

# ARHGEF7 promotes metastasis of colorectal adenocarcinoma by regulating the motility of cancer cells

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**Abstract.** Previous studies have shown that Rho guanine nucleotide exchange factor 7 (ARHGEF7) is implicated in cytoskeleton remodelling, which is important for cell motility and invasiveness, and exhibits frequent high-level genetic amplification in metastatic lesions of colorectal adenocarcinoma. Therefore, it was hypothesized that ARHGEF7 may be involved in the metastasis of colorectal adenocarcinoma. In the present study, it was demonstrated that the expression level of ARHGEF7 was significantly upregulated in colorectal adenocarcinoma tumor tissues compared with matched nontumorous tissues, and its expression level correlated with colorectal adenocarcinoma metastasis. *In vitro* assays showed that the overexpression of ARHGEF7 in CRC cells significantly enhanced cell migration and invasion, whereas the knockdown of ARHGEF7 in colorectal adenocarcinoma cells significantly decreased cell migration and invasion. *In vivo* assays showed that the overexpression of ARHGEF7 in CRC cells facilitated tumor metastasis, whereas the knockdown of ARHGEF7 in CRC cells significantly inhibited tumor metastasis. Furthermore, it was demonstrated that ARHGEF7 promoted cell motility by regulating the actin cytoskeleton. Finally, according to ReMARK guidelines for reporting prognostic biomarkers in cancer, it was found that a high expression of ARHGEF7 was significantly correlated with lymph node, mesenteric and distant metastasis. Patients

with colorectal adenocarcinoma with a high expression of ARHGEF7 had shorter disease-free survival (DFS) and shorter overall survival (OS) rates, compared with those with a low expression of ARHGEF7, as determined by the Kaplan-Meier method with a log-rank test. Cox regression analysis showed that a high expression of ARHGEF7 was an independent risk factor for DFS and OS rates in colorectal adenocarcinoma.

## Introduction

Colorectal cancer (CRC) is the third most common type of cancer in men and women and is the fourth leading cause of cancer-associated mortality (1). The incidence of CRC is rapidly increasing in China, which is a serious threat to the health of the population (2). Colorectal adenocarcinoma accounts for 98% of cancer types among newly diagnosed cases. Advances in surgical technology and conception of treatment have been made, and optimization of treatment for patients with colorectal adenocarcinoma has led to an increase of survival rate at 5 and 10 years (3). However, metastasis remains the main cause of mortality and poor prognosis (4-6). The metastases produced by carcinomas are a result of a complex succession of the invasion-metastasis cascade (7). The fact that cancer cells gain cell motility and invasiveness is considered a critical step contributing to the metastasis of cancer (8). However, the mechanisms by which tumor cells become metastatic remain to be fully elucidated.

Rho guanine nucleotide exchange factor 7 (ARHGEF7), a guanine nucleotide exchange factor for Rho GTPases, functions in cell migration, attachment and cell spread (9-11). ARHGEF7 is implicated in cytoskeleton remodelling, which is important in cell migration (12,13). In other types of tumor, studies have shown that ARHGEF7 affects the motility of cells *in vitro* and invasion *in vivo* (14,15). Specially, it has been reported that the ARHGEF7 gene frequently exhibits high-level genetic amplification in metastatic lesions compared with primary sites in colorectal adenocarcinoma by SNP array (16). These data indicate that ARHGEF7 may be involved in colorectal adenocarcinoma metastasis. However, few investigations have focused on the role of ARHGEF7 in the metastasis of colorectal adenocarcinoma. Metastasis is the main contributor to the poor prognosis of patients (17).

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ARHGEF7 may be a prognostic biomarker and associated with colorectal adenocarcinoma metastasis.

The present study aimed to examine the expression of ARHGEF7 in colorectal adenocarcinoma. The role of ARHGEF7 in colorectal adenocarcinoma metastasis and its underlying molecular mechanism were examined using a series of *in vitro* and *in vivo* assays. Finally, whether the expression of ARHGEF7 is clinically relevant in patients with colorectal adenocarcinoma was determined according to ReMARK guidelines for the reporting of prognostic biomarkers in cancer (18).

## Materials and methods

**Colorectal adenocarcinoma samples.** The present study was approved by the Ethics Committee of the Institutional Review Boards of the First Affiliated Hospital of Nanchang University (Nanchang, China) and Jiangxi Pingxiang People's Hospital (Pingxiang, China). Prior informed consent was obtained from all participants, and the study was performed in accordance with the Declaration of Helsinki and current ethical guidelines. Firstly, 30 pairs of frozen fresh colorectal adenocarcinoma tumor tissues and corresponding nontumorous colorectal tissues (NCTs) from 30 patients were collected, and another five matched liver metastatic nodules (LMNs), tumor tissues and NCTs from five patients were collected following surgical resection at the Department of General Surgery, the First Affiliated Hospital of Nanchang University between July 2016 and January 2017. These tissues were used to screen the mRNA and protein expression of ARHGEF7. Secondly, another two sets of samples were used for prognostic analysis according to ReMARK guidelines for reporting prognostic biomarkers in cancer (18). Formalin-fixed, paraffin-embedded paired colorectal adenocarcinoma samples (including tumors and NCTs) obtained from 180 patients undergoing radical surgical resection at the Department of General Surgery, the First Affiliated Hospital of Nanchang University between January 2007 and December 2009 were designated as the training set. Another sample cohort containing 150 samples, including tumors and NCTs, from patients who underwent resection between July 2007 and July 2010 at the Department of General Surgery, Jiangxi Pingxiang People's Hospital was designated as the validation set. The inclusion criteria for the samples enrolled in the study were as follows: Collection from patients with sporadic CRC, histopathologically diagnosed as adenocarcinoma by hematoxylin and eosin (H&E) staining; had completed clinicopathologic and follow-up data; were without neoadjuvant chemotherapies or distant metastasis prior to surgery. Patients with hereditary CRC were excluded from the study.

