Pathology & Oncology Research (2020) 26:1615–1623
https://doi.org/10.1007/s12253-019-00737-7

ORIGINAL ARTICLE

ALKBH5 Holds Prognostic Values and Inhibits the Metastasis of Colon Cancer

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Received: 19 February 2019 / Accepted: 27 August 2019 / Published online: 10 September 2019

Abstract

N^6-methyladenosine (m^6A) demethylase ALKBH5 is best known for modulating transcript modification in plenty of human malignancies, but its role in the progression of colon cancer is not well understood. In the present study, we identified the tumor repressive role of ALKBH5 in colon cancer. ALKBH5 was downregulated in human colon cancer tissues, where its decreased expression significantly correlated with distant metastasis and American Joint Committee on Cancer (AJCC) stage. ALKBH5 was also an independent prognostic indicator of overall survival and disease-free survival in colon cancer patients. Furthermore, functional studies established that overexpression of ALKBH5 inhibited colon cancer cells invasion in vitro and metastasis in vivo. These results indicated that ALKBH5 significantly inhibits tumor progression and serves as a potential therapeutic target for colon cancer.

Keywords ALKBH5 · Colon cancer · Invasion · Metastasis

Introduction

As the third leading cause of cancer-related deaths worldwide, colon cancer is one of the most aggressive malignancies [1, 2]. Distant metastasis is responsible for major cause of death in colon cancer patients [3]. Critical genes are known to be involved in the tumor metastasis, however, optimal prognostic biomarkers for anti-metastasis strategies have not been established up to now. Therefore, the discovery of novel markers involved in the invasion and metastasis of colon cancer and uncovering their roles are needed.

N6-methyl-adenosine (m6A) is the most prevalent internal chemical modification of messenger RNAs (mRNAs) in higher eukaryotes [4]. This modification is catalyzed by the m6A methyltransferases METTL3 (methyltransferase like 3) or METTL14 (methyltransferase like 14) [5, 6], and erased by FTO (fat-mass and obesity-associated protein) or ALKBH5 (alkB homologue 5) [7, 8]. In mammals, the effects of mRNA m6A modification on biological processes include metabolism [9], fertility [10], stemness maintenance [11] and immunomodulation [12]. As the critical RNA demethylase, ALKBH5 has been reported to play a regulatory role in the development of several cancers. It inhibits pancreatic cancer progression, but maintains tumorigenicity of glioblastoma stem-like cells [13, 14]. Additionally, ALKBH5 mediated m6A modification is also involved in the metastasis of breast cancer [15]. In fact, the disease-associated expression and function of ALKBH5 have remained unclear. Its definite role in colon cancer has never been studied.

In the present study, we discovered that ALKBH5 was downregulated in colon cancer tissues compared with that in the paired adjacent normal mucosa at both mRNA and proteins levels. Low ALKBH5 expression suggested poor outcomes in colon cancer. ALKBH5 inhibited the invasion and metastasis of colon cancer cells both in vitro and in vivo.

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Materials and Methods

Patients and Tissues Samples

A total of 96 colon cancer patients were enrolled. Among them, 60 patients who underwent surgery without prior chemotherapy or radiotherapy at the Department of General Surgery, The first affiliated hospital of Anhui University of Science and Technology. 60 colon cancer tissues and paired normal mucosa of these patients were fixed in formalin, and embedded in paraffin. Another 36 paired fresh colon cancer tissues were obtained within 6 months before initiating this study. The diagnoses were confirmed by professional pathologists. This study was approved by the Institutional Review Boards of The first affiliated hospital of Anhui University of Science and Technology and all of the patients provided informed consent.

Cell Culture and Transfection

The Hct116, RKO, SW620 and HCT8 human colon cancer cell lines and a normal intestinal mucous epithelium cell line NCM460 were purchased from the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM, Hyclone, Logan, UT, USA) with 10% FBS (Gibco, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Gibco) and used within 6 months. ALKBH5 was overexpressed or knocked down by transfecting with lv-ALKBH5 or shRNA ALKBH5 plasmids, respectively.

