Clinical significance and biological role of KLF17 as a tumour suppressor in colorectal cancer

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Abstract. It has been reported that kruppel-like factor 17 (KLF17) acts as a tumour suppressor in several tissues and cancer cells, however, the molecular roles, the underlying mechanisms and clinical significance of KLF17 in colorectal cancer (CRC) have not been completely elucidated. In the present study, KLF17 protein expression was detected in 140 primary CRCs and paired adjacent non-tumour tissues using immunohistochemistry with tissue microarrays. The KLF17 mRNA expression was determined in 4 CRC cell lines and 20 pairs of the aforementioned tissues using reverse transcription quantitative polymerase chain reaction. The correlation between KLF17 expression and clinicopathologic characteristics was determined. Next, the functions of KLF17 in CRC were examined by cell proliferation, colony formation, adhesion, invasion and mouse xenograft assays. Methylation-specific PCR and bisulfite sequencing PCR were also carried out to investigate the promoter methylation status of KLF17 in CRC cells and tissues and explore the effects of lentiviral-mediated RNAi of UHRF1 on the methylation and expression of KLF17. The results revealed that KLF17 expression was abnormally decreased in CRC and associated with lymph node metastasis and unfavorable overall survival. Moreover, ectopic KLF17 expression suppressed CRC cell growth and invasion *in vitro* and *in vivo*. In addition, the downregulation of KLF17 was associated with the hypermethylation of the CpG nucleotides on the KLF17 promoter. The knockdown of the epigenetic regulator UHRF1 reduced the methylation level of the KLF17 promoter and inhibited CRC cell adhesion, invasion and epithelial-mesenchymal transition by upregulating KLF17. The present findings indicated that KLF17 may act as a tumour suppressor gene in CRC and a potential independent prognostic biomarker in CRC patients. UHRF1 can suppress KLF17 expression through the hypermethylation of its promoter in CRC. These results offer insights into the KLF17 expression regulation in CRC and suggest an inhibitory effect of KLF17 on tumourigenesis.

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Introduction

As one of the most prevalent types of cancer, colorectal cancer (CRC) is a considerable contributor to cancer mortality worldwide (1). In spite of recent improvements in surgical techniques, dosing and scheduling of adjuvant therapy, the overall prognosis of CRC patients varies substantially depending on tumour progression. Accordingly, elucidating the molecular mechanisms underlying tumour development, as well as identifying novel related factors, are crucial for the development of new diagnostic and therapeutic methods against CRC.

Kruppel-like factor 17 (KLF17) belongs to the KLF family of transcription factors, which consists of 17 members. The KLF family members play important roles in multifarious cellular processes, including tumour development (2). Recent studies have investigated the downregulation of KLF17 in multiple human malignancies, including breast, liver, stomach and esophageal cancer, suggesting the potential of KLF17 as a tumour suppressor in cancerous cells (3-6). Furthermore, KLF17 may be involved in the regulation of epithelial-mesenchymal transition (EMT), which has been identified as a critical process in the progression of cancer to the metastatic state (7). Moreover, KLF17 has been revealed to be regulated by epigenetic mechanisms such as DNA methylation, and identified as a possible prognostic biomarker for CRC (6,8). However, the clinical significance and regulatory mechanism of KLF17 remains unclear in CRC and needs to be further explored.

The aim of the present study was to explore the clinical significance of KLF17 expression in CRC and determine whether KLF17 inhibited CRC cell growth and metastasis, as well as the possible underlying molecular mechanism.

Materials and methods

Clinical samples and database. A total of 140 patients with CRC undergoing curative surgeries between 2014 and 2016 at the Tenth People's Hospital (Shanghai, China) were included in this study. No patients received preoperative chemotherapy or radiotherapy. The paired adjacent non-cancerous samples were obtained at a distance of at least 10 cm from the tumour lesion. Demographic and clinicopathological characteristics, including age, sex, tumour location, histologic differentiation and TNM stage, were collected based on the UICC criteria. Survival data were collected through follow-up visits and telephone interviews. This study was approved by The Ethical Committee of the Tenth People's Hospital.

Tissue microarray (TMA) construction and immunohistochemistry (IHC). The TMA slides included 140 CRC and matched adjacent non-cancerous tissues. All tissues were formalin-fixed and paraffin embedded, and diverted 2 mm cores from representative areas into recipient block microarrays.

For IHC, the sections were mounted onto slides, dewaxed in xylene, and then treated for 30 min with methanol containing 1% hydrogen peroxide to block endogenous peroxidases. A primary rabbit anti-human KLF17 polyclonal antibody (dilution, 1:100; cat. no. ab84196; Abcam) was used, according to the manufacturer's instructions. For immunostaining, a peroxidase-conjugated goat anti-rabbit secondary antibody (OriGene Technologies, Inc.) was used, according the manufacturer's instructions. IHC staining was assessed by two independent investigators under light microscopy. KLF17 expression was quantified by evaluating the percentage of positive tumour cells (9). Positive expression was defined as ≥5% stained cells in a sample (10).

Cell culture. The human colorectal cancer cell lines LoVo, SW620, HT29 and Caco-2 cells were purchased from the American Type Culture Collection. The HT29 cell line was recently authenticated in Microread Gene Technology by performing a short tandem repeat (STR) profiling analysis. All cells were cultured in recommended medium supplemented with 10% fetal bovine serum. For the demethylation experiments, 2 μM 5-Aza-dC (Merck KGaA) was added to the cell medium and the exponentially growing cells were treated for 72 h.

Lentiviral infection and transient transfection. The recombinant lentiviral vector pLV.0-KLF17 overexpressing KLF17 (lenti-KLF17) and control recombinant lentivector carrying a scramble construct and green fluorescent protein (lenti-GFP) were obtained from GeneCopoeia, Inc. RNA interference recombinant lentiviral vector for UHRF1 (lenti-shF1) was described in our previous study (11). Virus packaging was performed in 293T cells, following standard procedures, and viral supernatant was used to infect LoVo and SW620 cells. KLF17 small interfering RNA (si-F17) and scramble oligonucleotide used as a negative control (si-NC) were purchased from Thermo Fisher Scientific, Inc. and transfected into the Lenti-shF1-infected LoVo cells using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.) to knockdown the expression of KLF17.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). The mRNA level was quantified by RT-qPCR using the Quantitect SYBR Green PCR kit (Qiagen AB). β-Actin was used as the internal control to normalize the results. Primers used in this experiment were as follows: KLF17 forward, 5'-GCTGCCCAGGATAACGAGAAC-3' and reverse, 5'-ATC TCTGCGCTGTGAGGAAAG-3'; UHRF1 forward, 5'-CAA GATTGAGCGGCCGGGTGAAGG-3' and reverse, 5'-TGA GGGGCGGGTCCAGGCAGTAGA-3'; E-cadherin forward, 5'-CGAGAGCTACACGTTCACGG-3' and reverse, 5'-GGG TGTCGAGGGAAAAATAGG-3'; vimentin forward, 5'-AGT CCACTGAGTACCGGAGAC-3' and reverse, 5'-CATTTC ACGCATCTGGCGTTC-3'; β-actin forward, 5'-CCTGTA CGCCAACACAGTGC-3' and reverse, 5'-ATACTCCTGCTT GCTGATCC-3'. Amplification conditions were 94˚C for 2 min followed by 40 cycles of 94˚C for 45 sec, 60˚C for 40 sec and 72˚C for 40 sec. The $2^{-\Delta\Delta Cq}$ method was used to calculate the difference in expression (12).

