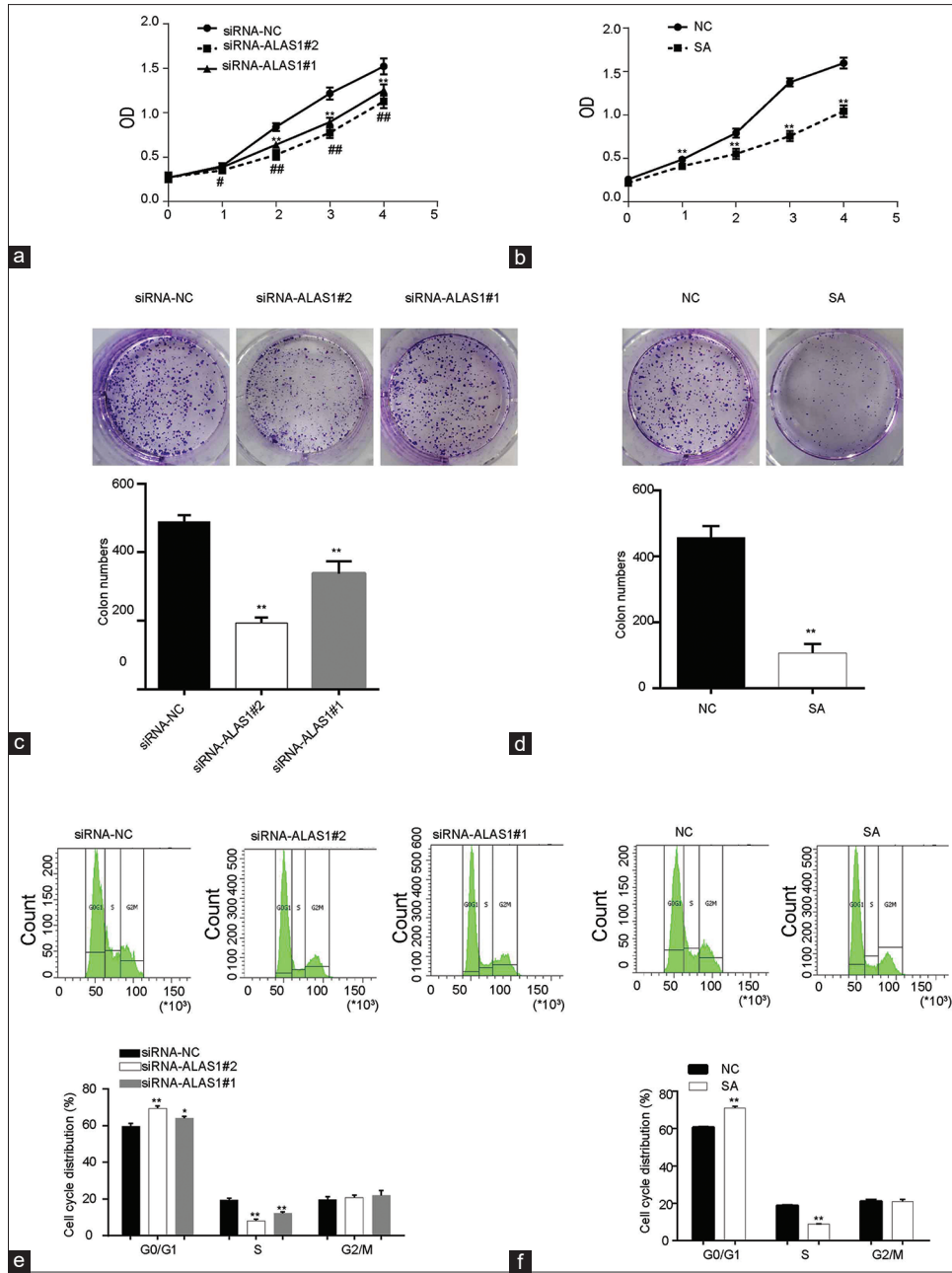
Figure 3: The effect of inhibition of ALAS1 activity on cell proliferation. (a and b) Cell count kit-8 (cck-8) assay showed that the proliferation activity of HCT116 cells was significantly inhibited after inhibition of ALAS1 activity. (c and d) The plate cloning experiments showed that the number of cell colony formations was significantly reduced after inhibition of ALAS1 activity. (e and f) Flow cytometry showed that inhibition of ALAS1 activity significantly inhibited mitosis in HCT116 cells. $**P < 0.01$; $^{\#\#}P < 0.01$