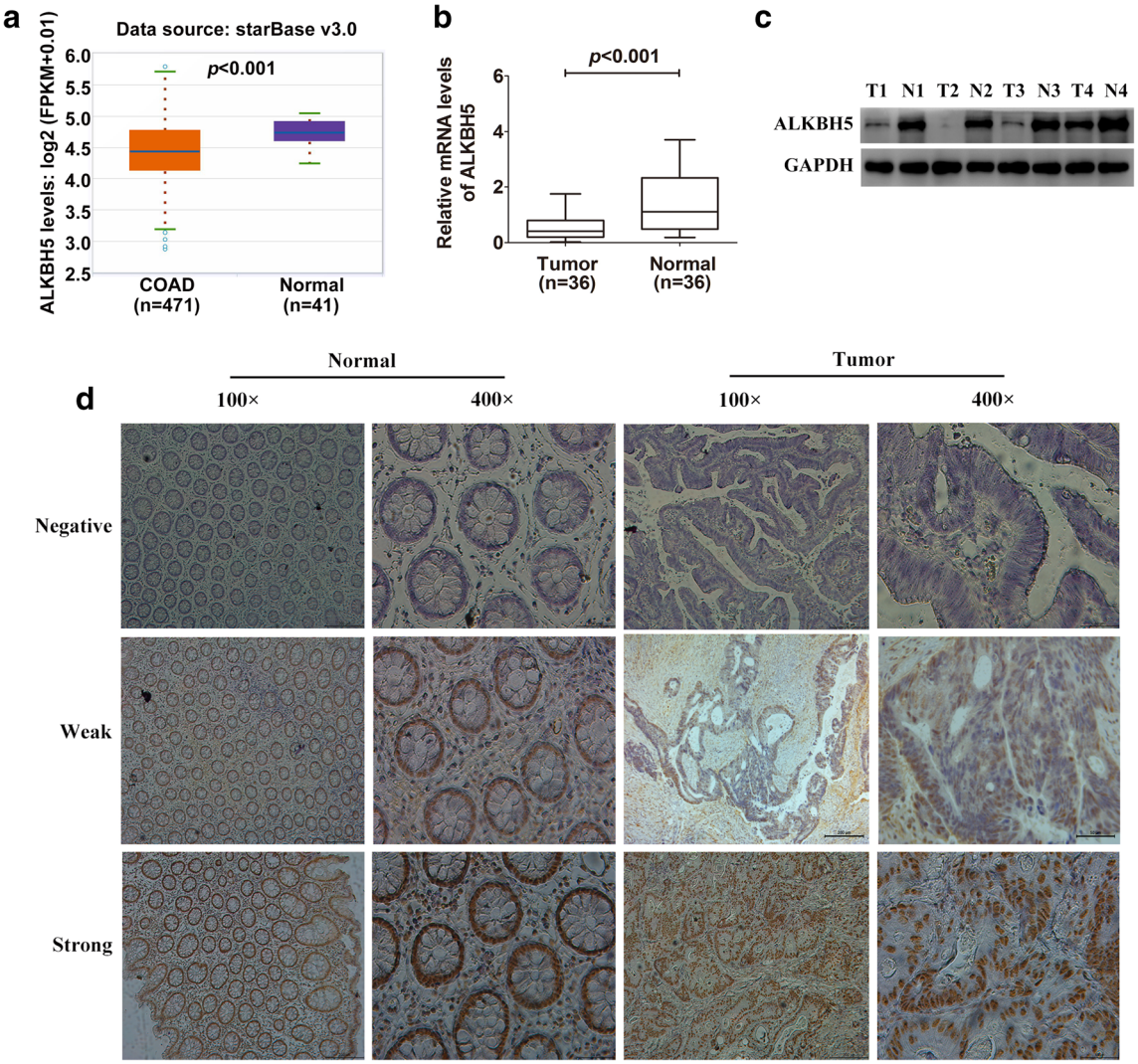

Fig. 1 ALKBH5 was downregulated in colon cancer tissues. a ALKBH5 mRNA levels in colorectal cancer tissues and normal mucosal tissues from the TCGA database. **b** Relative expression of ALKBH5 mRNA in 36 paired colon cancer tissues and normal mucosa tissues. **c** Representative western blot analyses of ALKBH5 protein in colon cancer tissues and paired normal mucosal tissues. **d** Negative, weak or strong staining of ALKBH5 in 60 paired colon cancer tissues via immunohistochemistry