**Prognostic evaluation.** All patients were regularly followed-up by trained and experienced researchers. The follow-up period was defined as the interval between the date of surgery and that of the patient's mortality or distant metastasis or the last follow-up. The median follow-up was 62.3 months (range 6.0-100.0 months) for the training set and 60.0 months (range 6.4-100.0 months) for the validation cohort. Patient mortality from other causes were treated as censored cases. Following surgery, all patients had regular

clinical examination, serial monitoring of carcinoembryonic antigen (CEA) levels at 1-month interval, had computed tomography (CT) or magnetic resonance imaging (MRI) scan at a 3-month interval, and had investigation by colonoscopy at the 1-year interval. Recurrence or metastasis was diagnosed by clinical examination, serial CEA level, and CT or MRI or positron emission tomography-CT. Disease-free survival (DFS) was defined as the length of time following resection during which a patient survived without signs of recurrence or metastasis. Overall survival (OS) was defined as the interval between surgery and mortality, or between surgery and the last observation for surviving patients. Data for conventional clinical and pathological variables were also collected for analysis, including sex, age, serum CEA level, tumor differentiation, tumor site, tumor size, tumor grade, lymphatic vessel/vessel/neuron infiltration, mesenteric tumor deposit formation, and tumor-node-metastasis (TNM) stage. The follow-up data were regularly updated in the database for each patient. Patients alive at the end of follow up or those who succumbed to mortality from causes without sign of recurrence or metastasis were censored.

**Cell lines.** The FHC normal colorectal mucosal cell line, and the HCT116, HT-29, SW480, SW620, LoVo cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). Short tandem repeat (STR) DNA fingerprinting was used to authenticate all cell lines prior to commencement of the study. All cell lines were routinely cultured with RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare Life Sciences, Logan, UT, USA), and maintained in a 5% CO<sub>2</sub> humidified incubator at 37°C.

**Vector construction and transfection.** The lentiviral vector (LV) encoding short hairpin RNAs (shRNAs) for ARHGEF7 knock-down, and the LV encoding the ARHGEF7 gene were purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The sequences of four shRNAs for ARHGEF7 knockdown were as follows: ARHGEF7-shRNA-Seq1, sense, 5'-ACAAGTCC TCAGTTCCTTAGTGACTCTA-3'; ARHGEF7-shRNA-Seq2, sense, 5'-CCACCATAAAGCCTCATTCAGTGCCATCT-3'; ARHGEF7-shRNA-Seq3, sense, 5'-CCTGAACGGAAGCCT TCAGATGAGGAGTT-3'; and ARHGEF7-shRNA-Seq4, sense, 5'-TACGGCCATTGCAGACCAGTGAGAAGTTA-3'. The LoVo cells were transfected with LVs encoding the shRNAs, and the HCT116 cells were transfected with LVs encoding the human ARHGEF7 gene. An empty vector was used as the negative control and was designated as LV-control. The LV vectors were transfected into the CRC cells with an appropriate multiplicity of infection of 50. At 48 h post-transfection, 3.0 µg/ml puromycin (OriGene Technologies, Inc.) was added, and the cells were incubated for 2 weeks to select the stably transfected cells. The overexpression or downregulated expression of ARHGEF7 was confirmed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analyses. The inhibitory efficiencies of the four shRNAs were validated and ARHGEF7-shRNA-Seq2 was used for subsequent experiments due to highly effective inhibition of the expression of ARHGEF7 in LoVo cells.

**RT-qPCR analysis.** Total RNA was extracted from the cell lines ( $\sim 1 \times 10^7$  cells) or fresh frozen tumor specimens (50-100 mg) using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The RNA was then reverse transcribed to obtain cDNA using the universal cDNA synthesis kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's protocol. The aliquots of double-stranded cDNA were subjected to RT-qPCR. RT-qPCR analysis was performed using the SYBR<sup>®</sup>-Green Realtime PCR Master Mix assay kit (Toyobo Co., Ltd.) according to the manufacturer's protocol; the assay was performed on a PRISM 7300 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The cycling parameters were as follows: Initial denaturation at 95°C for 2 min, followed by 95°C for 15 sec, 55-60°C for 15 sec and 72°C for 15 sec for 50 cycles, and a final extension step at 72°C for 5 min. The primers for ARHGEF7 were as follows: Forward, 5'-CGCAAACCTGAACGGAAGCCTT-3' and reverse, 5'-GTTTTGGCGCTGGTGCAGTAAG-3'. GAPDH was used as a control using the following primers: Forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and reverse, 5'-TGGTGAAGACGCCAGTGGGA-3'. The results were analyzed using the  $2^{-\Delta\Delta C_q}$  method with the following formula:  $\Delta\Delta C_q = \Delta C_{q_{\text{tumor}}} - \Delta C_{q_{\text{NCT}}}$ ;  $\Delta C_q = C_{q_{\text{ARHGEF7}}} - C_{q_{\text{GAPDH}}}$  (19).

**Western blot analysis.** Total proteins were extracted using RIPA lysis buffer. Protein concentration was then determined using the bicinchoninic acid method, and equal quantities of protein (20-60  $\mu\text{g}$ ) from each sample were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes (EMD Millipore, Bedford, MA, USA). The blotted membranes were blocked with 5% skimmed milk for 30 min at 25°C and incubated with primary antibodies (mouse anti-ARHGEF7; 1:400; cat. no. sc-136035; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and then an appropriate HRP-conjugated goat anti-mouse secondary antibody (1:1,000; cat. no. 04-18-06; KPL, Inc., Gaithersburg, MD, USA) in order. Bands were detected with enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc.).  $\beta$ -actin protein was also determined using the specific mouse anti- $\beta$ -actin antibody (1:1,000; cat. no. A1978, Sigma; EMD Millipore) as a loading control. Protein expression levels were quantified using BandScan software 4.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and defined as the ratio of target protein relative to  $\beta$ -actin.

