



RESEARCH ARTICLE

N6-methyladenosine demethylase ALKBH5 suppresses colorectal cancer progression potentially by decreasing *PHF20* mRNA methylation

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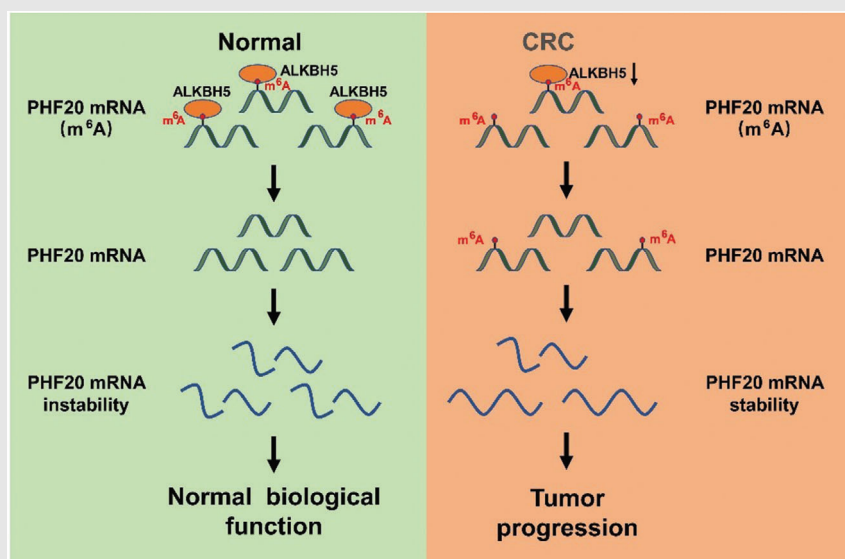
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Graphical Abstract



- ALKBH5 expression is down-regulated in CRC and plays a crucial tumor suppressive role.
- ALKBH5 inhibits the stability of *PHF20* mRNA by removing the m⁶A modification.
- Targeting the ALKBH5-mediated m⁶A modification of *PHF20* mRNA may be a promising therapeutic strategy for intervention and treatment of CRC.

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Abstract

Background: As the most widespread mRNAs modification, N6-methyladenosine (m⁶A) is dynamically and reversibly modulated by methyltransferases and demethylases. ALKBH5 is a major demethylase, and plays vital roles in the progression of cancers. However, the role and mechanisms of ALKBH5 in colorectal cancer (CRC) is unclear.

Results: Herein, we discovered that in CRC, downregulated ALKBH5 was closely related to poor prognosis of CRC patients. Functionally, our results demonstrated that knockdown of ALKBH5 enhanced the proliferation, migration and invasion of LOVO and RKO in vitro, while overexpression of ALKBH5 inhibited the functions of these cells. The results also demonstrated that knockdown of ALKBH5 promoted subcutaneous tumorigenesis of LOVO in vivo, while overexpression of ALKBH5 suppressed this ability. Mechanistically, results from joint analyses of MeRIP-seq and RNA-seq indicated that *PHF20* mRNA was a key molecule that was regulated by ALKBH5-mediated m⁶A modification. Further experiments indicated that ALKBH5 may inhibit stability of *PHF20* mRNA by removing the m⁶A modification of *PHF20* mRNA 3'UTR.

Conclusions: ALKBH5 suppresses CRC progression by decreasing *PHF20* mRNA methylation. ALKBH5-mediated m⁶A modification of *PHF20* mRNA can serve as a hopeful strategy for the intervention and treatment of CRC.

KEYWORDS

ALKBH5, colorectal cancer, m⁶A modification, *PHF20*

Zhen Zhang and Ling Wang contributed equally to this work.

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1 | INTRODUCTION

As one of the most common malignant tumours, colorectal cancer (CRC) ranks the third with regard to incidence and the second in terms of mortality worldwide.¹ It was reported that there would be about 2 million new CRC cases, and 0.9 million CRC-associated deaths in 2020.¹ Although comprehensive treatment methods have improved, the prognosis of CRC remains poor. The molecular mechanisms of CRC progression need to be explored.

N⁶-methyladenosine (m⁶A) is reported to be the most widespread mRNAs modification in eukaryotic cells. M⁶A modification of mRNAs regulates its metabolic process, including splicing, transport, stability and translation of mRNAs.^{2,3} Recent studies have demonstrated that the m⁶A modification of mRNAs is dynamically and reversibly regulated by methyltransferases and demethylases. The methyltransferase complex consists of METTL3 (methyltransferase-like 3), METTL14 (methyltransferase-like 14) and additional adaptor molecules.^{4,5} Demethylases include FTO (fat mass and obesity-associated protein) and ALKBH5 (alkB homologue 5).^{6,7} Lots of studies have demonstrated that methyltransferases and demethylases are frequently dysregulated among various cancers, making it vital roles in the progression of cancers.^{8,9}

ALKBH5 is one of two RNA demethylases, which is able to remove the m⁶A modification on RNAs.⁷ ALKBH5 is dysregulated in many tumours, such as hepatocellular cancer, pancreatic cancer and gastric cancer.¹⁰ With regard to CRC, ALKBH5 may promote cancer cell motility by demethylating the lncRNA NEAT1.¹¹ However, ALKBH5 has been reported to be downregulated in CRC tissues and positively associated with overall survival and disease-free survival.¹² Therefore, the role and mechanisms of ALKBH5 in CRC need to be further studied.

Herein, we identified that downregulated ALKBH5 was closely associated with the poor prognosis of CRC patients. ALKBH5 significantly inhibited the proliferation, migration and invasion abilities of LOVO and RKO in vitro, and suppressed the subcutaneous tumorigenicity of LOVO in vivo. Mechanistically, *PHF20* mRNA was a key downstream molecule of ALKBH5. ALKBH5 might inhibit the stability of *PHF20* mRNA via removing the m⁶A modification of *PHF20* mRNA 3'UTR, thereby suppressing the progression of CRC.

2 | MATERIALS AND METHODS

2.1 | Patient samples and cell lines

CRC tumour tissues and tumour-adjacent normal tissues were collected from Peking University People's Hospital. All colorectal tissues were pathologically confirmed.

Six human colon cancer cell lines (RKO, SW480, HCT116, HCT8, LS174T, LOVO) were purchased from the Cell Resource Center of Peking Union Medical College (China). RKO, SW480, HCT116, HCT-8, LS174T and LOVO were cultured in MEM, IMDM, RPMI 1640, MEM and F12K, respectively. All the cell lines were cultivated in the corresponding medium containing 10% FBS (Gibco, USA) in 5% CO₂ environment at 37°C.

2.2 | Establishment of stable knock-down and overexpression cells

Three siRNAs of *ALKBH5* (siALKBH5-1: ACAAGTACTTCTTCGGCGA, siALKBH5-2: GCGCCGTCATCAACGACTA, siALKBH5-3: CTGA-GAACTACTGGCGCAA) and two siRNAs of human *PHF20* (siPHF20-1: CCCGAGAAATACACCTGTTAT, siPHF20-2: ATGTGCCACTGATGATAAAC) were synthesized by RiboBio (China). In addition, the two most efficient sequences for *ALKBH5* were used to construct lentiviral shRNA plasmids. The lentiviral plasmid expressing shALKBH5 or shNC, overexpressing *ALKBH5* or an empty vector were purchased from GeneCopoeia (USA). The packaging plasmid, envelope plasmid and target plasmid were transfected to the 293T cells to obtain the lentivirus using the Lenti-Pac HIV Expression Packaging Kit (GeneCopoeia, USA). Then, the lentivirus was used to infect LOVO and RKO.

2.3 | Immunohistochemical staining (IHC)

Immunohistochemical staining (IHC) was performed, as previously described.¹³ ALKBH5 staining index score (0–12) was calculated using the staining intensity multiplying by the percentage of ALKBH5 positive staining. The staining intensity is divided into four grades (negative: 0; weak: 1; moderate: 2; strong: 3), and the percentage of positive staining is divided into five grades (<5%: 0; 5%–25%: 1;

26%–50%: 2; 51%–75%: 3; >75%: 4). Anti-ALKBH5 (Sigma, USA) was used. All scores were independently evaluated by two pathologists.