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNAs from the 36 paired tissues and cultured cell lines were extracted by using RNAiso Plus (Takara Bio, Dalian, China) and reverse transcription reactions were carried out using random primers. Then the SYBR Premix Dimmer Eraser kit (Takara, Dalian, China) based on the Applied Biosystems 7300 RT-PCR system (Applied Biosystems, Foster City, CA) was utilized to conduct real-time PCR. All segments were performed according to the manufacturer's instructions. The related primers were listed as follows: ALKBH5 forward 5'-ATCCT CAGGA AGACA AGATT AG-3′, and reverse 5'-TTCTC TTCCT TGTCC ATCTC-3′; GAPDH forward 5'-GGAGC GAGAT CCCTC CAAAA T-3′, and reverse 5'-GGCTG TTGTC ATACT TCTCA TGG-3′. The relative ALKNH5 mRNA expression was calculated by using the $2^{-\Delta\Delta Ct}$ method.

Western Blot Analysis and Immunohistochemistry (IHC)

Western blot and IHC procedures were performed as previously described [16]. Antibodies against ALKBH5 and GAPDH were purchased from Abcam company. For western blot analyses, quantification based on grayscale analysis was performed with Image-Pro Plus 6.0 software. For IHC analyses, the staining area was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%) on the basis of the percentage of positively stained cells. The staining intensity for ALKBH5 was scored as 0 (no staining), 1 (weak staining), 2 (moderated staining), and 3 (strong staining). The final staining scores which is the product of the extension scores and intensity, were divided into 2 groups as followed: 0–3, low expression; ≥4, high expression.

In Vitro Invasion Assay and In Vivo Metastatic Assay

Invasion assay was carried out by using Transwell chamber with inserts of 8-μm pore size (Corning Costar) as described previously [17].

For the animal studies, 6-week-old male athymic BALB/c nude mice were implanted with modified ALKBH5 expressing Hct116 or RKO cells (10^5/ml, 200 ml) via lateral tail vein injection. All the mice were killed after 6 weeks, and the lungs were subjected to H&E staining. All animal experiments were performed in accordance with the Anhui University of Science and Technology Animal Care Guidelines.

Statistical Analysis

All statistical analyses were carried out with SPSS 19.0 Software package (SPSS, Chicago, IL). Student's t test or

Table 1 Expression of ALKBH5 in 60 paired of colon cancer tissues

Tissue samples	No. of patients	ALKBH5 expression		*P* value
		Low $n = 55$ (%)	High $n = 65$ (%)	
Normal tissues	60	15 (27.3)	45 (69.2)	<0.001*
Tumor tissues	60	40 (72.7)	20 (30.8)	

*$p < 0.05$ represents the p value with significant difference

Table 2 Association between ALKBH5 expression and clinicopathological characteristics in colon cancer

Characteristics	$n = 60$ (%)	ALKBH5 expression		*P* value
		Low $n = 40$ (%)	High $n = 20$ (%)	
Age (years)				0.582
<65	33 (55.0)	21 (52.5)	12 (60.0)	
≥65	27 (45.0)	19 (47.5)	8 (40.0)	
Gender				0.711
Male	25 (41.7)	16 (40.0)	9 (45.0)	
Female	35 (58.3)	24 (60.0)	11 (55.0)	
Location				0.280
Right	19 (31.7)	15 (37.5)	4 (20.0)	
Others	41 (68.3)	25 (62.5)	16 (80.0)	
pT stage				0.131
T1	4 (6.7)	1 (2.5)	3 (15.0)	
T2	21 (35.0)	12 (30.0)	9 (45.0)	
T3	25 (41.7)	19 (47.5)	6 (30.0)	
T4	10 (16.6)	8 (20.0)	2 (10.0)	
pN stage				0.076
N0	33 (55.0)	18 (45.0)	15 (75.0)	
N1	9 (15.0)	7 (17.5)	2 (10.0)	
N2	18 (30.0)	15 (37.5)	3 (15.0)	
pM stage				0.011*
M0	49 (81.7)	29 (72.5)	20 (100)	
M1	11 (18.3)	11 (27.5)	0 (0)	
AJCC stage				0.017*
I/II	32 (53.3)	17 (42.5)	15 (75.0)	
III/IV	28 (46.7)	23 (57.5)	5 (25.0)	
Differentiation				0.226
Well	21 (35.0)	17 (42.5)	4 (20.0)	
Moderate	27 (45.0)	16 (40.0)	11 (55.0)	
Poor	12 (20.0)	7 (17.5)	5 (25.0)	
Vessel invasion				0.348
No	37 (61.7)	23 (57.5)	14 (70.0)	
Yes	23 (38.3)	17 (42.5)	6 (30.0)	