**Immunohistochemistry (IHC).** The detailed IHC procedures were performed as described previously (20). The paraffin-embedded tissues were sectioned into 4- $\mu\text{m}$  slides. These slides were dewaxed, tissues were rehydrated, and antigen retrieval was performed using microwave-pretreated EDTA buffer (1 mM; pH 8.0) for 10 min. Following blocking, the slides were incubated for ARHGEF7 antibody (mouse anti-ARHGEF7 1:200; cat. no. sc-136035; Santa Cruz Biotechnology, Inc.) at 4°C overnight. The slides were incubated with biotin-labeled secondary and streptavidin-peroxidase (cat. no. SP-9002; Zhongshan Goldenbridge Biotechnology Co., Ltd., Beijing, China) for 30 min at 37°C. The samples were developed using 3,3'-diaminobenzidine substrate (cat. no. ZLI-9018) and counterstained with hematoxylin (cat. no. ZLI-9609) (both from Zhongshan Goldenbridge Biotechnology Co., Ltd.). Negative

controls were without primary antibody incubation during the procedure. The staining was determined in a blinded manner by two independent pathologists. The final immunostaining score (IS) was defined by the consistency of the grading by two pathologists. The expression levels of ARHGEF7 were scored based on staining intensity (SI) and percentage of positive cells (PP) using the IS as described previously (21). The SI was classified into four grades: 0, negative; 1, weak; 2, moderate; 3, strong. The PP was defined into five categories: 0, 0% positive cells; 1, 0-25% positive cells; 2, 25-50% positive cells; 3, 50-75% positive cells, and 4, 75-100% positive cells.  $IS = SI \times PP$ .

**Cell proliferation and colony formation assays.** Methyl thiazolyl tetrazolium (MTT) assays were used to determine the level of cell proliferation. For the MTT assays,  $5 \times 10^3$  cells were seeded into each well of 96-well plates. Three repeated wells for each group were detected every time. Fresh medium (100  $\mu\text{l}$ ; 0.5 mg/ml) containing MTT (Sigma; EMD Millipore) was added into each well and incubated at 37°C for 4 h. The medium was then replaced with 100  $\mu\text{l}$  DMSO and shaken at room temperature for 10 min. The absorbance was measured at 570 nm. For the colony formation assays, 500 cells were seeded into 35-mm dishes (Corning Incorporated, Corning, NY, USA) and cultured in 5% CO<sub>2</sub> for 2 weeks at 37°C. The number of colonies per dish was counted following staining with crystal violet (Beyotime Institute of Biotechnology, Jiangsu, China). Only positive colonies (diameter >40  $\mu\text{m}$ ) in the dishes were counted by inverted microscope (TE-2000S; Nikon Corporation, Tokyo, Japan) and compared (22). These experiments were performed in triplicate.

**Transwell assays.** Transwell migration and invasion assays were used to determine cell motility and invasion ability separately. For the Transwell invasion assays, the upper chamber of the insert was plated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). For the Transwell migration assays, the upper chamber of the insert was without Matrigel. Briefly, following preincubation with Mitomycin-C (10  $\mu\text{g}/\text{ml}$ ) for 1 h at 37°C to suppress cell proliferation,  $\sim 1 \times 10^5$  cells in serum-free medium were placed into the upper chamber of the insert. Following incubation in 5% CO<sub>2</sub> at 37°C for 24 h, the cells in the upper chamber were removed with cotton swabs, fixed in 20% methanol, and then stained with a solution containing 0.1% crystal violet (Beyotime Institute of Biotechnology). The number of cells that adhered to the lower membrane of the inserts was counted. For each experimental group, the assays were performed in triplicate, and five random fields of view were selected for analysis.

**Adhesion assay.** Cell-extracellular matrix (ECM) adhesion and cell-cell adhesion assays were used to analyze the adhesive ability of the cells. For the cell-ECM adhesion assay, a 96-well plate was coated with fibronectin at 37°C for 1 h and washed twice with washing buffer (0.1% BSA in DMEM; HyClone; GE Healthcare Life Sciences). The plates were blocked in blocking buffer (0.5% BSA in DMEM) at 37°C in a CO<sub>2</sub> incubator for 60 min. The cells (100  $\mu\text{l}$ ) at a density of  $\sim 1 \times 10^5/\text{ml}$  were added into each well of a 96-well plate and cultured at 37°C. Five wells for each group were detected at