2.4 | RT-qPCR

RT-qPCR was performed, as previously described.¹³ The primer sequences are listed in Table S1.

2.5 | Western blot

Western blot assays were performed, as previously described.¹³ Primary antibodies included anti-ALKBH5 (Sigma, USA), anti-PHF20 (CST, USA) and anti-GAPDH (CST, USA).

2.6 | Cell function experiments

To perform the cell proliferation assays, we seeded 3×10^3 cells into a 96-well plate. Next, 10- μ l CCK8 solution was added into each well of the plate. After incubation for 2 h, the absorbance at 450 nm was detected to assess cell proliferation. Moreover, cell proliferation was assessed for five consecutive days after cells seeded.

To conduct the colony formation assays, we seeded 5×10^2 cells into a 6-well plate. After cultivated for 10 days, the clones were fixed in 4% paraformaldehyde solution for 25 min. Then, the clones were stained with .1% crystal violet solution for 25 min. Last, the clones were counted after washed with PBS three times.

To perform the migration assays, we seeded 1×10^5 cells into the upper chambers (Corning, USA) and added medium with 10% FBS to the lower chambers. To perform the invasion assays, the upper chambers were coated with Matrigel (Sigma, USA). After cultivated for 48 h, the cells migrating below the chambers were fixed, stained and counted under a microscope.

2.7 | Animal experiments

Female Balb/c nude mice (6–8 weeks) were purchased from the Experimental Animal Center of Military Medical Sciences (China). We injected 1×10^6 cells subcutaneously in the flanks of mice and measured the length (L) and width (W) of tumour every 2 days. Tumour volume (V) was calculated as follows: $V = \frac{1}{2}L \cdot W^2$. The mice were euthanized at 16th days after the injection of cancer cells. Then, the subcutaneous tumours were removed and weighted.

2.8 | RNA stability assays

Tumour cells were treated with actinomycin D (Sigma, USA) at 5 μ g/ml. The cells were collected after incubated for 0, 3 or 6 h, and then RNA was extracted to perform RT-qPCR assays as described earlier. The half-life of mRNA was calculated to assess its stability.

2.9 | mRNA m⁶A quantification

The mRNA was purified from total RNA using the GenElute mRNA Miniprep Kit (Sigma, USA). The m⁶A modification level of mRNA was evaluated utilizing the EpiQuik m⁶A RNA methylation quantification kit (EpiGentek, USA). In brief, 200-ng mRNA was incubated with capture antibody and then incubated with detection antibody. The m⁶A modification level of mRNA was quantified colourimetrically by measuring the absorbance at 450 nm.

2.10 | Luciferase reporter assays

The wild type and m⁶A sites mutated PHF20 were constructed into the luciferase reporter vector (GeneCopoeia, USA). The sequences of the wild type and m⁶A sites mutated PHF20 are listed in Table S2. Cells were transfected with siNC or siALKBH5. The cells were re-seeded into a 24-well plate after incubated with siRNA for 48 h and then were transfected with *PHF20* 3'UTR-WT or *PHF20* 3'UTR-MUT. The cells were lysed after cultivated for 24 h. Firefly and Renilla luciferase activities in cell lysates were analysed utilizing the Dual-Glo Luciferase Assay system (Promega, USA).

2.11 | RNA sequencing

Total RNA was isolated with a TRIzol reagent (Invitrogen, CA). A total of 1- μ g RNA was used to perform RNA sequencing. Sequencing libraries were generated using the NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, USA). Sequencing was performed based on the Illumina NovaSeq platform. Differentially expressed genes were identified utilizing the DESeq2 R package ($|\log_2(\text{fold change})| > 0$ and $p < 0.05$).

2.12 | MeRIP-qPCR

The m⁶A modification of mRNA was quantified using the Magna MeRIP m⁶A Kit (Millipore, Germany). In brief, 10%

of mRNA purified from total RNA was saved as inputs. Magna ChIP Protein A/G Magnetic Beads were washed and then incubated with 5- μ g anti-m⁶A antibody with rotation for 30 min at room temperature. The antibody-beads mixed with mRNA were incubated at 4°C with RNase inhibitors for 2 h. Then, the methylated mRNAs were eluted and purified utilizing the RNeasy mini kit (QIAGEN, Germany). RT-qPCR was used to determine m⁶A enrichment by normalizing to the input.

2.13 | Statistical analysis

All results are presented as mean \pm SD. The SPSS 17.0 was used for data analysis. The χ^2 test was utilized to analyse the correlation between ALKBH5 and clinicopathological features of patients. Kaplan–Meier analysis was used to construct survival curves, and the differences of different groups were estimated by log-rank test. For all continuous variables, Student's *t*-test or ANOVA was performed to evaluate the differences. A *p* value of .05 was considered statistically significant.

3 | RESULTS

3.1 | ALKBH5 was downregulated in CRC

To evaluate the expression of *ALKBH5* in cancers, we initially detected mRNA expression of *ALKBH5* in 23 solid cancers in TCGA (The Cancer Genome Atlas) datasets. In 12 solid cancers, *ALKBH5* was significantly dysregulated in comparison to adjacent normal tissues (Figure 1A). As shown in Figure 1A, compared to adjacent normal tissues, *ALKBH5* was significantly downregulated in seven solid cancers (CRC, GBM, KICH, KIRP, PRAD, THCA and UCEC) and upregulated in five solid cancers (CHOL, HNSC, KIRC, LIHC and LUSC). We discovered that, compared with adjacent normal tissues, *ALKBH5* was significantly reduced in tumour tissues in TCGA-CRC cohort. We also downloaded the RNA sequencing data of CRC from GEO (Gene Expression Omnibus) datasets. The results confirmed the reduction of *ALKBH5* mRNA in CRC tissues (GSE39582 and GSE87211; Figure 1B). Furthermore, RT-qPCR showed that *ALKBH5* mRNA expression in CRC tissues was significantly lower than that in adjacent normal tissues (Figure 1C). We also evaluated ALKBH5 protein expression in CRC. IHC assays indicated that ALKBH5 protein expression was significantly decreased in CRC tissues (Figure 1D,E). What is more, the m⁶A quantification assays of mRNA implied that m⁶A modification levels of mRNAs were higher in CRC tissues (Figure 1F).

3.2 | Loss of ALKBH5 predicted worse prognosis of CRC patients

In order to explore the clinical value of *ALKBH5*, we analysed the correlation between *ALKBH5* and clinicopathological features of CRC patients at the mRNA and protein levels. The results indicated that, at the mRNA levels, downregulated *ALKBH5* was significantly associated with distant metastasis (*p* = .025) and late clinical stage (*p* = .034) (Table 1; Figure 2A,B). Consistently, at the protein levels, downregulated ALKBH5 was significantly related to distant metastasis (*p* = .042) and late clinical stage (*p* = .024) (Table 2; Figure 2D,E). The Kaplan–Meier analysis indicated that CRC patients with a loss of ALKBH5 had shorter overall survival both at the mRNA (Figure 2C, *p* = .026) and protein (Figure 2F, *p* = .048) levels.

3.3 | Knock-down of ALKBH5 enhanced growth and metastasis of colon cancer cells

Next, we explored the effect of ALKBH5 on colon cancer cell function. We initially identified *ALKBH5* mRNA expression in five colon cancer cells, and noted that *ALKBH5* was elevated in LOVO and RKO cells, compared to other colon cancer cell lines (Figure 3A). Three specific siRNAs were utilized to knock-down *ALKBH5* in LOVO and RKO cells (Figure 3B,C). Furthermore, two siRNAs (siALKBH5-2, siALKBH5-3) with high knock-down efficiency were utilized to construct knock-down plasmids (shALKBH5-2, shALKBH5-3) for subsequent experiments. We constructed two stable cell lines using two independent *ALKBH5*-knock-down lentiviruses. The knock-down efficiency was validated both at the mRNA and protein levels (Figure 3D–F). Subsequently, the CCK-8 assays revealed that the knock-down of ALKBH5 markedly enhanced cellular growth, as well as the viability of LOVO and RKO cells (Figure 3G). It was confirmed that the knock-down of ALKBH5 significantly increased the colony formation abilities of LOVO and RKO cells through the colony formation assays (Figure 3H). Additionally, we assessed migration and invasion abilities of LOVO and RKO cells using the transwell assays. The results indicated that the knock-down of ALKBH5 drastically elevated cell migration and invasion abilities (Figure 3I,J).