Western blotting. Total protein was extracted from cells and tissues with the Total Protein Extraction kit (EMD Millipore), according to the manufacturer's instructions. The protein content was determined using a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories, Inc.) with bovine serum albumin as the standard. Proteins (20 μg) were separated by 8% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore). The membranes were blocked in 5% non-fat milk in Tris-buffered saline with 0.05% Tween-20 at room temperature for 2 h, then probed with antibodies against KLF17 (cat. no. ab 84196; 1:1,000 dilution), UHRF1 (cat. no. ab 57083; 1:2,000 dilution), E-cadherin (cat. no. ab 40772; 1:1,000 dilution; all from Abcam), vimentin (cat. no. 5741; 1:500 dilution; Cell Signalling Technology, Inc.) and β-actin (cat. no. A2228, 1:5,000 dilution; Sigma-Aldrich; Merck KGaA) overnight at 4˚C. After washing, membranes were incubated at 37˚C for 1 h with the horseradish peroxidase-conjugated secondary antibody (cat. no. A0208, 1:1,000 dilution; Beyotime Institute of Biotechnology, Haimen, China). The blotted proteins were visualized with an enhanced chemiluminescence (ECLTM plus Western Blotting Detection Kit; GE Healthcare) and images were obtained by using the chemiluminescent image analyzer (LAS-4000; Fujifilm). The density of protein band was quantified using Quantity One v4.4 software (Bio-Rad Laboratories). Relevant protein expression levels were defined as the ratio of the density of the specific band for a target protein to that of the band for β-actin.

Cell proliferation assay. Cell proliferation rates were detected by MTT assay. Cells ($5x10^3$) were seeded in 96-well plates and cultured for 24, 48, 72 or 96 h. MTT (20 μl) was then added into the plates and the cells were treated for another 2 h at 37˚C. Following the dissolution of the crystals using dimethyl sulphoxide (DMSO), the optical density was determined using a microplate reader (BioTek Instruments, Inc.).

Adhesion assay. CRC cells were placed on 24-well plates covered with fibronectin (BD Biosciences) and cultured for 24 h. The medium was then removed and the adherent cells were fixed and counted under a fluorescence microscope

(Olympus Corp.). Five random visual fields were selected on each insert at a magnification of x200.

Cell invasion assays. Next, $5x10^4$ cells were seeded in a Transwell chamber (Corning, Inc.) and covered with Matrigel (BD Biosciences). The chambers were then filled with complete RPMI-1640 medium and incubated for 24 h. The cells on the top surface of the membranes were removed, whereas those invading from the upper to the lower surface were fixed and stained with 0.1% crystal violet for 1 h at room temperature. Five random visual fields were captured at a magnification of x200.

Tumourigenicity and liver metastasis assay in nude mice. Six-week-old female athymic BALB/c nude mice (n=60; Chinese Academy of Sciences) were maintained under specific pathogen-free conditions, using a 10-h light/14-h dark cycle at a temperature of 25±1˚C and relative humidity of 40-60% with free access to food and water.

For the tumourigenicity assay, 30 mice were randomly divided into three groups on average and subcutaneously injected in the right flank with $1.5x10^6$ LoVo (blank), lenti-KLF17- or lenti-GFP-infected cells in 100 μl PBS. The length and width of the tumours was measured daily and the volume was calculated using the formula $V=(LxW^2)/2$. Tumour volumes were observed for 28 days and the growth curves of the tumours for each group were plotted. The mice were then euthanized by inhalation of a lethal dose of isoflurane (concentration of 5%) in a closed chamber and the resected tissues were collected for RNA detection and IHC.

A nude mouse model with CRC cells described previously was used for the liver metastasis assay (13). First, a total of 30 nude mice were randomly divided into three groups on average. After the mice were anesthetised by inhalation of isoflurane, a small left abdominal incision was performed to expose the spleen. Next, $1x10^6$ LoVo (blank), lenti-KLF17- or lenti-GFP-infected cells in 100 μl PBS were injected into the spleen of the mice from the three groups. Splenectomy was performed 10 min later, and the abdomen was closed. The mice were euthanized 6 weeks later, their livers were examined for metastasis and the resected tissues were collected for RNA detection and IHC.

All animal experiments were performed according to protocols approved by the Ethics Committee of Animal Experiments of the Tenth People's Hospital Affiliated to Tongji University.

DNA extraction and methylation analysis. The CGI analysis software (www.urogene.org) was used to find typical CGIs in the promoter region of KLF17. Two different methods were used to determine the DNA methylation status of the KLF17 CGI in CRC. For bisulfite sequencing PCR (BSP), the genomic DNA was bisulfite-converted using the EZ DNA methylation kit (Zymo Research, Corp.) and amplified using the following bisulfite PCR primers: 5'-TTTGTTGTTTAGGTTGGAGTG TAAT-3' and 5'-AATCACTTAAAATCAAAAATTTAAAAC C-3'. The PCR products were cloned into pMD-18T (Takara Bio, Inc.) and the sequencing was performed in 10 positive clones. QUMA analyser software was used to detect and analyse the sequencing data. For methylation-specific PCR (MSP), following treatment with the DNA methylation kit, the genomic DNA was amplified using the following primers: Methylated, 5'-TTTAGGTTGGAGTGTAATGGC-3' and 5'-ATTAACCAAACGTAATAACGCGTA-3'; Unmethylated, 5'-GTTGTTTAGGTTGGAGTGTAATGGT-3' and 5'-AAT TAACCAAACATAATAACACATA-3'.

Statistical analysis. Data are presented as the mean ± SD of at least three independent experiments. Comparisons between the two groups were performed by Student's t-test, Dunnett's t-test, or the Mann-Whitney U test, where appropriate, and comparisons among multiple groups were performed by a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. The relationship between the expression of KLF17 and clinicopathological features was calculated using χ^2 and Fisher's exact tests. Kaplan-Meier curves and log-rank analysis evaluated the effects of KLF17 expression on overall survival. Multivariate cox regression survival analysis with the stepwise backwards (Wald) method was used to discover independent prognostic factors. $P<0.05$ was considered to indicate a statistically significant difference. SPSS version 13 (SPSS, Inc.) was used for statistical analysis.

Results

Downregulation of KLF17 in CRC tissue samples and highly metastatic cells. Positive KLF17 staining by IHC was observed in the nucleus of the CRC (Fig. 1A-a and e) and adjacent non-cancerous cells (Fig. 1A-b and -f). The IHC staining scores of 140 CRC and matched adjacent non-cancerous tissues are included in Table SI. Among the 140 paired tissues, the percentage of KLF17 positive cells in CRC (25.7%, 36/140) was statistically lower than that in the adjacent non-cancerous samples (72.9%, 102/140; $P<0.001$).

Next, RT-qPCR was performed to detect the KLF17 mRNA expression in 20 paired CRC and adjacent non-tumour tissues; the results revealed a significantly decreased KLF17 expression in CRC tissues, as compared to the adjacent normal tissues (Fig. 1B). In addition, the highly metastatic CRC LoVo and SW620 cells exhibited a lower KLF17 expression, as compared to the lowly metastatic Caco- 2 and HT29 cells using RT-qPCR detection (Fig. 1C).