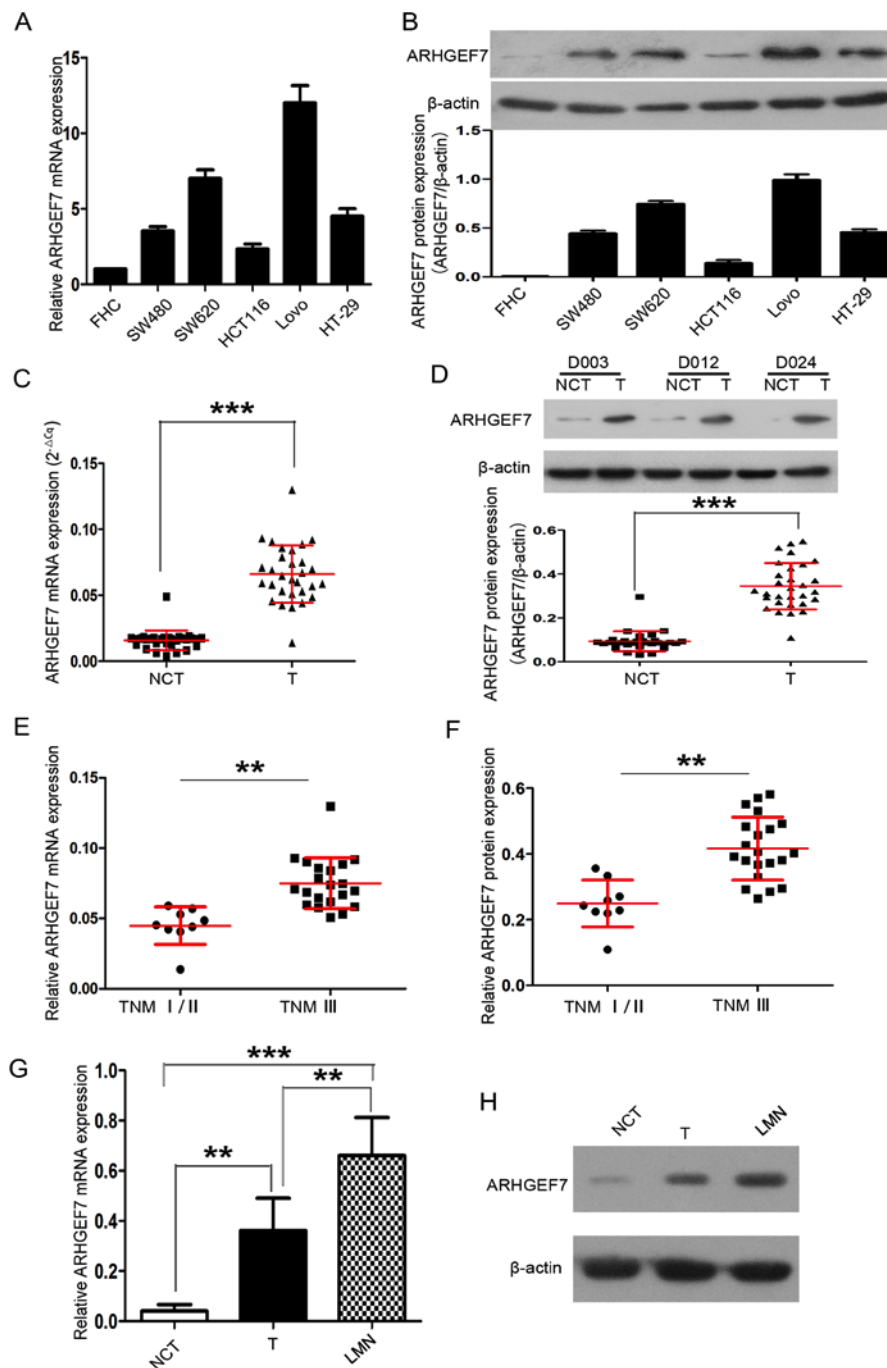


Figure 1. ARHGEF7 is overexpressed in colorectal adenocarcinoma and associated with metastasis. ARHGEF7 was significantly upregulated in colorectal adenocarcinoma cell lines. (A) RT-qPCR analysis of ARHGEF7 mRNA showed that, compared with the FHC normal colorectal mucosal cell line, mRNA expression of ARHGEF7 was elevated in HCT116, HT-29, SW480, SW620, LoVo colorectal adenocarcinoma cell lines. (B) Western blot results showed ARHGEF7 protein was overexpressed in HCT116, HT-29, SW480, SW620 and LoVo cells relative to FHC cells. Expression of ARHGEF7 was significantly upregulated in colorectal adenocarcinoma tissues. (C) RT-qPCR was used to analyze mRNA expression of ARHGEF7 in colorectal adenocarcinoma T tissues (n=30) and corresponding NCTs (n=30). RT-qPCR results showed that the mRNA expression level of ARHGEF7 was significantly higher in T tissues than in NCTs. (D) Western blot results showed that the expression of ARHGEF7 was higher in T tissues than in NCTs. Upper panel: Representative western blot of randomly selected samples (D003, D012 and D024); lower panel: Scatter plot for ARHGEF7 expression in T tissues and NCTs. (E) mRNA and (F) protein expression of ARHGEF7 in colorectal adenocarcinoma tissues from advanced stage (TNM III) patients were significantly higher than that in those from early stage patients (TNM I/II). Expression of ARHGEF7 progressively increased from NCTs, to T, to LMNs from the same patient (n=5). (G) RT-qPCR analysis of ARHGEF7 mRNA in NCTs, T tissues and LMNs.  $P=0.003$  NCT vs. T;  $P<0.001$  NCT vs. LMN;  $P=0.005$  T vs. LMN (P-values determined by analysis of variance and further SNK test). (H) Representative images of western blot results showed that expression of ARHGEF7 progressively increased from NCTs, to T, to LMNs from the same patient.  $^{**}P<0.01$ ;  $^{***}P<0.001$ . ARHGEF7, Rho guanine nucleotide exchange factor 7; T, tumor; NCT nontumorous tissues; TNM, tumor-node-metastasis; LMN, liver metastatic nodule; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

60, 90 or 120 min. The medium was entirely removed, and unbound cells were washed away with PBS. Fresh medium (100  $\mu$ l; 0.5 mg/ml) containing MTT (Sigma; EMD Millipore)

was added into each well and incubated at 37°C for 4 h. The medium was then replaced with 100  $\mu$ l of DMSO and shaken at room temperature for 10 min. The absorbance was



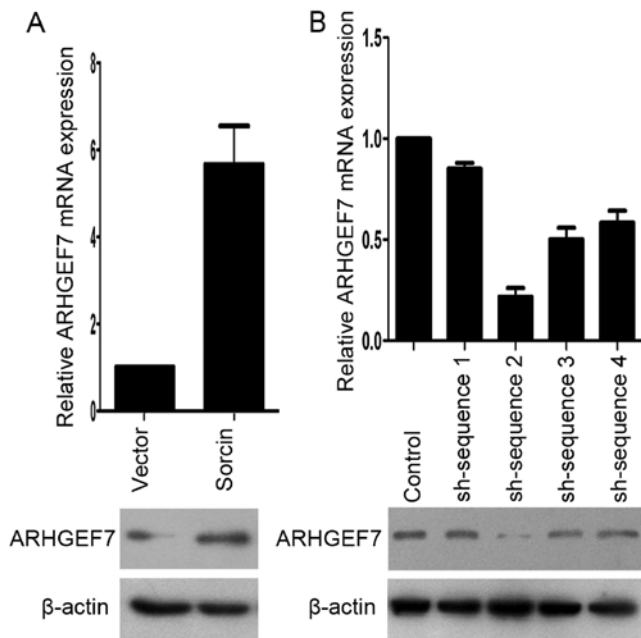


Figure 2. Validation of the expression of ARHGEF7 in colorectal adenocarcinoma cell lines with ARHGEF7 overexpression or knockdown. (A) ARHGEF7 was upregulated by ARHGEF7-expression LV particles in HCT116 cells. RT-qPCR and western blot analyses were used to evaluate the upregulation of exogenous ARHGEF7 in HCT116 cells. (B) ARHGEF7 was downregulated by shRNA-ARHGEF7 LV particles in LoVo cells. RT-qPCR and western blot analyses were used to assess the inhibitory efficiency of four designed ARHGEF7 shRNA LV particles and control. Expression of ARHGEF7 in control cell was set to 1. Relative expression levels of ARHGEF7 in these cells are shown in the diagram. ARHGEF7-shRNA-Seq2 reduced ARHGEF7 expression by >90% and was used for subsequent experiments due to its highly effective inhibitory efficiency. ARHGEF7, Rho guanine nucleotide exchange factor 7; shRNA, short hairpin RNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; LV, lentiviral.