To further determine whether ALKBH5 affects colon cancer cell function in vivo, we established tumour xenograft models. We found that tumour growth rate was faster (Figure 4C), and tumour volume and weight were increased (Figure 4A,B,D) when ALKBH5-knock-down LOVO cells were implanted (*n* = 5). Moreover, the expression of Ki-67 was also increased in the

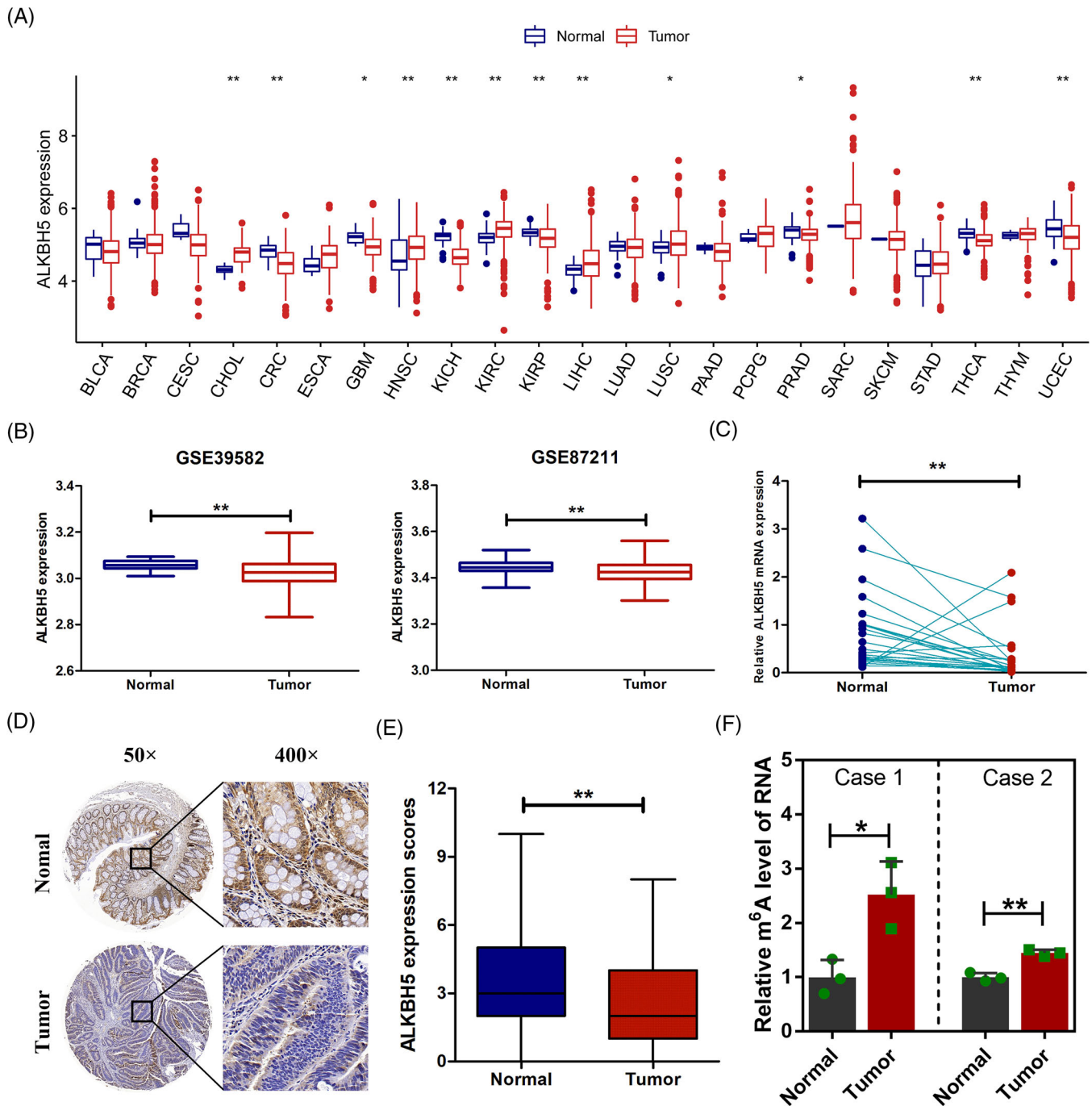


FIGURE 1 AlkB homologue 5 (ALKBH5) was downregulated in colorectal cancer (CRC). (A) *ALKBH5* mRNA expression in 23 solid cancers in The Cancer Genome Atlas (TCGA) databases. (B) *ALKBH5* mRNA expression in CRC in the Gene Expression Omnibus (GEO) databases. (C) RT-qPCR analysis of *ALKBH5* mRNA expression in 24 paired CRC tumours and adjacent normal tissues. (D) Representative immunohistochemical staining (IHC) images of ALKBH5 expression in CRC tumour and paired normal tissues. (E) Quantification of the IHC score of ALKBH5 in CRC tumour tissues ($n = 114$) and adjacent normal tissues ($n = 107$). (F) Relative quantitative analysis of global N6-methyladenosine (m⁶A) levels of mRNA in CRC tumour tissues and adjacent normal tissues. Statistical significance was determined using Wilcoxon test (A, B, E), paired Wilcoxon test (C) and *t*-test (F) (* $p < .05$, ** $p < .01$).

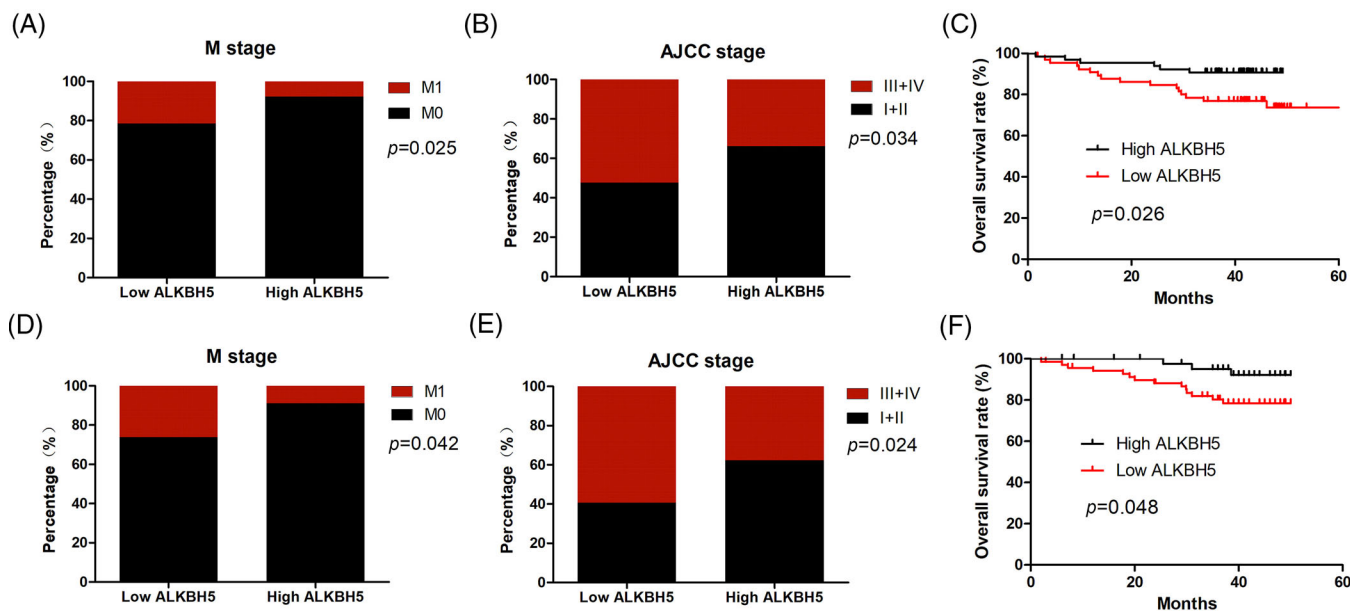


FIGURE 2 Loss of alkB homologue 5 (ALKBH5) predicted worse prognosis of colorectal cancer (CRC) patients. (A) The relationship between *ALKBH5* mRNA expression and metastasis (M) stage. (B) The relationship between *ALKBH5* mRNA expression and AJCC stage. (C) The Kaplan–Meier analysis of overall survival (OS) for CRC patients based on *ALKBH5* mRNA expression. (D) The relationship between ALKBH5 protein expression and M stage. (E) The relationship between ALKBH5 protein expression and AJCC stage. (F) The Kaplan–Meier analysis of OS for CRC patients, based on ALKBH5 protein expression. Statistical significance was determined using χ^2 test (A, B, D and E) and log-rank tests (C and F).

shALKBH5 groups in comparison to the shNC groups (Figure S1).