ALAS1 is the rate-limiting enzyme for haem biosynthesis. The increase in ALAS1 expression can promote the synthesis of haem. Experimental studies in the past have demonstrated that haem can directly bind to and control the activities of a wide array of cellular regulators, such as the transcriptional factor Bach1,[15] the haem-regulated eIF2a kinase,[16,17] the ras-ERK signaling pathway,[13] and the essential miRNA processing factor DGCR8.[18] Haem is a core element of mitochondrial function and a signalling molecule that regulates diverse molecular and cellular processes and plays a key role in the whole process of oxygen metabolism.[19-21] Haem serves as an important prosthetic group for many enzymes involved in haemoglobin, myoglobin and mitochondrial respiratory chain processes.[10,19] Moreover, haem degradation products are highly expressed in tumour tissues and play an important role in tumour development.[6,7,22]

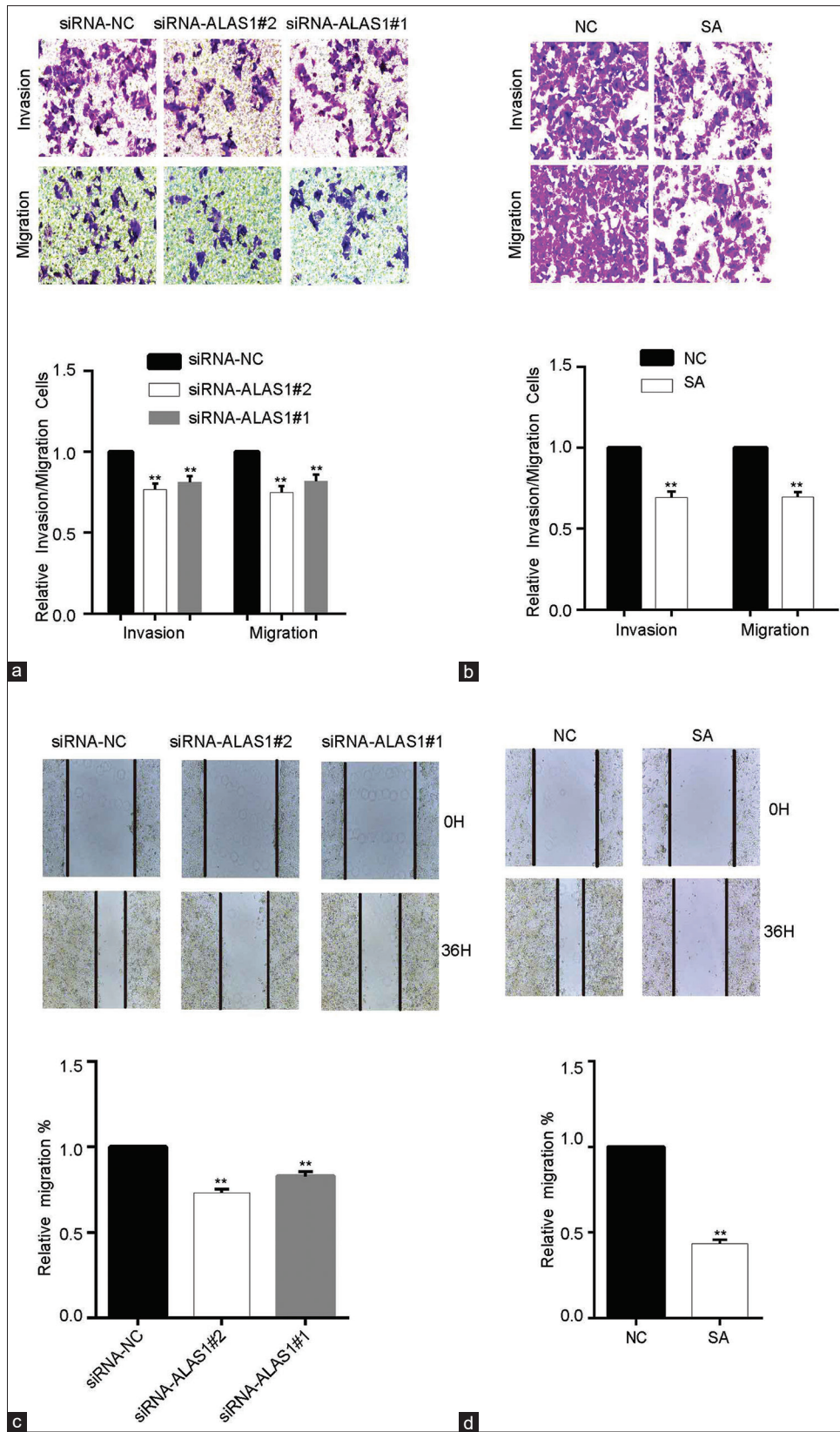Therefore, abnormal expression of ALAS1 may be involved in tumourigenesis by affecting the level of haem.