measured at 570 nm. For the cell-cell adhesion assay, subconfluent cell layers (~70-80%) were rinsed twice with  $\text{Ca}_2^-$  and  $\text{Mg}_2^-$ -free PBS and detached by incubation in HBSS containing 1 mmol/l EDTA at 37°C for 20 min. Subsequently, single cells (100  $\mu\text{l}$ ) at density of  $\sim 1 \times 10^5/\text{ml}$  were added into a 96-well plate (Costar; Corning Incorporated) with a fully confluent single cell layer, and cultured at 37°C for 0-120 min. The unbound cells were washed from the wells, and were collected and quantified using an inverted microscope (TE-2000S; Nikon Corporation). The adhesion rate was determined by counting representative aliquots from each sample on a hemacytometer. The percentage of adhesion was quantified at 60, 90 or 120 min as:  $N_0 - N_t / N_0 \times 100$ , where  $N_t$  is the total number of unbound cells at the incubation time  $t$ , and  $N_0$  is the total number of cells.

**Cellular cytoskeleton analysis.** Rhodamine-conjugated phalloidin was used to analyze cell cytoskeleton. The cells grown on cover slides were fixed, and then incubated with rhodamine-conjugated phalloidin (1:200; cat. no. CA1610; Solarbio Science and Technology Co., Ltd., Beijing, China). Following staining with DAPI (1:200; cat. no. C1002; Beyotime Institute of Biotechnology), images of the slides were captured using an inverted fluorescence microscope (TE-2000S; Nikon Corporation).

**GTPase activity assays.** In brief, the cells were grown to ~80% confluence in regular culture medium, were serum-starved for 24 h and were stimulated with 10% FBS for 5 min. GTP-bound Ras-related C3 botulinum toxin substrate 1 (Rac1), cell division cycle 42 (Cdc42) and total protein were detected using G-LISA Rac1 and G-LISA Cdc42 Activation Assay Biochem kits (Cytoskeleton, Inc., Denver, CO, USA) according to the manufacturer's protocol.

**Metastatic assays in an *in vivo* orthotopic model.** The mice were provided by the Animal Institute of Nanchang University and were housed under specific pathogen-free conditions: Temperature, 25°C; relative humidity, ~40%; lighting, 10 h/day with fluorescent lights. The mice received *ad libitum* access to sterilized food and water. The experiments were performed according to the protocols approved by the Medical Experimental Animal Care Commission. For the *in vivo* metastatic assays, the orthotopic model in mice was constructed (23). Briefly,  $1 \times 10^6$  cells were injected into the subserosal layer of the sigmoid colon of male BALB/c nude mice weighing ~16-20 g (5 weeks old). The mice were sacrificed following 7 weeks of cell inoculation. Following necropsy, tumors growing in the colon and peritoneum were excised. All livers and macroscopically enlarged mesenteric lymph nodes were harvested, fixed with 10% phosphate-buffered neutral formalin, sectioned serially, and stained with H&E for defining the presence of metastatic disease.

**Statistical analysis.** All data were analyzed using the statistical software SPSS 18.0 for Windows (SPSS, Inc., Chicago, IL, USA). The differences between groups were analyzed using Student's *t*-test between two groups or by one-way analysis of variance in more than two groups when the variance was homogeneous. If the variance was not homogeneous, the differences between groups were analyzed using the Mann-Whitney U test between two groups or Kruskal-Wallis H test in more than two groups.  $\chi^2$  analysis was used to analyze the correlation between the expression of ARHGEF7 and clinicopathologic features, and the presence of metastasis between two groups. Survival curves were constructed with the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards regression model was established to identify independent factors for OS and DFS rates of patients. Receiver operating characteristics (ROC) curves were constructed to assess sensitivity, specificity, and respective areas under the curves with 95% CI. The cut-off value was assessed by the highest Youden index. All tests were two-tailed and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Expression of ARHGEF7 is significantly increased and associated with metastasis in colorectal adenocarcinoma.** Firstly, the expression of ARHGEF7 in colorectal adenocarcinoma cell lines was detected. Compared with the FHC normal colorectal mucosal cell line, ARHGEF7 mRNA and protein were expressed at high levels in the colorectal adenocarcinoma cell lines, including the HCT116, HT-29, SW480, SW620 and LoVo cell lines (Fig. 1A and B). The expression