KLF17 expression is inversely correlated with lymph node metastasis and adverse prognosis in CRC patients. The relationship between KLF17 expression and patient characteristics is presented in Table I. The low expression of KLF17 was correlated with lymph node metastasis ($P=0.036$; Table I) in CRC patients.

Kaplan-Meier analysis revealed that the 5-year overall survival rate was higher in patients with a high KLF17 expression, as compared to patients with a low KLF17 expression (75 vs. 56.7%; $P=0.019$; Fig. 1D). Moreover, univariate and multivariate analysis revealed that KLF17 expression is an independent prognostic biomarker for CRC (RR: 0.444, 95% CI: 0.216-0.912, $P=0.027$; Table II).

Ectopic KLF17 expression inhibits cell growth, invasion and EMT in CRC cells. Considering that lower KLF17 levels were associated with worse outcomes in CRC patients, the function of KLF17 in CRC cells was subsequently evaluated. In

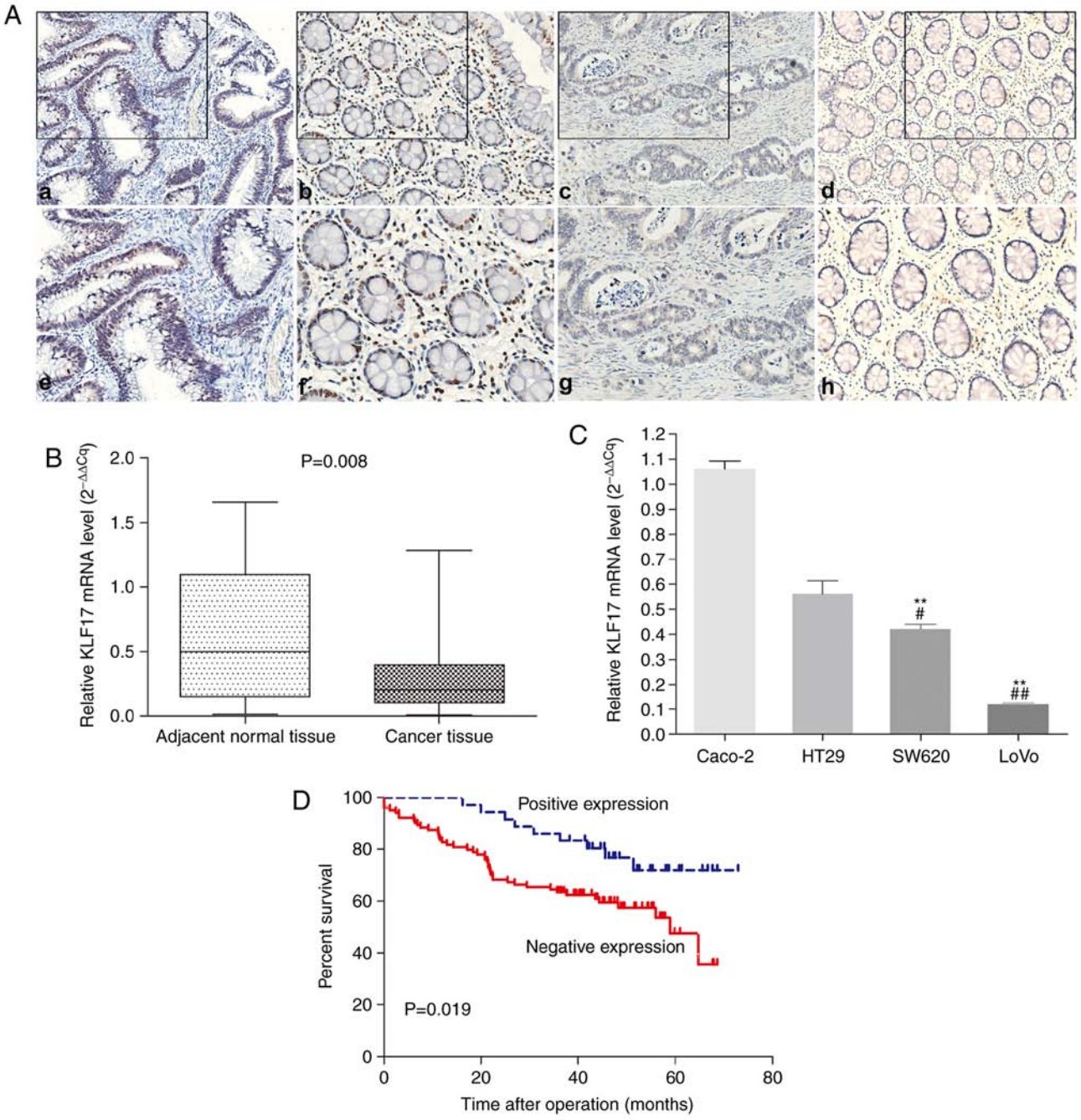

Figure 1. KLF17 expression is downregulated in CRC tissues and cell lines. (A) IHC staining of positive KLF17 expression in the nucleus of (a and e) CRC and (b and f) normal colonic epithelial cells, and negative KLF17 expression in (c and g) CRC and (d and h) normal colonic epithelial cells. (B) Quantification of KLF17 expression by RT-qPCR in matched CRC and adjacent non-tumour tissues. (C) Quantification of KLF17 expression by RT-qPCR in 4 CRC cell lines. **P<0.01 vs. Caco-2 cells; $^{\#}$P<0.05 vs. HT29 cells; $^{\#\#}$P<0.01 vs. HT29 cells. (D) Kaplan-Meier overall survival analysis based on the KLF17 expression levels of CRC patients. KLF17, kruppel-like factor 17; CRC, colorectal cancer.

accordance with the KLF17 mRNA level in different CRC cell lines (Fig. 1C), stable LoVo and SW620 cell lines were established through lentiviral infection that ectopically expressed KLF17.

As revealed in Fig. 2A-C, the expression levels of KLF17 were significantly increased in LoVo and SW620 cells following lentivirus-mediated KLF17 infection, as compared to cells infected with lenti-GFP control.

The MTT assays then revealed that the upregulation of KLF17 inhibited cell proliferation in LoVo and SW620 cells (Fig. 2D and E). Similarly, the potential of colony formation was substantially reduced in the KLF17-upregulated CRC cells (Fig. 2F and G).

To detect the effects of KLF17 expression on the invasion and metastasis capacities of these cells, adhesion and Transwell assays were performed and the results revealed that increased KLF17 expression significantly reduced the ability of adhesion and invasion in LoVo and SW620 cells (Fig. 2H-K).

KLF17 has been demonstrated to be a negative regulator of metastasis and EMT. It has been revealed to band directly to the promoters of genes such as E-cadherin and vimentin, which are involved in EMT, and inhibit their expression.

Table I. Association of KLF17 expression with clinicopathological features in CRC patients.

Clinicopathological features	No. of patients (%)	KLF17 expression		P-value
		Positive (n=36)	Negative (n=104)	
Age (years)				
<60	36	7	29	
≥60	104	29	75	0.381
Sex				
Female	56	18	40	
Male	84	18	64	0.244
Tumour location				
Right colon	49	14	35	
Left colon	48	10	38	
Rectum	43	12	31	0.632
Differentiation				
Well-Moderate	104	25	79	
Poor	36	11	25	0.508
Tumor size				
<5	82	17	65	
≥5	58	19	39	0.12
Depth of invasion				
T0-T2	12	3	9	
T3-T4	128	33	95	0.953
Nodal status				
N0	68	23	45	
N1-N2	72	13	59	0.036
Liver metastasis				
Absent	132	35	97	
Present	8	1	7	0.68
TMN stage				
0-II	65	18	48	
III-IV	75	18	56	0.703

KLF17, kruppel-like factor 17; CRC, colorectal cancer.