3.4 | Overexpression of ALKBH5 inhibited growth and metastasis of colon cancer cells

We also constructed stably transfected cell lines with *ALKBH5* overexpression utilizing a lentivirus vector, and RT-qPCR and Western blot assays validated the overexpression efficiency (Figure 5A–C). The CCK-8 assays revealed that the overexpression of ALKBH5 markedly repressed cellular growth and the viability of LOVO and RKO cells (Figure 5D). The colony formation assays confirmed that the overexpression of ALKBH5 resulted in a significant decrease of the colony formation abilities of LOVO and RKO cells (Figure 5E). Additionally, the overexpression of ALKBH5 drastically weakened cell migration and invasion by the transwell assays (Figure 5F,G). Then, our results of tumour xenograft models indicated that tumour growth rate was slower (Figure 6C), and that tumour volume and weight were decreased (Figure 6A,B,D) when LOVO cells overexpressing ALKBH5 were implanted ($n = 5$).

3.5 | ALKBH5 targeted PHF20

To identify the m⁶A-modified targets, we collected tumour tissues and normal tissues from six CRC patients to perform MeRIP-seq.¹⁴ We identified 1343 dysregulated m⁶A peaks in six pairs of tissues by MeRIP-seq (Figure 7A). Among them, 625 m⁶A peaks were hypermethylated in tumour tissues, whereas 718 m⁶A peaks were hypomethylated in tumour tissues (Figure 7A). Next, 625 hypermethylated m⁶A peaks were distributed among the transcripts of 265 genes, and 718 hypomethylated m⁶A peaks were distributed among the transcripts of 311 genes. Interestingly, we discovered that in the six pairs of tissues for MeRIP-seq, the expression of *ALKBH5* in tumour tissues was significantly lower than that in normal tissues (Figure 7B). We hypothesized that the transcripts of these 265 genes were regulated by ALKBH5. In order to further explore m⁶A modification targets regulated by ALKBH5, we performed RNA-seq in ALKBH5-knock-down LOVO cells and control cells. The results demonstrated that after ALKBH5 knocked down, 679 genes were differentially expressed, including 362 upregulated genes and 317 downregulated genes (Figure 7C,D). Combining MeRIP-seq and RNA-seq, we found 19 genes regulated potentially by ALKBH5 through m⁶A modification, including 11 upregulated genes (*PCDH7*, *QSOX1*, *LAMA5*, *CCDC69*, *LITAF*,

TABLE 1 Clinical characteristics of 130 colorectal cancer (CRC) patients according to alkB homologue 5 (ALKBH5) mRNA levels

	ALKBH5			p-Value
	All cases	Low expression	High expression	
Gender				
Female	61	33	28	.38
Male	69	32	37	
Age				
≤60	46	22	24	.714
>60	84	43	41	
Tumour size				
≤5	65	30	35	.384
>5	65	35	30	
Histological type				
Adenocarcinoma	118	58	60	.545
Others	12	7	5	
Tumour differentiation				
Well and moderate	104	53	51	.661
Poor	26	12	14	
Lymphovascular invasion				
Positive	71	36	35	.86
Negative	59	29	30	
T stage				
T1 + T2	21	8	13	.233
T3 + T4	109	57	52	
N stage				
N0	81	37	44	.295
N1 + N2	49	28	21	
M stage				
M0	111	51	60	.025*
M1	19	14	5	
AJCC stage				
I + II	74	31	43	.034*
III + IV	56	34	22	

*p-Value <.05.

MDK, *TGFBIII*, *ATP2B4*, *DUSP3*, *PHF20* and *PLIN3*) and 8 downregulated genes (*NPNT*, *KRT20*, *PCSK7*, *SAMD4A*, *NEDD4L*, *SLC35A3*, *SPTLC2* and *DNMIL*) after ALKBH5 knock-down (Figure 7E).

Then, we examined the expression of these 19 genes after ALKBH5 knock-down. The results of RT-qPCR displayed that among the 11 upregulated genes, 5 genes (*CCDC69*, *LITAF*, *TGFBIII*, *PHF20* and *PLIN3*) were upregulated both

TABLE 2 Clinical characteristics of 114 colorectal cancer (CRC) patients according to alkB homologue 5 (ALKBH5) protein levels

	ALKBH5			p-Value
	All cases	Low expression	High expression	
Gender				
Female	47	26	21	.341
Male	67	43	24	
Age				
≤60	41	30	11	.038*
> 60	73	39	34	
Tumour size				
≤5	77	47	30	.872
> 5	37	22	15	
Histological type				
Adenocarcinoma	112	67	45	.249
Others	2	2	0	
Tumour differentiation				
Well and moderate	93	58	35	.398
Poor	21	11	10	
Lymphovascular invasion				
Positive	53	30	23	.424
Negative	61	39	22	
T stage				
T1 + T2	19	9	10	.199
T3 + T4	95	60	35	
N stage				
N0	62	33	29	.082
N1 + N2	52	36	16	
M stage				
M0	92	51	41	.042*
M1	22	18	4	
AJCC stage				
I + II	56	28	28	.024*
III + IV	58	41	17	

*p-Value <.05.

in ALKBH5-knock-down LOVO and RKO cells (Figure 7F). In addition, we discovered that among the eight downregulated genes, only *PCSK7* was downregulated both in ALKBH5-knock-down LOVO and RKO cells (Figure 7G). Next, we examined the correlation between ALKBH5 and the six candidate genes in the TCGA database. Our results indicated that only *PHF20* was negatively correlated with ALKBH5 in CRC (Figure 7H). We also verified elevated protein expression of *PHF20* in ALKBH5-knock-down