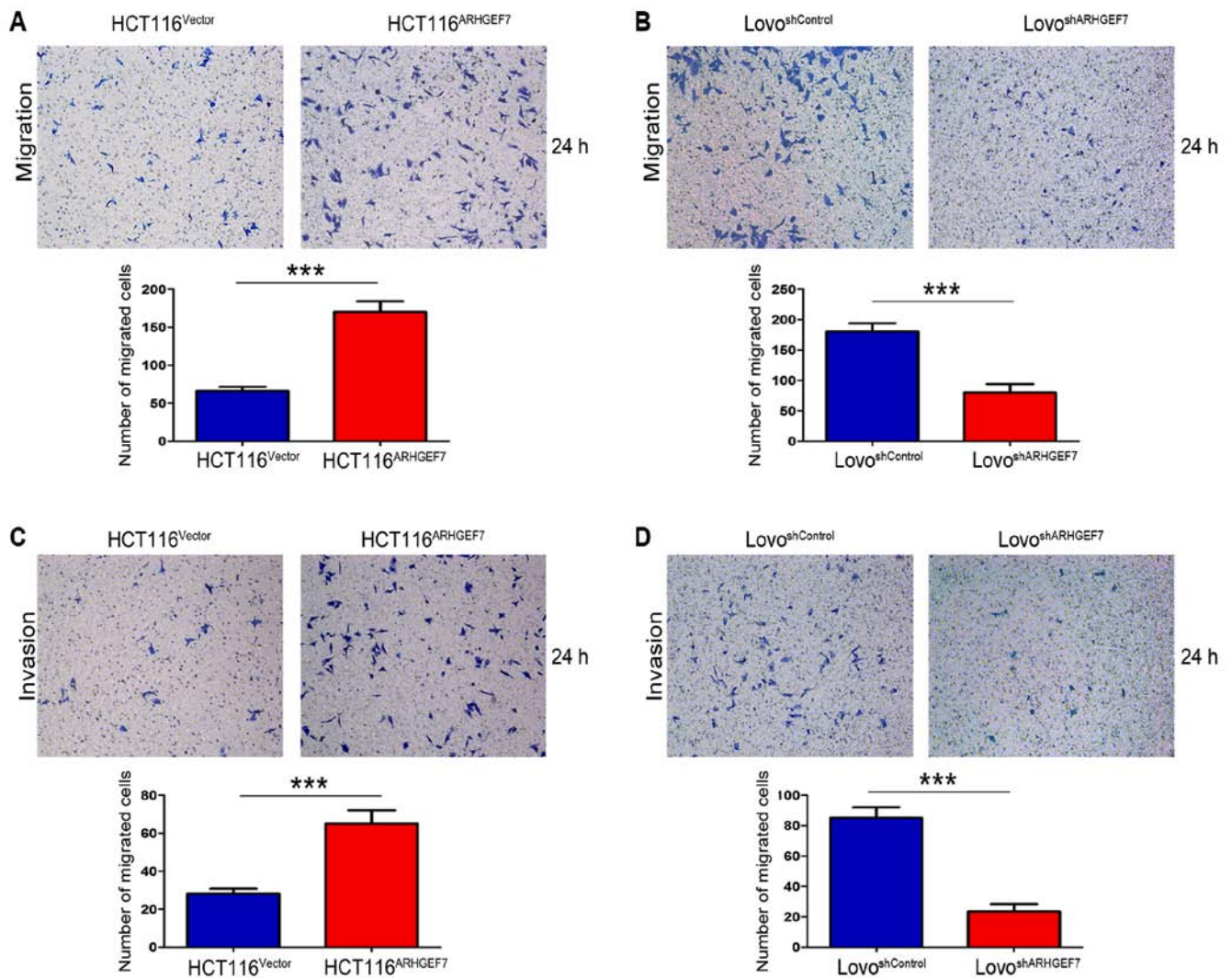


Figure 3. ARHGEF7 promotes colorectal adenocarcinoma cell migration and invasion *in vitro*. ARHGEF7 promoted colorectal adenocarcinoma cell migration *in vitro*. Transwell migration assays of (A) HCT116 cells with ARHGEF7 overexpression (HCT116<sup>ARHGEF7</sup>) or control (HCT116<sup>Vector</sup>), and (B) LoVo cells with ARHGEF7 knockdown (LoVo<sup>shARHGEF7</sup>) or control (LoVo<sup>shControl</sup>). ARHGEF7 promoted colorectal adenocarcinoma cell invasion *in vitro*. Original magnification, x100. Transwell invasion assays of (C) HCT116<sup>ARHGEF7</sup> and HCT116<sup>Vector</sup>, and (D) LoVo<sup>shARHGEF7</sup> and LoVo<sup>shControl</sup>. Original magnification, x100. Adhesion assays were used to determine the effect of ARHGEF7 on cell adhesion.

of ARHGEF7 was also determined in 30 paired colorectal adenocarcinoma samples. RT-qPCR analysis revealed that the mRNA expression of ARHGEF7 was markedly higher in tumor tissues than in corresponding NCTs ( $P < 0.001$ ; Fig. 1C). Western blot analysis also showed that the protein expression of ARHGEF7 was markedly higher in tumor tissues than in NCTs ( $P < 0.001$ ; Fig. 1D).

Subsequently, 30 primary tumors of different TNM stages were analyzed. The expression of ARHGEF7 in colorectal adenocarcinoma tissues was significantly higher in advanced stage patients (TNM stage III) than in early stage patients (TNM stage I/II) ( $P < 0.01$ ; Fig. 1E and F). The expression of ARHGEF7 was further analyzed in LMNs, primary tumors and corresponding NCTs from the same patient ( $n=5$ ), and the results showed that the expression of ARHGEF7 was progressively increased from NCT to tumor to LMN at the mRNA (Fig. 1G) and protein (Fig. 1H) levels. The LMNs had the highest expression of ARHGEF7, whereas NCTs had the lowest expression (Fig. 1G and H).

Taken together, these data confirmed that the expression of ARHGEF7 was significantly elevated in colorectal adenocarcinoma tissues and may be associated with metastasis in colorectal adenocarcinoma.

*ARHGEF7 promotes colorectal adenocarcinoma cell motility and invasion in vitro.* To determine the role of ARHGEF7 in the metastasis of colorectal adenocarcinoma, ARHGEF7 was overexpressed in HCT116 cells (Fig. 2A) and was stably knocked down in LoVo cells (Fig. 2B) according to the expression level of ARHGEF7 and biological characteristics of CRC cell lines. The Transwell migration assays showed that the overexpression of ARHGEF7 significantly increased motility of HCT116 cells (Fig. 3A), whereas the downregulation of ARHGEF7 decreased the motility of LoVo cells (Fig. 3B). Similar results were observed in the Transwell invasion assays (Fig. 3C and D). Adhesion assays showed that ARHGEF7 knockdown suppressed cell-ECM adhesion and enhanced cell-cell adhesion (Fig. 3E and F), whereas the