Therefore, the expression of certain EMT biomarkers was next investigated in stable LoVo and SW620 cells with KLF17 overexpression to evaluate the effects of KLF17 on EMT in CRC cells. As revealed in Fig. 3, ectopic KLF17 expression led to the upregulation of epithelial biomarker E-cadherin and the downregulation of mesenchymal biomarker vimentin, indicating that KLF17 represses EMT in CRC cells.

Ectopic KLF17 expression suppressed the tumour growth and metastasis of CRC in nude mice. In the following experiment, a nude tumour model was established to verify the *in vitro* findings as aforementioned. As revealed in Fig. 4A, the mouse group subcutaneously injected with the lenti-KLF17-infected LoVo cells exhibited a lower proliferation capacity, as compared to the control groups. The tumour volume in mice injected with lenti-KLF17-infected LoVo cells was considerably smaller than that in mice injected with LoVo cells or lenti-GFP-infected LoVo cells. At the end of the experiment, the tumour weight was significant lower in the group with lenti-KLF17-infected LoVo cells (0.118±0.021 g), as compared to the groups with LoVo (0.304±0.032 g) and lenti-GFP-infected LoVo (0.282±0.051 g) cells (Fig. 4B). Using antibodies against Ki-67, a standard biomarker for cellular proliferation, IHC was further performed, and the results revealed that lenti-KLF17-transfected LoVo cells displayed increased levels of KLF17 and decreased levels of Ki-67, as compared to the control groups (Fig. 4C).

In addition, RT-qPCR and western blotting were performed to detect the mRNA and protein levels in tumour specimens when the mice were euthanized (Fig. 4D-F). The increased KLF17 expression in lenti-KLF17-infected mice confirmed the successful transmission of KLF17 by lentivirus-mediated infection.

The tumour metastasis potential of lenti-KLF17-infected LoVo cells was analyzed using a nude mice model of metastasis. As revealed in Fig. 4G and H, more hepatic metastatic

Table II. Cox proportional hazard regression model analysis (n=140)

Variables	Categories	RR (95% CI)	Wald χ^2	P-value
Univariate analysis				
Age (years)	≥60 vs. <60	0.900 (0.496-1.633)	0.121	0.728
Sex	Male vs. female	0.840 (0.491-1.438)	0.404	0.84
Differentiation	Poor vs. well-moderate	1.273 (0.709-2.289)	0.653	0.419
Tumor size	≥5 vs. <5	1.056 (0.614-1.814)	0.038	0.845
Depth of invasion	T3-T4 vs. T0-T2	1.161 (0.419-3.217)	0.082	0.775
Nodal status	N1-N2 vs. N0	2.140 (1.212-3.779)	6.872	0.009
Metastasis to other organs	Present vs. absent	5.191 (2.309-11.670)	15.876	<0.001
TNM stage	III-IV vs. 0-II	2.257 (1.267-4.019)	7.639	0.006
KLF17 expression	Positive vs. negative	0.432 (0.211-0.887)	5.223	0.022
Multivariate analysis[a]				
KLF17 expression	Positive vs. negative	0.444 (0.216-0.912)	4.889	0.027
Metastasis to other organs	Present vs. absent	3.785 (1.634-8.771)	9.64	0.002
TNM stage	III-IV vs. 0-II	1.925 (1.058-3.505)	4.596	0.032

[a]Forward LR stepwise elimination procedure. KLF17, kruppel-like factor 17; LR, linear regression.

nodules were found in the lenti-GFP and blank control groups than in the lenti-KLF17 group. In addition, the higher KLF17 expression level in the lenti-KLF17-infected group confirmed that synthetic KLF17 was successfully delivered into the LoVo cells (Fig. 4I).

UHRF1-regulated promoter methylation suppresses KLF17 expression in CRC. It has been reported that KLF17 can be silenced by the methylation of its promoter (6), as the methylation of CpG islands (CGIs) in promoters suppresses reciprocation with transcription factors and inhibits gene expression (14). Therefore, it was investigated whether promoter methylation in CRC is associated with the downregulation of KLF17. Using CGI analysis software (www.urogene.org), a typical CGI in the promoter region of KLF17 was revealed (Fig. 5A).

To determine whether promoter methylation directly downregulated KLF17, LoVo cells were treated with methylation inhibitor 5-aza-dc, and then BSP analysis was performed. Following demethylation, it was observed that the methylation of KLF17 promoter decreased (Fig. 5B) and the expression of KLF17 was restored (Fig. 5C). MSP was next used to analyze the CGI methylation level of KLF17 in human CRC. As revealed in Fig. 5D, methylated PCR products were detected in 93.3% (28/30) and 53.3% (16/30) of CRC and paired normal mucosa specimens, respectively. The methylation level of CRC tissue samples was evidently higher than that of corresponding normal tissue samples (P=0.001).

In our previous study, UHRF1 was highly expressed in CRC and revealed to play an essential role in CRC carcinogenesis (11). Given the potential of UHRF1 as a DNA methylation regulator and that CGI exists on the KLF17 promoter, we questioned whether the overexpression of UHRF1 is an underlying mechanism of KLF17 DNA methylation in CRC. Lentiviral-mediated RNAi of UHRF1 was then carried out to knock down the UHRF1 expression of LoVo cells (lenti-shF1), and BSP analysis was performed to detect the CGI methylation status. As revealed in Fig. 5E and F, the depletion of UHRF1 decreased CpG methylation and elevated expression of KLF17 in CRC.

Rescue experiments were then performed to inspect whether UHRF1 promotes CRC progression through the downstream KLF17 gene using lenti-shF1-infected LoVo cells co-transfected with KLF17 small interfering RNA. It was revealed that the decreased adhesion, invasion and EMT of CRC cells induced by UHRF1 inhibition could be partially rescued by KLF17 silencing (Fig. 6). Collectively, the present results indicated that KLF17 may be a potent downstream gene of UHRF1 and its downregulation by UHRF1 can be caused by DNA methylation.

Discussion

The KLF transcription factor family proteins have vital functions in many physiological processes and cancer development, including proliferation, invasion and metastasis. Increasing evidence has revealed that KLF4, KLF6 and KLF9 were downregulated (15-17), but KLF5 upregulated in CRC specimens, as compared to normal epithelium specimens (18). Moreover, decreased KLF4 expression was correlated with lymph node metastasis and poor survival in CRC patients (19).

As an inhibitor of EMT and a potential tumour suppressor gene in several types of cancer (6,20-22), KL17 has been reported to be negatively correlated with a poor outcome in lung (23), liver (3), gastric (4) and papillary thyroid cancer (24).