*$p < 0.05$ represents the p-values with significant differences

one-way ANOVA was used to analyze the between-group differences. The relative gene expression levels and clinicopathologic parameters were compared by Fisher's exact test or a Chi-square test. Survival curves were plotted by the Kaplan-Meier method with log-rank test. Cox regression was utilized to estimate the univariate and multivariate hazard ratios for the variables.

Results

ALKBH5 is Significantly Downregulated in Colon Cancer at both mRNA and Protein Levels

We checked the mRNA levels of ALKBH5 in colon adenocarcinoma (COAD) samples by analyzing the public dataset from starBase v3.0 project (http://starbase.

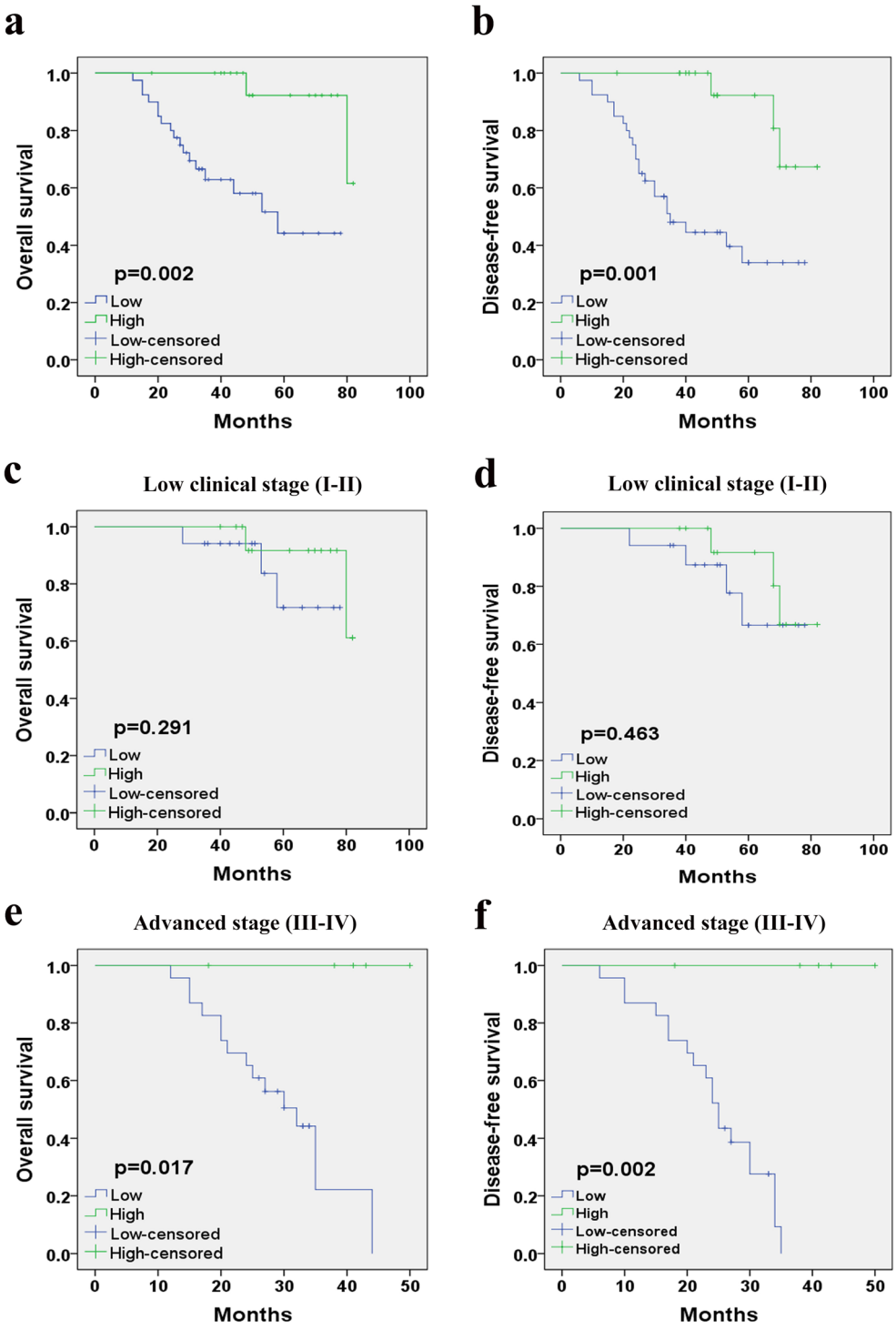

Fig. 2 Low ALKBH5 expression predicts poor prognosis in patients with colon cancer. a–b Impact of ALKBH5 expression on the OS ($p = 0.002$) and DFS ($p = 0.001$) of patients with colon cancer. **c–d** No differences were found in low-stage colon cancer patients between high ALKBH5 expression group and low ALKBH5 expression group. **e–f** The advanced-stage patients with low ALKBH5 expression had shorter OS and DFS compared with high expression group

sysu.edu.cn/panCancer.php). The results showed that ALKBH5 was downregulated in colon cancer tissues than in the normal tissues (Fig. 1a). We also randomly selected 36 paired fresh colon cancer tissues and normal mucosal tissues to further validate the expression of ALKBH5 mRNA, and found that attenuation of ALKBH5 was a frequent event in colon cancer tissues (Fig. 1b). Subsequently, the protein expression of ALKBH5 was also evaluated in the same 36 paired samples. Western blot analysis showed that ALKBH5 protein level was significantly lower in cancer tissues compared with that in the paired adjacent normal mucosa ($P < 0.01$, Student's t test) (Fig. 1c). These findings confirmed the decreased ALKBH5 expression at both the transcriptional and translational levels.

Association of ALKBH5 Expression with Clinicopathological Parameters of Colon Cancer Patients