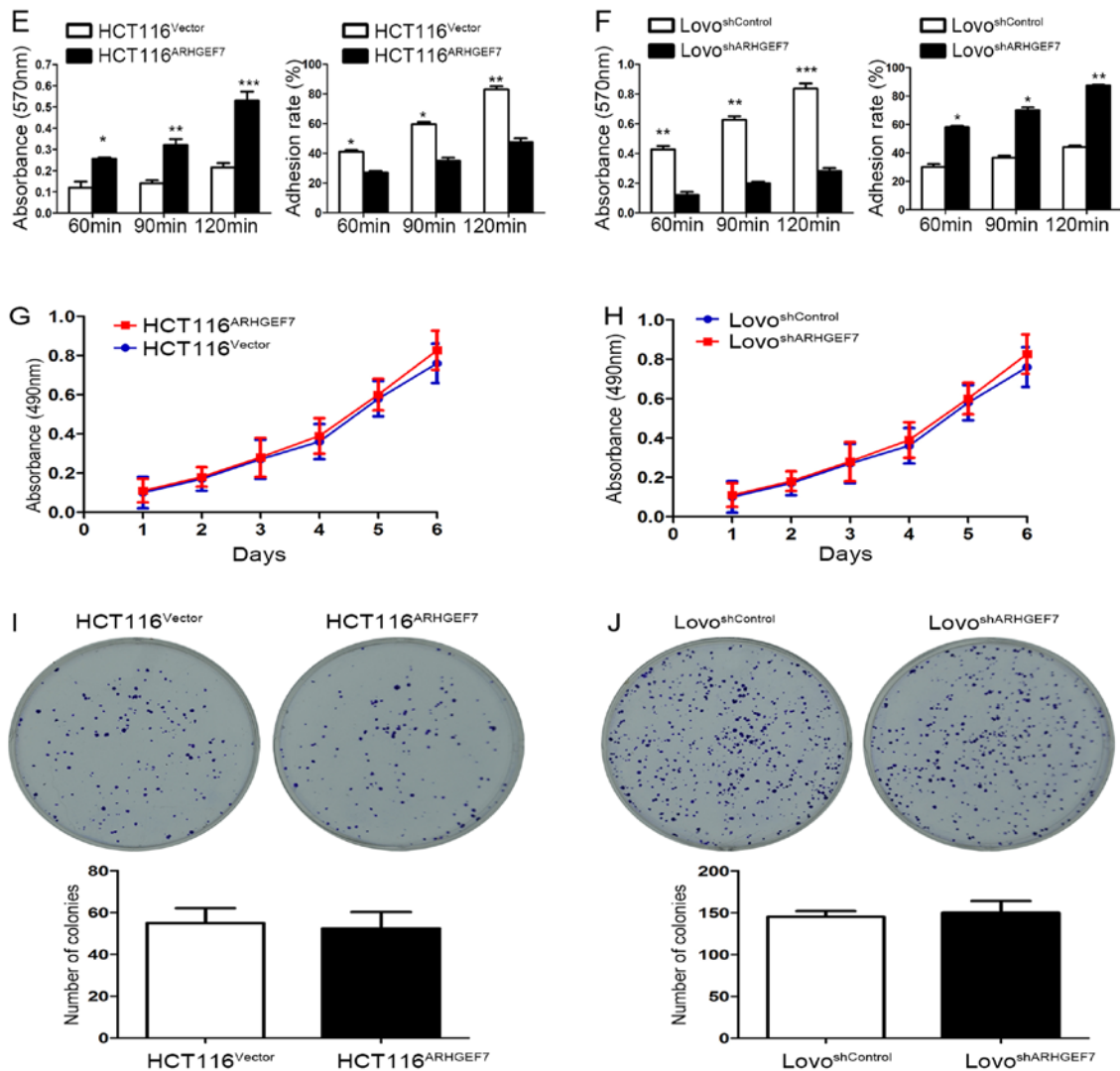


Figure 3. Continued. (E) ARHGEF7 knockdown suppressed cell-ECM adhesion and enhanced cell-cell adhesion. (F) Overexpression of ARHGEF7 increased cell-ECM adhesion and inhibited cell-cell adhesion. Methyl thiazolyl tetrazolium assays were performed to assess the effect of ARHGEF7 on (G) HCT116 and (H) LoVo cell proliferation. Colony formation assays were used to determine the proliferation of (I) HCT116 and (J) LoVo cells. Neither overexpression of ARHGEF7 in HCT116 cells or ARHGEF7 knockdown in LoVo cells affected colony formation. \* $P<0.05$ ; \*\* $P<0.01$ ; \*\*\* $P<0.001$ . Data are presented as the mean  $\pm$  standard deviation. ARHGEF7, Rho guanine nucleotide exchange factor 7; shRNA, short hairpin RNA; ECM, extracellular matrix.

overexpression of ARHGEF7 increased cell-ECM adhesion and inhibited cell-cell adhesion (Fig. 3E and F). Subsequently, the effect of ARHGEF7 on the proliferation of CRC cells was examined. The MTT assays showed that the overexpression of ARHGEF7 had minimal effect on HCT116 cell proliferation (Fig. 3G). Similarly, the downregulation of ARHGEF7 did not affect LoVo cell proliferation (Fig. 3H). The colony formation assays also showed that the capacity of colony formation of HCT116<sup>ARHGEF7</sup> cells was almost equal to that of HCT116<sup>Vector</sup> cells (Fig. 3I). The colony formation capacity of the LoVo<sup>shSocrin</sup> cells was similar to that of the LoVo<sup>shControl</sup> cells (Fig. 3J). These data indicated that ARHGEF7 was involved in promoting colorectal adenocarcinoma cell motility and invasion *in vitro*.

*ARHGEF7 enhances the invasion and metastasis of colorectal adenocarcinoma cells in vivo.* The role of ARHGEF7 in colorectal adenocarcinoma was further evaluated using *in situ* xenograft metastasis mice models. Following 7 weeks of cell inoculation, the tumor volume of orthotopic

mice colon tumors was analyzed. Compared with orthotopic implantation of HCT116<sup>Vector</sup> cells, mice with implantation of HCT116<sup>ARHGEF7</sup> cells had the similar tumor incidence (Table I) and tumor volume in the colon ( $P>0.05$ ; Fig. 4A). However, mice with implantation of HCT116<sup>ARHGEF7</sup> cells had increased peritoneum incidence of regional mesenteric lymph node metastasis ( $P=0.031$ ; Fig. 4A; Table I) and liver metastasis ( $P=0.038$ ; Fig. 4C and D; Table I) than mice with implantation of HCT116<sup>Vector</sup> cells. In the mice implanted with LoVo cells, the tumor incidence and volume of orthotopic colon tumors derived from the LoVo<sup>shSocrin</sup> cells was not significantly smaller than that of orthotopic colon tumors derived from LoVo<sup>shControl</sup> cells ( $P>0.05$ ; Fig. 4B; Table I). However, mice receiving implantation of LoVo<sup>shSocrin</sup> cells showed lower incidence of regional mesenteric lymph node metastasis ( $P=0.010$ ; Fig. 4B; Table I) and liver metastasis ( $P=0.031$ ; Fig. 4C and D; Table I) than the control. Taken together, these results demonstrated that ARHGEF7 promoted colorectal adenocarcinoma metastasis *in vivo*.