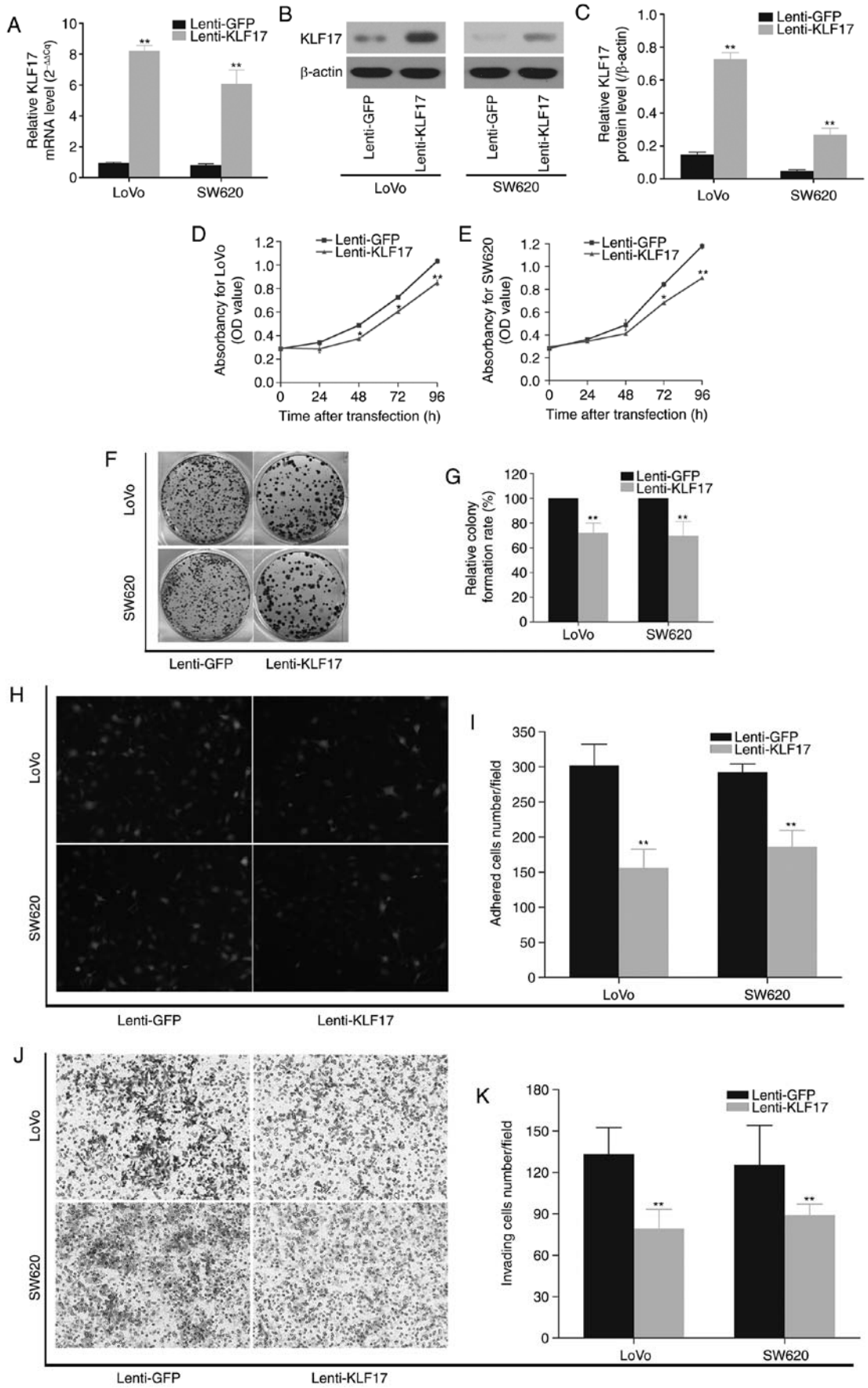

Figure 2. Ectopic KLF17 expression inhibits cell proliferation, invasion and metastasis in CRC cells. (A) RT-qPCR and (B and C) western blotting revealed an increased expression of KLF17 in CRC cells infected with lenti-KLF17. (D and E) MTT assay in (D) LoVo and (E) SW620 cells revealed that the infection of cells with lenti-KLF17 suppressed cell proliferation, as compared to the control group. (F and G) Colony formation was performed to analyse cell proliferation. Overexpression of KLF17 suppressed (H and I) adhesion and (J and K) invasion abilities in LoVo and SW620 cells. *P<0.05 and **P<0.01 vs. the lenti-GFP group. Results are presented as the mean ± SD from three independent experiments. KLF17, kruppel-like factor 17; CRC, colorectal cancer.

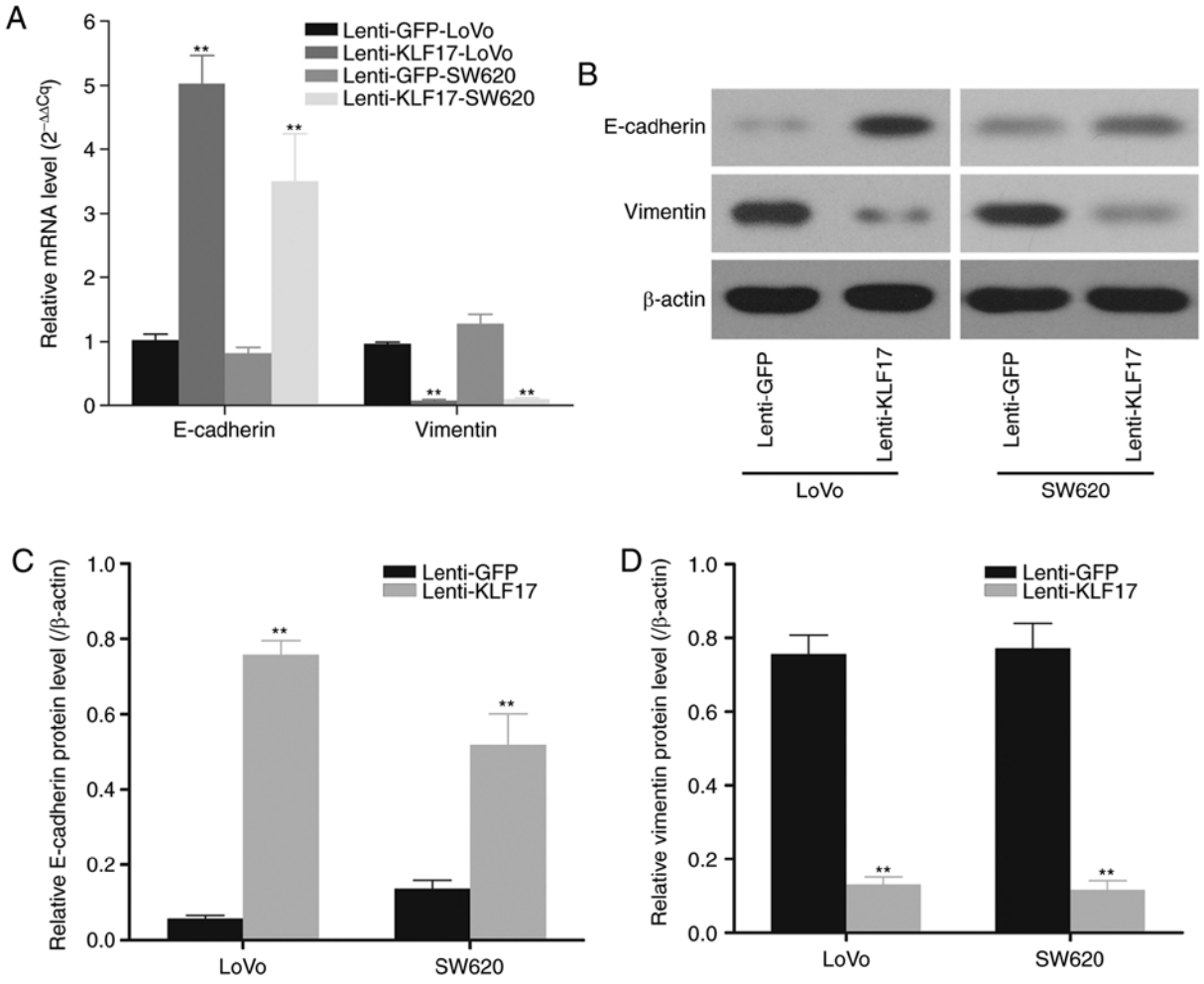

Figure 3. Ectopic KLF17 expression inhibits EMT in CRC cells. Quantification of E-cadherin and vimentin expression by (A) RT-qPCR and (B-D) western blotting in LoVo and SW620 cells infected with lenti-KLF17. Results are presented as the mean ± SD from three independent experiments. **P<0.01. KLF17, kruppel-like factor 17; EMT, epithelial-mesenchymal transition; CRC, colorectal cancer.