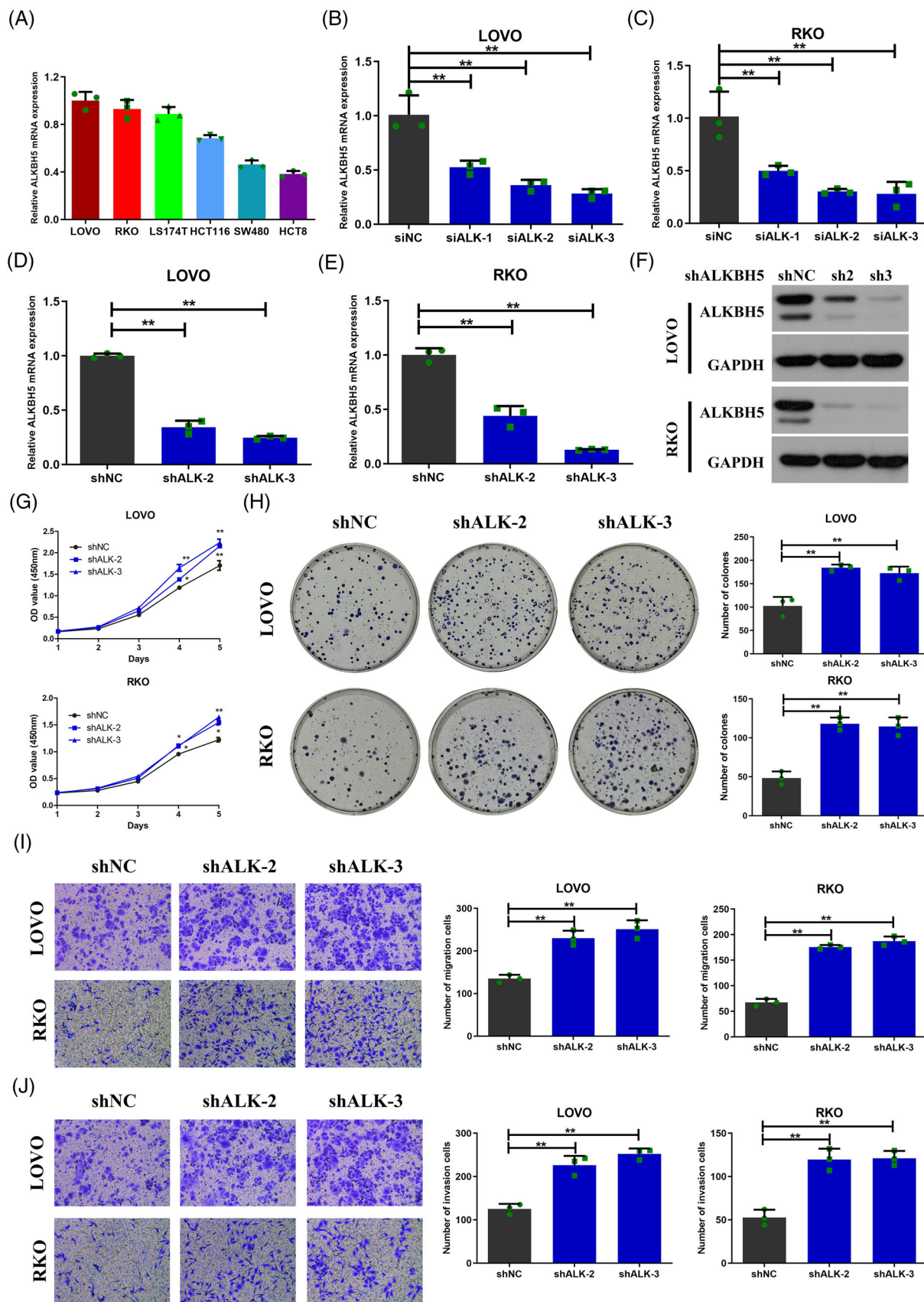


FIGURE 3 Knock-down of alkb homologue 5 (*ALKBH5*) enhanced proliferation, migration and invasion of colon cancer cells in vitro. (A) *ALKBH5* mRNA expression in five colon cancer cells. (B and C) *ALKBH5* mRNA expression in LOVO (B) and RKO (C) cells infected with three independent siRNAs targeting *ALKBH5*. (D and E) *ALKBH5* mRNA expression in LOVO (D) and RKO (E) cells infected with two

independent ALKBH5-knock-down lentiviruses. (F) ALKBH5 protein expression in LOVO and RKO infected with two independent ALKBH5-knock-down lentiviruses. (G and H) The proliferation ability of LOVO and RKO cells with ALKBH5 knock-down determined by CCK8 (G) and colony formation (H) assays. (I and J) The migration (I) and invasion (J) ability of LOVO and RKO cells with ALKBH5 knock-down, as determined by transwell assays. Statistical significance was determined using ANOVA (* $p < .05$, ** $p < .01$).

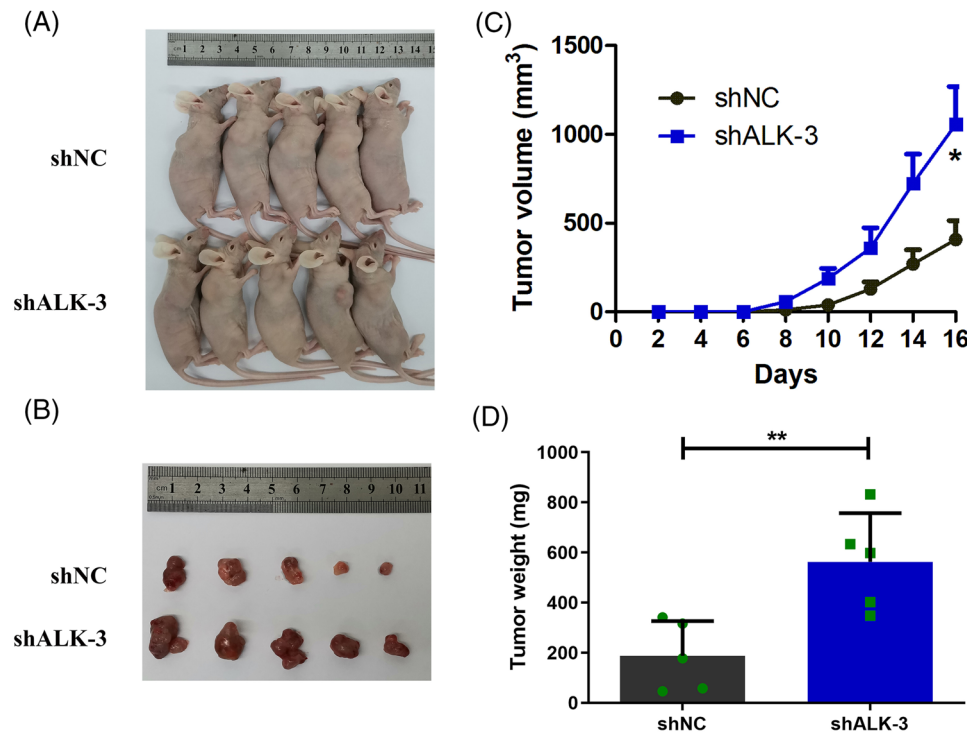


FIGURE 4 Knock-down of alkB homologue 5 (ALKBH5) enhanced the proliferation of colon cancer cells in vivo. (A and B) Tumour xenograft models were constructed using ALKBH5-knock-down LOVO cells ($n = 5$). (C) Tumour formation and size in the tumour xenograft model were monitored every 2 days. (D) Tumour weights were measured from sacrificed mice. Statistical significance was determined using t -test (* $p < .05$, ** $p < .01$).

cells compared to control cells by Western blot assays (Figure 7I). Moreover, visualization analysis showed that compared to normal tissues, the m⁶A peaks of *PHF20* mRNA were more abundant in tumour tissues (Figure 7J). Thus, *PHF20* may be a key molecule regulated by ALKBH5 through m⁶A modification.

3.6 | Loss of ALKBH5 increased the stability of *PHF20* mRNA to facilitate CRC progression

As a demethylase, ALKBH5 is able to remove m⁶A modification from mRNA. The m⁶A quantification assays of mRNA indicated that ALKBH5 loss significantly increased the m⁶A modification level of mRNAs (Figure 8A). The MeRIP-qPCR assays demonstrated that ALKBH5 loss significantly increased the m⁶A modification level of *PHF20* mRNA (Figure 8B). Next, we predicted the m⁶A sites of *PHF20* mRNA utilizing the SRAMP database

(<http://www.cuilab.cn/sramp>) and BERMP database (<http://www.bioinfo.org/bermp>). We discovered that the m⁶A methylation sites on *PHF20* mRNA were mainly located in the 3'UTR, and that there were eight possible m⁶A methylation sites. According to the m⁶A methylation sites on *PHF20* mRNA, we constructed a luciferase reporter vector (Figure 8C). The luciferase reporter assays illustrated that ALKBH5 loss significantly enhanced the expression of the wild-type *PHF20* 3'UTR plasmid, but that there was no significant effect on *PHF20* 3'UTR plasmid with m⁶A mutation sites (Figure 8D).