Next, to explore whether ALKBH5 expression was associated with the clinicopathological characteristics, another 60 paired colon cancer tissues were used. ALKBH5 protein levels were divided into high expression group and low expression group in relation to the immunohistochemistry (IHC) staining scores (Fig.1 d). As shown in Table 1, 72.7% (40/60) cases showed low ALKBH5 expression in colon cancer tissues. These results suggested significant differences of ALKBH5 expression between the normal and cancer tissues. Moreover, ALKBH5 expression was significantly associated with pM stage ($P = 0.011$) and AJCC (American Joint Committee on Cancer) stage ($P = 0.017$) (Table 2).

The Prognostic Value of ALKBH5 for Colon Cancer Patients

Overall survival (OS) and disease-free survival (DFS) of 60 colon cancer patients were analyzed by Kaplan-Meier method with log-rank test, and striking differences were observed between the low ALKBH5 expression group and the high expression group (Fig. 2a, b). Interestingly, despite no differences were found in low-stage (TNMI/II, $n = 32$) colon cancer patients (Fig. 2c, d), low ALKBH5 expression significantly correlated with a worse OS and DFS in advanced-stage (TNM III/IV, $n = 28$) patients (Fig. 2e, f). Additionally, univariate and multivariate analyses indicated that downregulation of ALKBH5 was an independent prognostic indicator for OS and DFS in colon cancer patients (Tables 3 and 4). These findings revealed that ALKBH5 holds significant clinical values in colon cancer patients.

Effects of ALKBH5 on the Invasion and Metastasis of Colon Cancer Cells Both In Vitro and In Vivo

To investigate the functional roles of ALKBH5 on colon cancer cells, one normal colon mucosa epithelial cell line (NCM460) and four colon cancer cell lines (Hct116, RKO, SW620 and HCT8) were used to evaluate the expression level of ALKBH5. All the cancer cell lines showed lower ALKBH5 expression at both mRNA and protein levels when compared with NCM460 cell line (Fig. 3a, b). The Hct116 and RKO cell lines were selected for the further experiments. Then, ALKBH5 overexpressed plasmid (Lv-ALKBH5) and downregulated plasmid (sh-ALKBH5) were transfected into Hct116 or RKO cells, respectively. As a result, ALKBH5 was overexpressed or knocked down at both mRNA and protein levels when compared with their controls (Fig. 3c, d). In the transwell invasion assays, overexpression of ALKBH5