However, the impact of KLF17 on CRC development has not been fully elucidated. In the present study, it was demonstrated that KLF17 downregulation was associated with lymph node metastasis and may be an independent prognostic factor for CRC. In addition, a lower KLF17 expression was observed in the highly metastatic LoVo and SW620 CRC cell lines than that in the lowly metastatic CRC cell lines, indicating that KLF17 could play a negative regulatory role in CRC progression and metastasis.

Considering its higher transfection efficiency and more sustained long-term gene expression, lentivirus-mediated ectopic expression of KLF17 in 2 CRC cell lines was used in this study to further investigate the biological potential of KLF17 in the development of CRC (25). Our *in vitro* functional experiments revealed that KLF17 overexpression inhibited the proliferation and colony formation capacity of CRC cells. The *in vivo* tumourigenicity assay in nude mice verified that KLF17 overexpression led to a significant decrease in tumour growth in CRC. In an earlier study, Cai *et al* revealed that forced KLF17 expression in lung cancer cells inhibited the growth rate and colony formation in a time-dependent manner (23). In a later study, Ye *et al* reported that the loss of KLF17 enhances the proliferation of papillary thyroid cancer cells (24). Furthermore, Ali *et al* observed that ectopic KLF17 expression in breast cancer cells containing mutant p53 reduced cell proliferation, while the depletion of KLF17 promoted cell growth and decreased the apoptotic level of adriamycin-treated breast cancer cells (20).

A major cause of cancer mortality, metastasis, is a complex process consisting of multiple steps (26). EMT is considered the key process for cancer metastasis. During this cellular process, the epithelial features are lost and mesenchymal features are acquired for the epithelial cells, leading to the elimination of cell connection and an increase in cell migration and invasion (27). Using both mouse and cell models, Gumireddy *et al* (7) observed that KLF17 knockdown resulted in EMT and spindle-like and fibroblastic morphology of breast cancer cells. Furthermore, KLF17 has been revealed to inhibit EMT and cancer metastasis by controlling related genes (28). The lower KLF17 expression was associated with the alteration of EMT-related gene expression in HCC patients. Specifically, the depletion of KLF17 altered the expression of E-cadherin, vimentin and ZO-1 in HepG2 cells (29). Sun *et al* (3) revealed that KLF17 directly binds to the promoter of vimentin, ZO-1 and fibronectin, suggesting that KLF17 is an upstream regulator of those EMT-related genes.