To assess the effect of ALKBH5 on *PHF20* mRNA stability, we treated colon cancer cells with actinomycin D and found that ALKBH5 loss improved stability of *PHF20* mRNA and prolonged its half-life (Figure 8E). Furthermore, our data showed that *PHF20* knock-down contributed to a significant inhibition of the proliferation, colony formation, migration and invasion of LOVO (Figure S2). Then we carried out rescue experiments to determine whether ALKBH5 had an effect on the

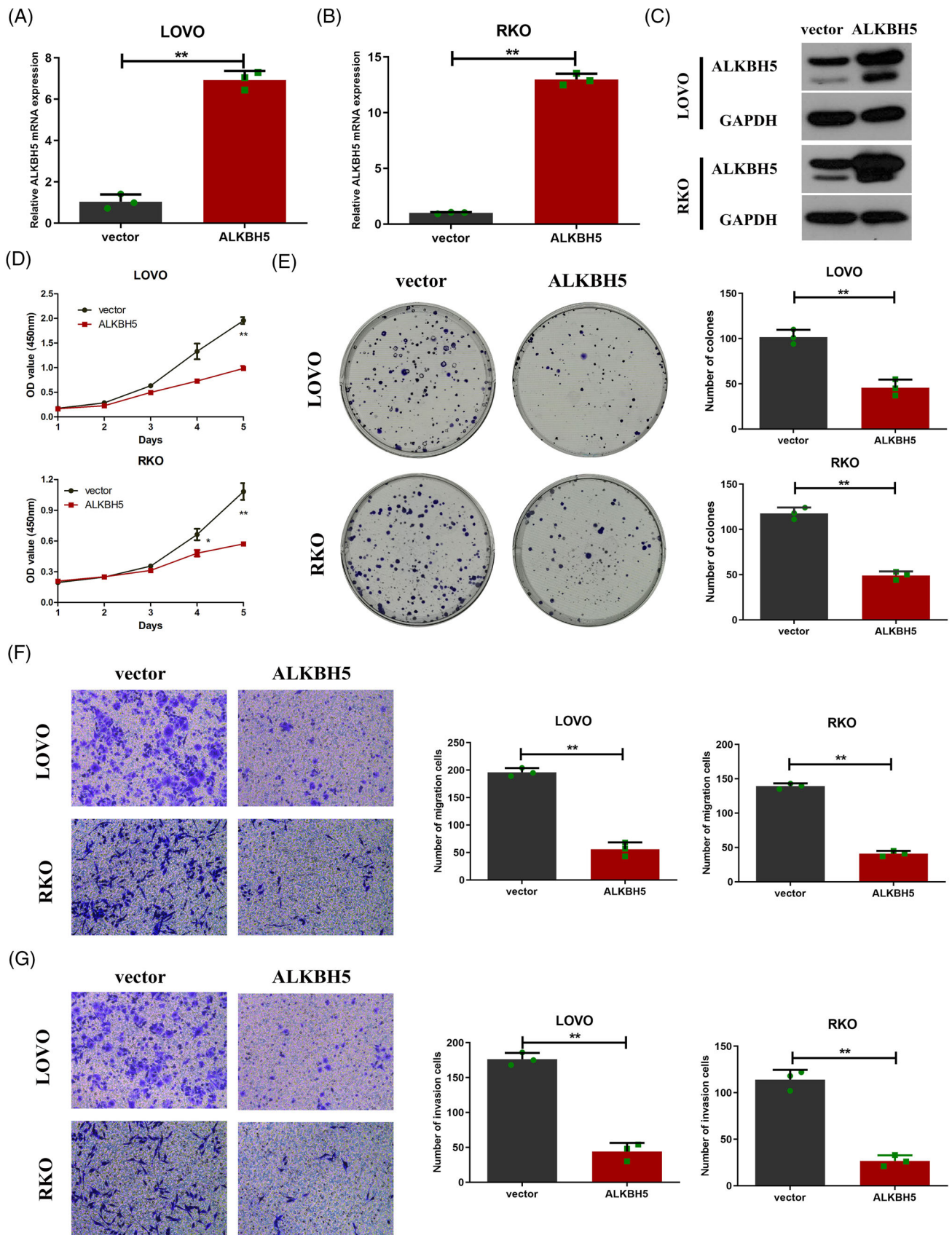


FIGURE 5 Overexpression of alkB homologue 5 (ALKBH5) inhibited proliferation, migration and invasion of colon cancer cells in vitro. (A and B) *ALKBH5* mRNA expression in LOVO (A) and RKO (B) cells infected with ALKBH5-overexpression or control vector lentiviruses. (C) ALKBH5 protein expression in LOVO and RKO cells infected with ALKBH5-overexpression or control vector lentiviruses. (D and E) The

proliferation ability of LOVO and RKO with ALKBH5 knock-down, as determined by CCK8 (D) and colony formation (E) assays. (F and G) The migration (F) and invasion (G) ability of LOVO and RKO cells with ALKBH5 knock-down, as determined by transwell assays. Statistical significance was determined using *t*-test (* $p < .05$, ** $p < .01$).

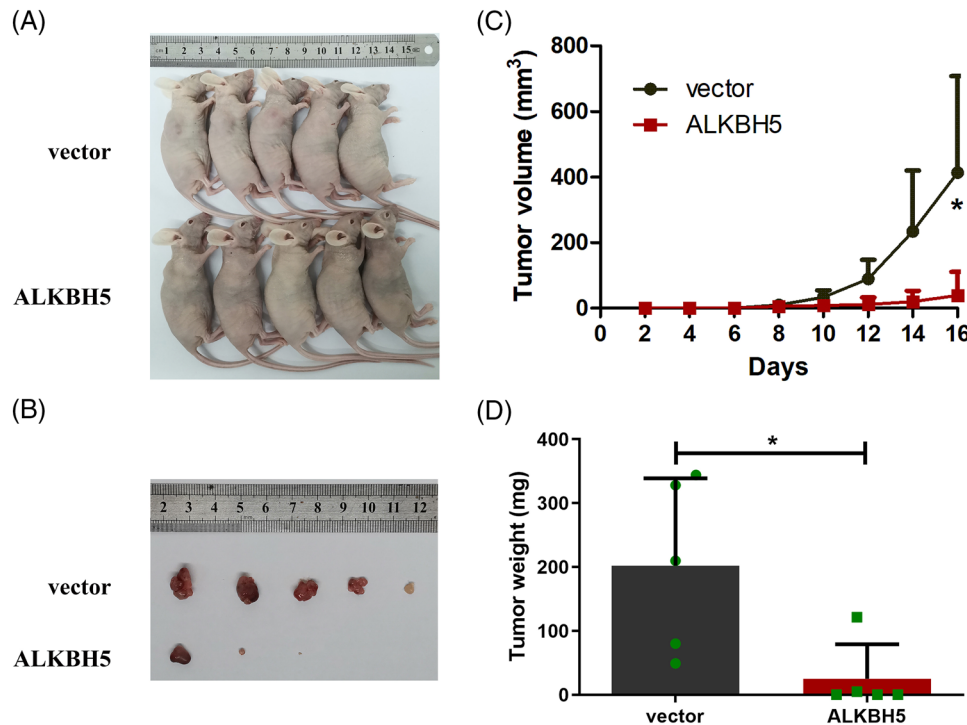


FIGURE 6 Overexpression of alkB homologue 5 (ALKBH5) inhibited the proliferation of colon cancer cells in vivo. (A and B) Tumour xenograft models were constructed using *ALKBH5*-overexpression LOVO cells ($n = 5$). (C) Tumour size and formation in the tumour xenograft models were monitored every 2 days. (D) Tumour weights were measured from sacrificed mice. Statistical significance was determined using *t*-test (* $p < .05$).

biological function of colon cancer cells by regulating PHF20. It showed that PHF20 knock-down inhibited the shALKBH5-mediated enhancement of the proliferation, colony formation, migration and invasion of LOVO and RKO (Figure 8F–I).

4 | DISCUSSION

M⁶A modification of RNA is a new research direction in epigenetics. Our previous study has first performed MeRIP-seq in CRC and discovered dysregulated m⁶A peaks of transcripts.¹⁴ Herein, the m⁶A quantification assays of mRNA revealed higher levels of mRNA m⁶A modification in CRC tumour tissues than that in adjacent normal tissues. It indicated that m⁶A modification regulated by methyltransferase and demethylase was dysregulated in CRC.