Figure 4: The effect of inhibition of ALAS1 activity on cell migration and invasion. (a and b) Compared with the control group, the ability of cell migration and invasion was significantly decreased after inhibition of ALAS1 activity. (c and d) The wound healing experiment showed that after inhibiting ALAS1 activity, the wound healing was slow and the cell migration area was significantly reduced. **$P < 0.01$

Zhang Li *et al.* found that the expression of ALAS1 was elevated in non-small cell lung cancer cells. In this study, qRT-PCR, WB and immunofluorescence were used to detect the expression of ALAS1 in CRC tissues and the adjacent mucosa. We also studied the effects of ALAS1 on cell proliferation, migration and invasion by a CRC cell line *in vitro*. We found that the mRNA and protein levels of ALAS1 were significantly elevated in most CRC tissues. Moreover, the expression level of ALAS1 was related to the depth of tumour invasion, N staging and tumour size, suggesting that ALAS1 plays an important role in the development of CRC.

To further elucidate the oncogenic role of ALAS1 in CRC, we investigated the effect of ALAS1 on the viability of CRC cell lines *in vitro*. Recent evidence has shown that reducing ALAS1 activity in NSCLC cell lines by SA inhibited cell proliferation, colony formation, migration and tumourigenic function. Our findings suggest that knockdown of ALAS1 in HCT116 cell lines inhibited cell growth rate, colony formation, migration and cell cycle induction in G0/G1, which were in line with those reported previously. Consistent with previous reports, our data show that ALAS1 plays an important role in the proliferation and migration of CRC cell lines. The above experimental results indicate that ALAS1 may be involved in the occurrence and development of CRC, and is expected to become a new biomarker for the diagnosis or treatment of CRC.

This study had some limitations, such as small sample size, and limited research on related molecular mechanisms. Therefore, we will further expand the sample size of patients to gain new insight into the diagnosis and treatment of CRC. In conclusion, we found that the transcription level and translation level of ALAS1 were significantly elevated in CRC. The upregulation of ALAS1 was closely related to the depth of tumour invasion, N stage, and tumour size. Further analysis showed that inhibition of ALAS1 activity could inhibit the proliferation and metastasis of CRC cells, suggesting that ALAS1 might be a novel target for the treatment of CRC.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient(s) has/ have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

Conflicts of interest

There are no conflicts of interest.

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