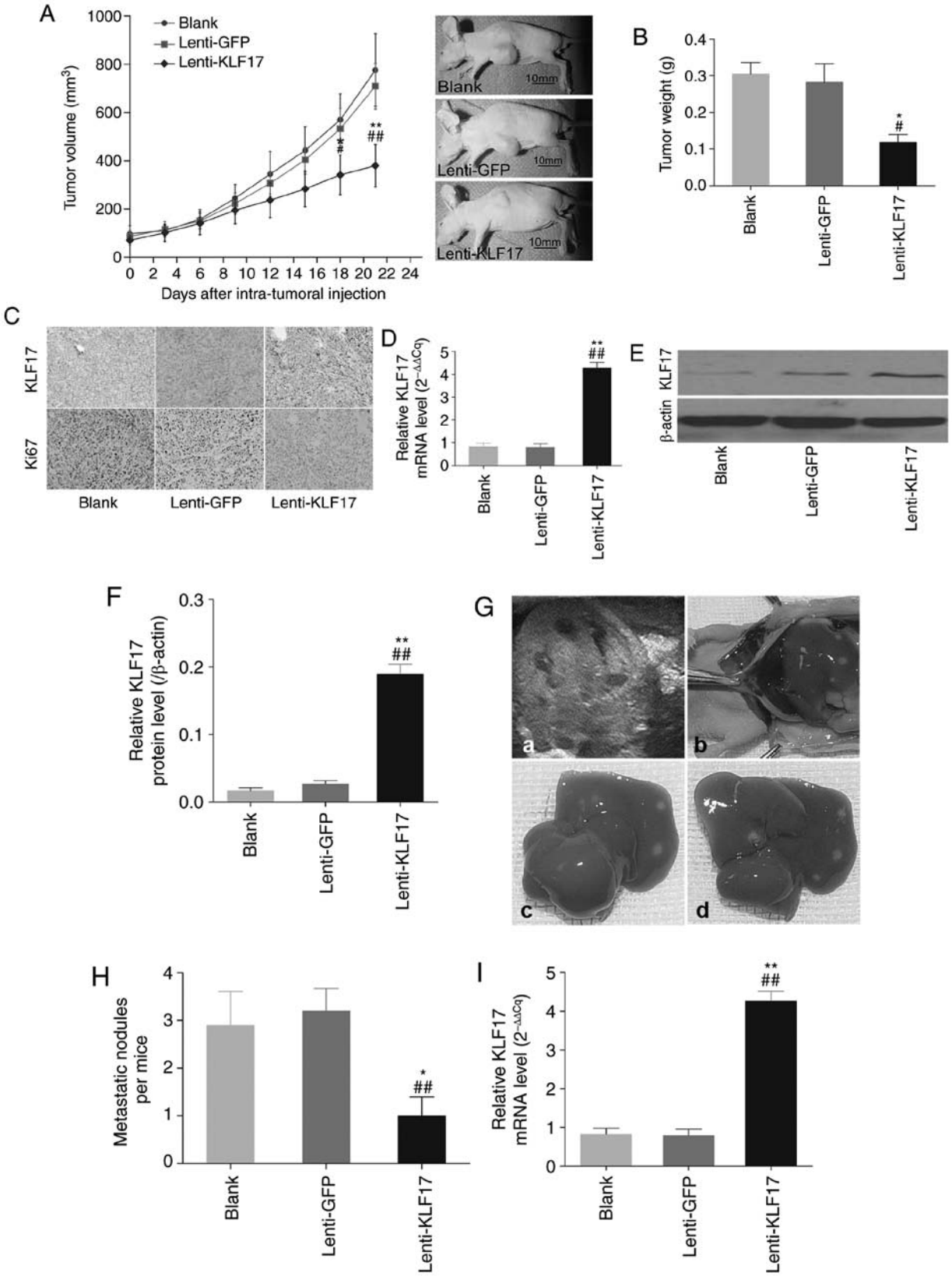

Figure 4. Ectopic KLF17 expression suppresses CRC cell proliferation and metastasis in nude mice. (A) The tumour volume growth curve of LoVo cells, lenti-GFP- or lenti-KLF17-infected LoVo cells. Over 18 days, lenti-KLF17 infection significantly inhibited tumour growth, as compared with the control groups. *P<0.05 and **P<0.01 vs. the blank group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. the lenti-GFP group. (B) Decreased tumour weights were observed in the lenti-KLF17 group, as compared with the other two groups. *P<0.05 vs. the blank group; $^{\#}$P<0.05 vs. the lenti-GFP group. (C) IHC analysis revealed increased KLF17 and decreased Ki-67 expression in the tumours of lenti-KLF17-infected LoVo cells, as compared to the other two groups. (D-F) Quantification of KLF17 expression via (D) RT-qPCR and (E and F) western blotting in the lenti-KLF17-infected group, as compared to other control groups. **P<0.01 vs. the blank group; $^{\#\#}$P<0.01 vs. the lenti-GFP group. (G-a) Ultrasonic image and (G-b-d) gross liver samples from LoVo liver metastatic nude mice. (b) Liver specimen *in vivo*. (c) Diaphragmatic surface of isolated liver specimen. (d) Visceral surface of isolated liver specimens. (H) Number of metastatic hepatic nodules in three mice groups. *P<0.05 vs. the blank group; $^{\#\#}$P<0.01 vs. the lenti-GFP group. (I) Relative KLF17 mRNA levels in three mice groups. **P<0.01 vs. the blank group; $^{\#\#}$P<0.01 vs. the lenti-GFP group. KLF17, kruppel-like factor 17; CRC, colorectal cancer; GFP, green fluorescent protein.

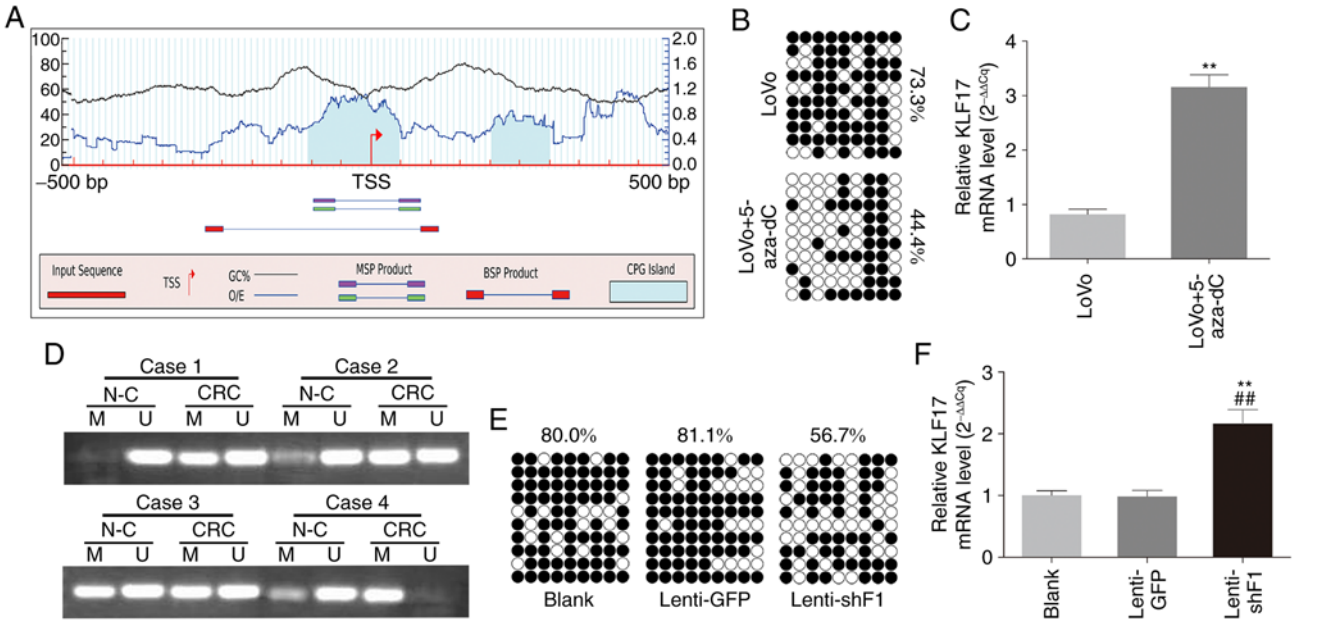

Figure 5. Promoter methylation regulates by UHRF1 suppresses KLF17 expression in CRC. (A) Predicted CGIs in the KLF17 promoter, with the positions of TSS. (B) Methylation levels of KLF17 CGIs detected by BSP in LoVo and 5-aza-dC-treated LoVo cells. Black circle, methylated; white circle, unmethylated. (C) Expression of KLF17 mRNA in LoVo and 5-aza-dC-treated LoVo cells. **P<0.01. (D) Methylation status of KLF17 promoter measured by MSP in CRC and corresponding normal mucosa. M, methylated; U, unmethylated. (E) Methylation levels of KLF17 CGIs detected by BSP in LoVo cells, lenti-GFP- or lenti-shF1-infected LoVo cells. Black circle, methylated; white circle, unmethylated. (F) The expression of KLF17 mRNA in LoVo cells, lenti-GFP- or lenti-shF1-infected LoVo cells. **P<0.01 vs. the blank group; $^{\#\#}$P<0.01 vs. the lenti-GFP group. Results are presented as the mean ± SD from three independent experiments. KLF17, kruppel-like factor 17; CRC, colorectal cancer; CGIs, CpG islands; BSP, bisulfite sequencing PCR; MSP, methylation-specific PCR; GFP, green fluorescent protein.

Following lentivirus-mediated forced KLF17 expression, two CRC cell lines exhibited reduced adhesion and invasion capacities. In addition, an *in vivo* liver metastasis model further confirmed the *in vitro* results, in which ectopic KLF17 expression significantly depressed LoVo cell metastasis to the liver. Major EMT markers were then analysed in CRC cells by RT-qPCR and western blotting. As anticipated, ectopic KLF17 expression suppressed vimentin (the mesenchymal marker) expression but promoted that of E-cadherin (the epithelial marker) in CRC cell lines. Collectively, these results indicated that KLF17 may act as a negative tumour regulator by suppressing EMT progression in CRC.

Given the potential diagnostic and prognostic value of KLF17 and its function as a tumour suppressor during CRC tumourigenesis, the mechanisms controlling KLF17 expression should be fully elucidated. In a previous study, Sachdeva *et al* observed the 5-Aza-dC-mediated induction of KLF3, a member of the KLF family, in soft tissue sarcomas. As the DNA methylation inhibitor, 5-Aza-dC induced KLF3 expression by three-fold. Subsequently, experiments detected conserved CGI in both mouse and human KLF3 promoters and discovered that the downregulation of KLF3 in sarcoma occurs due to promoter hypermethylation (30). Aberrant DNA methylation patterns are commonly observed in many types of cancer, including CRC. Cancer-specific CGI methylation blocks the initiation of gene transcription and affects many genes in CRC; these modifications were considered to be a key component of tumorigenesis (31). In the present study, a typical CGI was revealed in the promoter region of KLF17, and its methylation led to the suppression of KLF17 expression. In accordance with our findings, another study demonstrated that the methylation of CGI in the promoter of KLF17 by UHRF1 decreased KLF17 expression in breast cancer (6). These results suggested that the CGI in the promoter of KLF17 may be a cancer-related CGI, and the regulation of UHRF1 to the expression of KLF17 by methylation may be a crucial component in the mechanism underlying tumourigenesis not limited to CRC.