Table 3 Univariate and multivariate analyses of overall survival in colon cancer

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age	1.569	0.619–3.981	0.343	–	–	–
Gender	0.687	0.277–1.703	0.418	–	–	–
Location	1.007	0.382–2.657	0.988	–	–	–
AJCC stage	21.495	4.304–107.339	<0.001*	18.147	2.480–132.792	0.004*
T stage	1.377	0.768–2.469	0.282	–	–	–
N stage	3.008	1.654–5.470	<0.001*	–	–	–
M stage	15.911	4.782–52.941	<0.001*	4.003	1.198–13.380	0.024*
Differentiation	0.779	0.415–1.465	0.439	–	–	–
Vascular invasion	1.452	0.582–3.623	0.424	–	–	–
ALKBH5	0.080	0.011–0.603	0.014*	0.115	0.014–0.958	0.046*

HR: hazard ratio; CI confidence interval

*$p < 0.05$ represents the p-values with significant differences

Table 4 Univariate and multivariate analyses of disease-free survival in colon cancer

Variable	Univariate analysis			Multivariate analysis		
	HR	95% CI	*P* value	HR	95% CI	*P* value
Age	1.311	0.606–2.838	0.492	–	–	–
Gender	0.634	0.293–1.372	0.247	–	–	–
Location	0.695	0.315–1.536	0.369	–	–	–
AJCC stage	4.016	2.250–7.167	<0.001*	18.584	3.933–87.799	<0.001*
T stage	1.539	0.942–2.516	0.085	–	–	–
N stage	3.014	1.820–4.990	<0.001*	–	–	–
M stage	12.360	4.227–36.143	<0.001*	3.162	1.071–9.337	0.037*
Differentiation	0.959	0.561–1.641	0.879	–	–	–
Vascular invasion	1.865	0.862–4.034	0.113	–	–	–
ALKBH5	0.152	0.045–0.515	0.002*	0.173	0.046–0.653	0.010*

HR: hazard ratio; CI confidence interval

*$p < 0.05$ represents the *p*-values with significant differences

impeded the invasive ability in Hct116 cells (Fig. 4a, b), while knockdown of ALKBH5 promoted the invasive ability in RKO cells (Fig. 4c, d). Next, the in vivo metastatic model was established by injecting the modified ALKBH5 expressing colon cancer cells into the tail veins of nude mice. As expected, a marked less number of lung metastatic nodules was observed in the ALKBH5 overexpressing group compared with the control group (Fig. 4e, f). Conversely, depletion of ALKBH5 significantly increased the lung metastases burden of colon cancer cells (Fig. 4g, h). These results confirmed that ectopic expression of ALKBH5 could inhibit colon cancer aggression in vitro and in vivo.

Discussion

Colon cancer cells exert distant invasive potential and metastatic abilities through the complex gene interactions [17]. Thus, discovering novel regulators and revealing the underlying mechanism of metastasis involved in colon cancer are necessary for developing therapeutic strategies. In the present study, we provided a new insight into the association between ALKBH5 expression and colon cancer.

Recent evidences have demonstrated that some clinicopathological features such as tumor regional lymph node stage, distant metastasis, and AJCC stage can be used as independent