The discovery of demethylase is an important evidence of the reversible regulatory mechanism of m⁶A modification of RNA. So far, ALKBH5 is discovered as one of the two

m⁶A demethylases. ALKBH5 is a highly conserved homologue of the AlkB family of dioxygenases dependent on α -ketoglutaric acid and iron(II).⁷ The AlkB family is a class of repair enzymes that can directly reverse an alkylation damage of DNA or RNA through oxidative dealkylation to remove alkyl adjunct on the base.^{15,16} ALKBH5 specifically catalyses the removal of m⁶A modification on ssRNAs and is localized at the nuclear speckles, which is conducive to its direct interaction with mRNA substrates, regulating mRNA export, RNA metabolism and mRNA processing factor assembly in nuclear speckle.¹⁷

It is reported that ALKBH5 plays a vital and specific role in cancer regulation. *ALKBH5* functions as an oncogene in many cancers, including glioblastoma, breast cancer and esophageal squamous cell cancer.^{18–20} *ALKBH5* also functions as a suppressor gene in hepatocellular cancer, pancreatic cancer, bladder cancer and so on.^{21–24} With regard to CRC, there are contrasting effects of ALKBH5 across different studies. Guo et al. indicated that ALKBH5 may promote cancer cell motility by demethylating the lncRNA NEAT1.¹¹ In contrast, Yang et al. illustrated that in

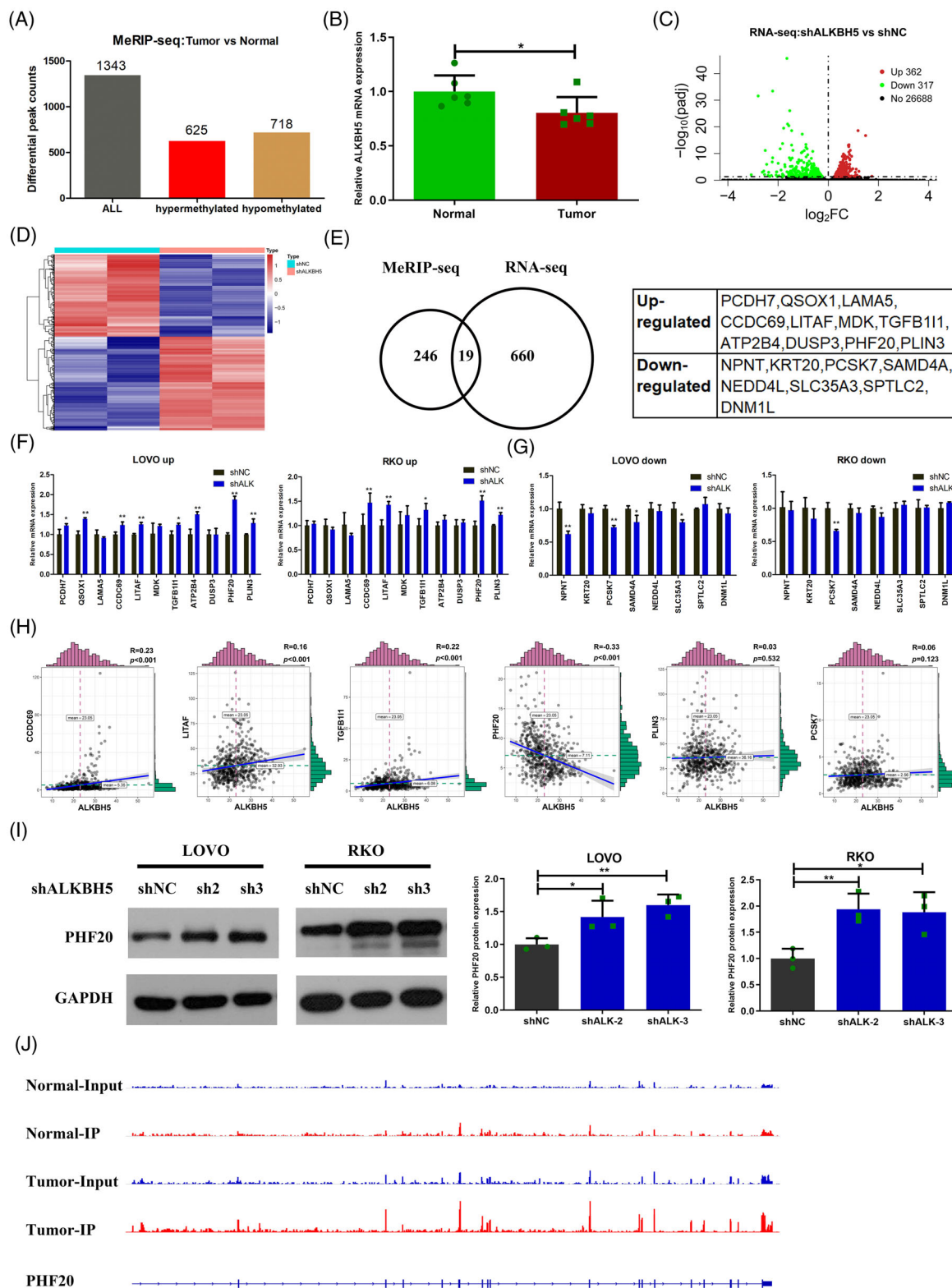


FIGURE 7 PHF20 was a downstream target of alkB homologue 5 (ALKBH5)-mediated N⁶-methyladenosine (m⁶A) modification. (A) MeRIP-seq results of tumour tissues and tumour-adjacent normal tissues showing the distribution of altered m⁶A peaks in six colorectal cancer (CRC) patients. (B) *ALKBH5* mRNA expression in the six paired tumour tissues and tumour-adjacent normal tissues. (C and D) Volcano plots (C) and heat map plots (D) indicating the differentially expressed genes in ALKBH5-knock-down cells, compared to control cells in RNA-seq. (E) Conjoint analysis of MeRIP-seq and RNA-seq data. (F) Validation of upregulated genes in ALKBH5-knock-down cells, compared to the control group. (G) Validation of downregulated genes in ALKBH5-knock-down cells, compared to the control group. (H) The relationship between ALKBH5 and six candidate genes (*CCDC69*, *LITAF*, *TGFB11*, *PHF20*, *PLIN3* and *PCSK7*) in The Cancer Genome Atlas (TCGA) database. (I) The protein expression of PHF20 in ALKBH5-knock-down and control cells. (J) Representative m⁶A peaks of *PHF20* mRNA in CRC. Statistical significance was determined using ANOVA (I) and *t*-test (B, F, G) (**p* < .05, ***p* < .01).