As a vital regulator of DNA methylation, UHRF1 has been revealed to be overexpressed and play an important role in the carcinogenesis of several types of cancer (32-34). In our previous study, UHRF1 expression was correlated with CRC progression and promoted the growth and metastasis of CRC (11). In the present study, it was further revealed that UHRF1 silences KLF17 expression through the methylation of CGI in the promoter of KLF17, and mediates cellular EMT and invasion in a KLF17-dependent manner in CRC cells. The results additionally elucidated the underlying mechanism of KLF17 downregulation and the regulation of UHRF1 in CRC carcinogenesis, invasion and metastasis.

Despite the considerable number of clinical specimens used in the present study, larger-scale prospective studies are required to further evaluate or confirm the potential of KLF17 as a novel biomarker for CRC. Moreover, additional extensive mechanistic studies are required to elucidate how KLF17 promoter methylation occurs and how KLF17 regulates downstream targets in CRC carcinogenesis and progression.

In summary, these data indicated that KLF17 is frequently silenced in CRC and may serve as a potential independent prognostic CRC biomarker. KLF17 reduced CRC EMT and suppressed tumour cell proliferation, adhesion, invasion and metastasis. Moreover, the knockdown of UHRF1 decreased the methylation level of the KLF17 promoter, triggered the

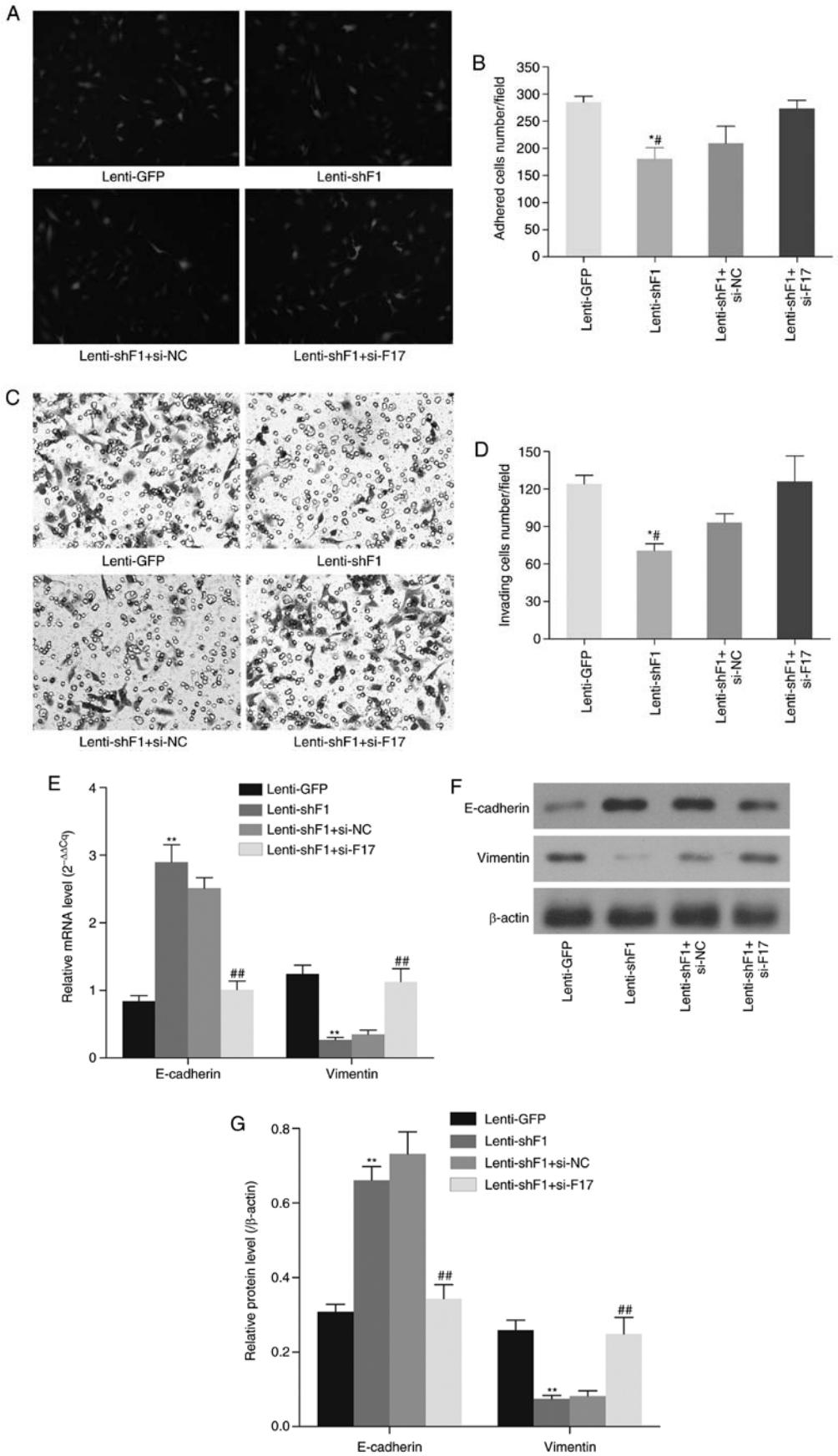

Figure 6. UHRF1 promotes adhesion, invasion and EMT in CRC through KLF17. (A) Representative images and (B) cell number record of an adhesion assay in the indicated cells. *P<0.05 vs. the lenti-GFP group; #P<0.05 vs. the lenti-shF1+si-F17 group. (C) Representative images and (D) cell count of invasion analysis in the indicated CRC cell lines. *P<0.05 vs. the lenti-GFP group; #P<0.05 vs. lenti-shF1+si-F17 group. (E) Quantification of E-cadherin and vimentin mRNA levels in the indicated CRC cell lines. **P<0.01 vs. the lenti-GFP group; ##P<0.01 vs. the lenti-shF1 group. (F and G) Quantification of E-cadherin and vimentin protein levels in the indicated CRC cell lines. Results are presented as the mean ± SD from three independent experiments. **P<0.01 vs. the lenti-GFP group; ##P<0.01 vs. the lenti-shF1 group. EMT, epithelial-mesenchymal transition; CRC, colorectal cancer; KLF17, kruppel-like factor 17; GFP, green fluorescent protein.

expression of KLF17 and reduced cell adhesion and invasion by inhibiting EMT in CRC. In combination, the present study offered insights into KLF17 expression regulation in CRC progression and suggested that making changes to this mechanism may represent a new therapeutic approach to blocking CRC development.

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Availability of data and materials

The data and materials used in the present study are available for research purposes.

Authors' contributions

FW and HQ designed the experiments and executed laboratory analysis. XJ, TYS, HL, CS and ZL conducted the experiments and contributed to the data collection. XJ, FW and HQ wrote the manuscript. All authors have approved the final version of the publication and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The research protocol was assessed and permitted by the Ethical Committee of the Tenth People's Hospital Affiliated to Tongji University. Written informed consent was obtained from all patients. All animal experiments were performed according to protocols approved by the Ethics Committee of Animal Experiments of the Tenth People's Hospital Affiliated to Tongji University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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