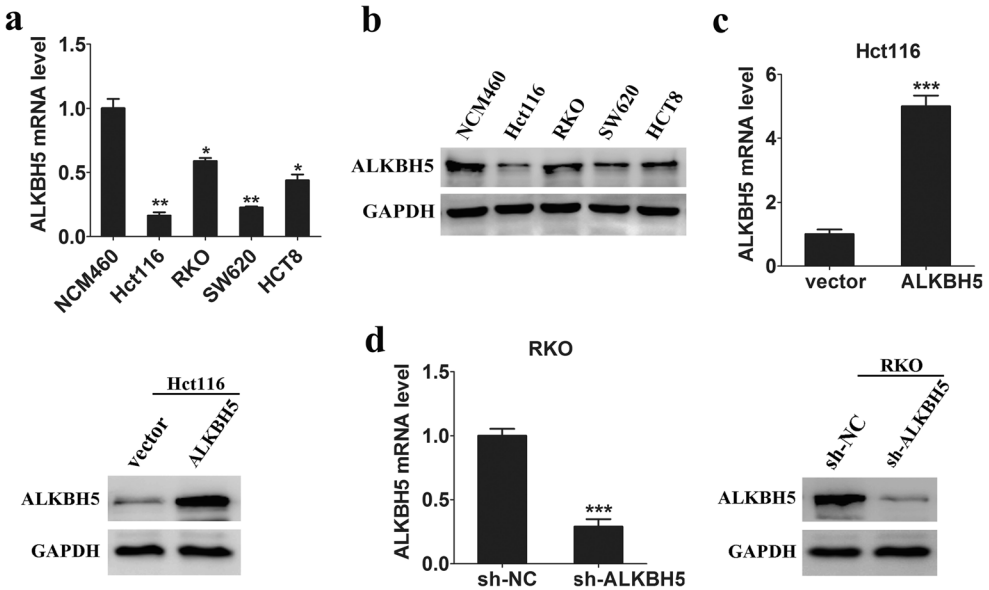

Fig. 3 Low expression of ALKBH5 in different cancer cell lines and transfection efficiency of ALKBH5. ALKBH5 expression was evaluated by using qRT-PCR (**a**) and Western Blot (**b**) in different cell lines, and all the cancer cell lines showed lower ALKBH5 expression at both mRNA and protein levels when compared with the normal colon mucosal cell line NCM460. **c** ALKBH5 was overexpressed at both mRNA and protein levels after transfecting ALKBH5 overexpressed plasmid into Hct116 cells. **d** ALKBH5 was knocked down at both mRNA and protein levels after transfecting ALKBH5 knockdown plasmid into RKO cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