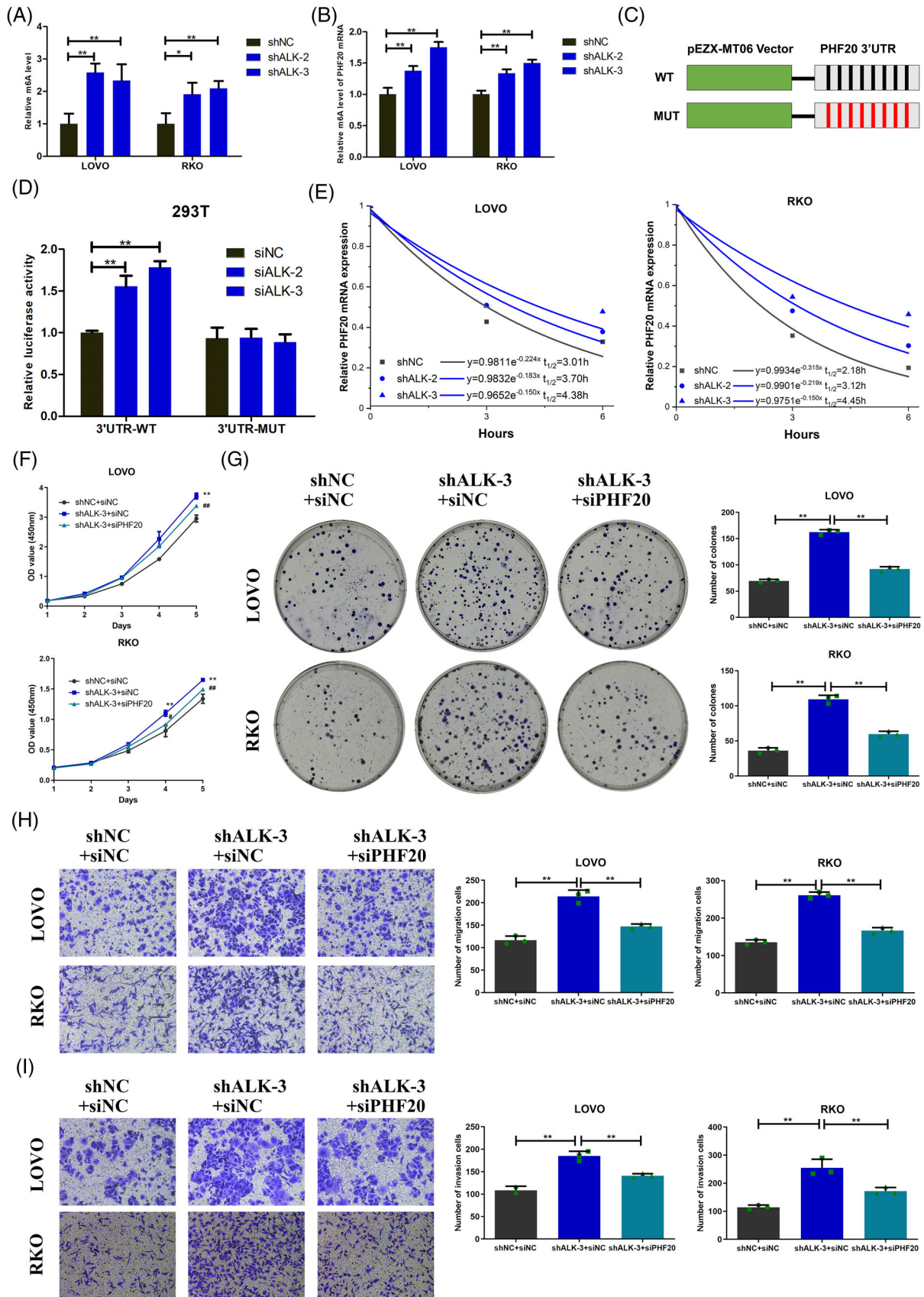


FIGURE 8 AlkB homologue 5 (ALKBH5) suppressed progression of colorectal cancer (CRC) by decreasing *PHF20* mRNA methylation. (A) Relative quantitative analysis of global N6-methyladenosine (m⁶A) levels of mRNA in ALKBH5-knock-down and control cells. (B) MeRIP-qPCR analysis of alterations in the m⁶A levels of *PHF20* mRNA in ALKBH5-knock-down and control cells. (C) Luciferase reporter

constructs containing the human PHF20 3'UTR that have m⁶A motifs or mutants (A–T mutation) m⁶A sites. Black represents A, and red represents T. (D) Relative luciferase activities of 293T cells that were co-transfected with plasmids containing wild-type or mutant PHF20 3'UTR and siRNAs containing siNC or siALKBH5. (E) RT-qPCR analysis of the decay rate of PHF20 mRNA after actinomycin D (5 μg/ml) treatment in LOVO or RKO cells after ALKBH5 inhibited. (F)–(I) The rescue experiment was utilized to determine whether ALKBH5 has an effect on proliferation (F and G), migration (H) and invasion (I) of LOVO and RKO cells by regulating PHF20. In (F), *: shALKBH5 + siNC versus shNC + siNC; #: shALKBH5 + siPHF20 versus shALKBH5 + siNC. Statistical significance was determined using ANOVA (**p* < .05, ***p* < .01, #*p* < .05, ###*p* < .01).

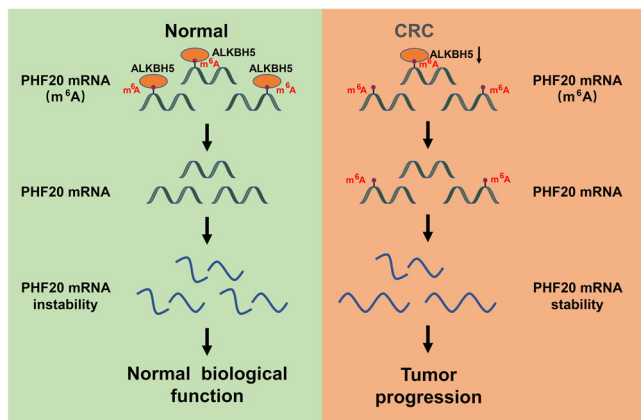


FIGURE 9 Schematic diagram of alkB homologue 5 (ALKBH5)-mediated removal of N6-methyladenosine (m⁶A) mRNA methylation, leading to the inhibition of *PHF20* mRNA stability and suppression of colorectal cancer (CRC) progression

CRC, ALKBH5 was downregulated and repressed tumour cell invasion in vitro and in vivo.¹² Previous studies were controversial, either drawing the opposite conclusion or with small samples validation. Therefore, a thorough and in-depth study is necessary. In our research, the regulatory mechanism of ALKBH5 was studied in depth from clinical samples to cellular mechanisms. We demonstrated that ALKBH5 was downregulated in a large CRC cohort and closely related to the poor prognosis of CRC patients. Furthermore, ALKBH5 was found to significantly inhibit the growth and metastasis of colon cancer cells in vitro and in vivo. These suggested the suppressive role of ALKBH5 in CRC.

The expression of ALKBH5 is not fully consistent across different tumours, which indicates the tissue-specific expression of ALKBH5. Therefore, it is necessary to explore downstream targets of ALKBH5. We comprehensively analysed the results of tissue sequencing and cell sequencing to screen out the downstream genes. Our data proved that PHF20 was an important downstream regulatory molecule of ALKBH5. ALKBH5 can remove the m⁶A modification of *PHF20* mRNA 3'UTR, leading to the inhibition effect on the stability of *PHF20* mRNA. IGF2BP1/2/3, as members of the m⁶A reading proteins, can recognize the m⁶A sites on 3' UTR of mRNAs and maintain RNA stability.²⁵ We knocked down IGF2BP1,

IGF2BP2 and IGF2BP3 in LOVO cells, respectively, and found that *PHF20* was decreased both in the mRNA and protein levels only when IGF2BP3 was knocked down by siRNA (Figure S3). Therefore, IGF2BP3 may be a reader protein targeting *PHF20* mRNA to reinforce its stability. We believe that after the m⁶A modification on *PHF20* mRNA is removed by ALKBH5, the function of IGF2BP3 recognizing the m⁶A modification and stabilizing *PHF20* mRNA is weakened.

PHF20 is a component of the H4K16 histone acetyltransferase MOF complex, which binds to methylated lysine residues on histones.^{26,27} Initially, PHF20 was identified as a tumour-associated antigen that elicited an immune response in the serum of glioblastoma patients.^{28–30} Furthermore, it was reported that PHF20 was highly expressed in many cancers, including nasopharyngeal cancer, lung cancer and CRC.^{31–36} PHF20 was upregulated in CRC, particularly in invasive CRC.³⁵ PHF20 reduced p53 accumulation and inhibited p53 transcriptional activity to p21 and Bax in response to DNA damage in CRC.³⁶ We found that PHF20 knock-down significantly suppressed the proliferation, colony formation, migration and invasion of LOVO. Moreover, our rescue experiments suggested that the knock-down of PHF20 inhibited the shALKBH5-mediated enhancement of colon cancer cellular functions in vitro. These results validate the tumour-promoting role of PHF20 in CRC.

5 | CONCLUSIONS

We determine that ALKBH5 expression is downregulated in CRC and plays a crucial tumour suppressive role. Mechanistically, ALKBH5 inhibits the stability of *PHF20* mRNA by removing the m⁶A modification (Figure 9). Our study contributes novel insights into the anticancer roles of ALKBH5-mediated m⁶A modification and suggests that targeting the ALKBH5-mediated m⁶A modification of *PHF20* mRNA can be a hopeful strategy for the intervention and treatment of CRC.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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