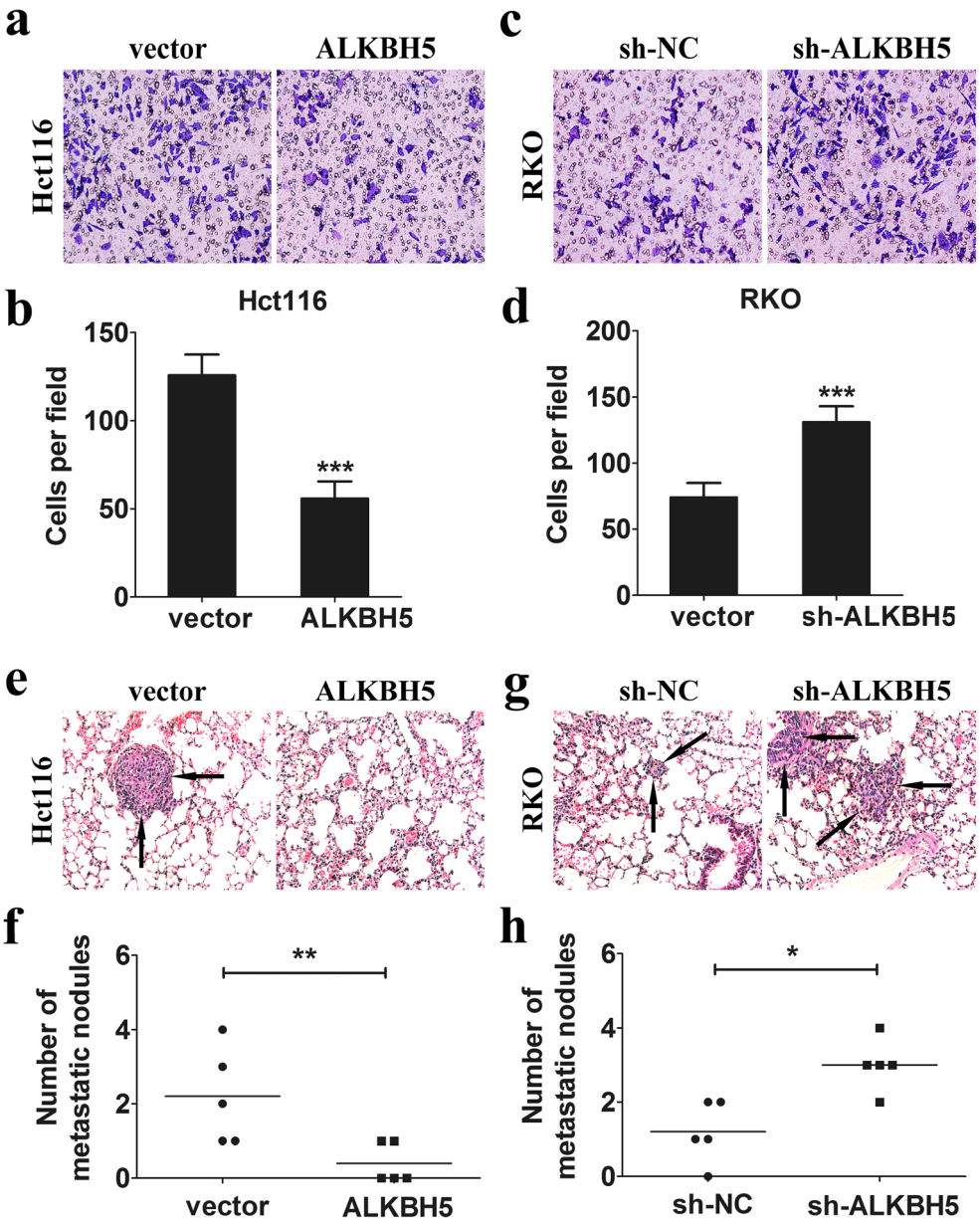

Fig. 4 Effects of ALKBH5 on the invasion and metastasis of colon cancer cells both in vitro and in vivo. Transwell invasion assays were performed both in empty vector-infected or ALKBH5 overexpressed plasmids infected Hct116 cells (**a**–**b**), and negative control or sh-ALKBH5 plasmids infected RKO cells (**c**–**d**), respectively. **e**–**f** A marked less number of lung metastatic nodules was observed in the ALKBH5 overexpressing group compared with the control group. **g**–**h** depletion of ALKBH5 significantly increased the lung metastases burden of colon cancer cells. (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$)

prognostic factors for clinical outcomes [18]. Moreover, plenty of biomarkers have been associated with survival, such as APC (adenomatous polyposis coli) [19], cell adhesion molecules [20], DNA mismatch repair genes [21], growth factors/receptors [22], and stem cell related genes [23]. Nevertheless, the optimal prognostic biomarkers for colon cancer have not been established up to now. To address whether or not ALKBH5 could possibly serve as a beneficial candidate biomarker for colon cancer patients, the prognostic value of ALKBH5 was explored. Our findings revealed that ALKBH5 expression levels was not only associated with distant metastasis and AJCC stage, but also strongly linked to the risk of tumor recurrence and survival. Univariate and multivariate analyses indicated that ALKBH5 expression alone could serve as an independent prognostic factor for OS and DFS in colon cancer.

RNA m6A methylation regulates cellular processes through a series of mechanisms, including alterations in RNA stability, secondary structure, subcellular localization, and translation efficiency [24]. This modification is reversible and can be demethylated by ALKBH5. Nowadays, the reports for abnormal regulation of ALKBH5 in human malignancies are limited. In this study, we verify for the first time that ALKBH5 inhibits colon cancer progression, the results of in vitro and in vivo assays highlighted the critical role of ALKBH5 in the development of colon cancer. It is noteworthy that ALKBH5 is known to exert crucial effects on pancreatic cancer and non-small cell lung cancer (NSCLC) by acting as a tumor suppressor [25], but be considered to play oncogenic roles in breast cancer and glioblastoma [14, 26]. In

other words, the roles of ALKBH5 in different cancers were different. Thus, further investigations are warranted to advance our understanding of the differences in potential regulatory mechanisms in different tumors.

In summary, ALKBH5 was considered as a novel tumor suppressor in inhibiting the invasion and metastasis of colon cancer. Despite that ALKBH5 mRNA was downregulated in our hospital cases, its expression in cancer tissues appeared to be lower, and the normal group was extremely variable. Moreover, another cohort consisting of 60 pairs of colon cancer tissues included only 11 M1 disease, and such a low metastatic disease group may lead to an unreasonable statistical result. Considering that our study was conducted in small-scale patients, a larger prospective clinical investigation are warranted to fully understand and exploit the prognostic and therapeutic value of ALKBH5.

Acknowledgements This work was supported by Science and Technology Project of Chongqing Education Committee (No.KJ1600210) and Shandong Province Natural Science Foundation of China (No. ZR2013HL054).

Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interests regarding the publication of this paper.

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