Table I. Human colon cancer cells implanted in the colon of nude mice.

Group	Tumor incidence	P-value	Incidence of lymph node metastasis	P-value	Hepatic metastasis	P-value
HCT116 <sup>Vector</sup>	6/8	0.450	0/8	0.031	0/8	0.038
HCT116 <sup>ARHGEF7</sup>	8/8		5/8		4/8	
LoVo <sup>shControl</sup>	8/8	0.500	8/8	0.010	8/8	0.031
LoVo <sup>shARHGEF7</sup>	7/8		2/8		3/8	

ARHGEF7, Rho guanine nucleotide exchange factor 7; sh, short hairpin RNA.

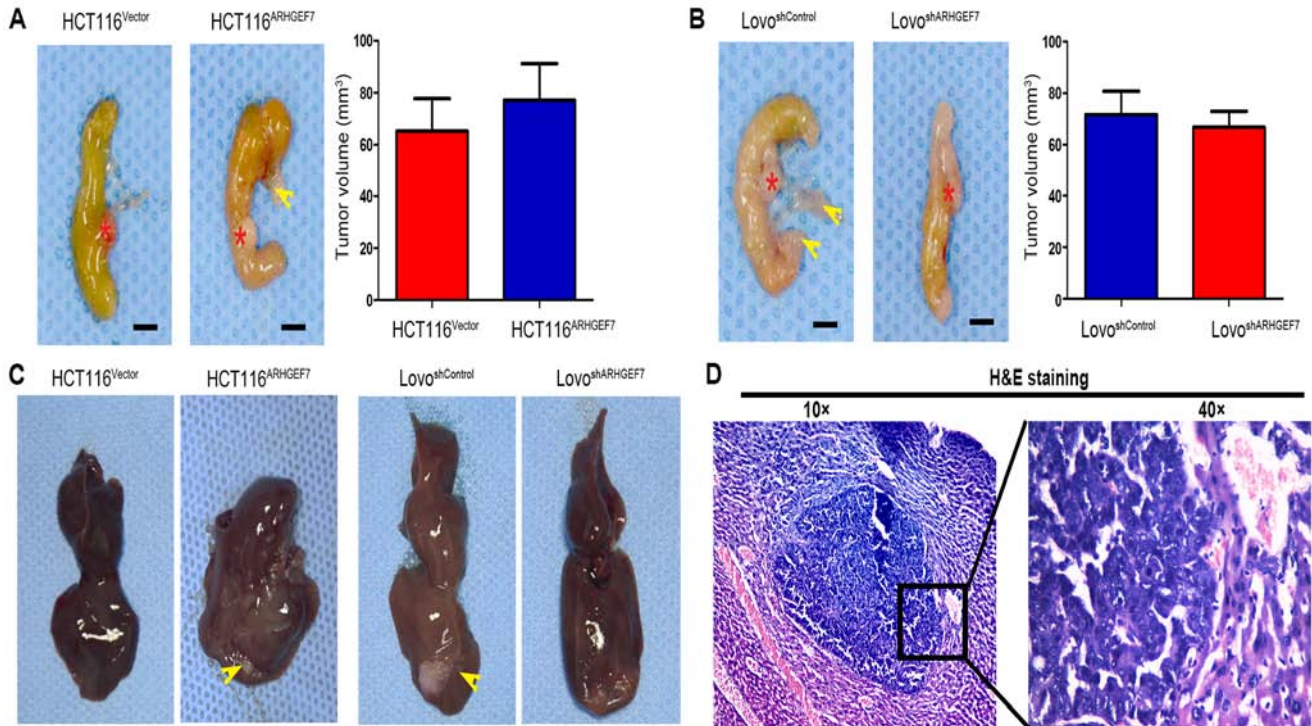


Figure 4. ARHGEF7 promotes colorectal adenocarcinoma cell metastasis *in vivo*. Representative orthotopic tumors from (A) HCT116<sup>ARHGEF7</sup> and (B) LoVo<sup>shARHGEF7</sup> cells and their control cells are shown. Red stars indicated orthotopic tumor; yellow arrow indicated metastatic lymph node. Tumor volumes of each group are shown and compared in the bar charts; ARHGEF7 exhibited minimal effect on colorectal adenocarcinoma cell growth *in vivo*. Representative images of (C) macrographic and (D) micrographic metastasis in the liver. ARHGEF7, Rho guanine nucleotide exchange factor 7; shRNA, short hairpin RNA; H&E, hematoxylin and eosin.

*ARHGEF7 promotes colorectal adenocarcinoma metastasis by regulating actin cytoskeleton rearrangements.* The above results showed that ARHGEF7 promoted colorectal adenocarcinoma metastasis by facilitating cell motility, which is associated with cytoskeletal reorganization by polymerization of filamentous actin (F-actin) (24). Therefore, the present study investigated the effect of ARHGEF7 on regulating the actin cytoskeleton in CRC cell motility by staining F-actin. F-actin immunofluorescence staining was used to analyze the cytoskeleton. The immunofluorescence analysis showed that, compared with the HCT116<sup>Vector</sup> cells, the appearance of F-actin fibers was present in the HCT116<sup>ARHGEF7</sup> cells, which had an increase in filopodia and lamellipodia (Fig. 5A). By contrast, the LoVo<sup>shARHGEF7</sup> cells exhibited loosely organized F-actin and shrinkable parallel bundles stress fibers relative to the LoVo<sup>shControl</sup> cells (Fig. 5B). ARHGEF7 is an identified guanine nucleotide exchange factor for Cdc42/Rac (10), which is involved in cell migration by the extension and maintenance of filopodia (25,26). The effect

of ARHGEF7 on the GTP/GDP-binding status of Rac1 and Cdc42 was determined. The results showed that GTP-Cdc42 and GTP-Rac1 activation was significantly increased in the ARHGEF7-overexpressing HCT116 cells (Fig. 5C and D). By contrast, in the LoVo cells, basal Rac1 and Cdc42 activities were marked reduced by ARHGEF7 knockdown (Fig. 5C and D). Therefore, these data indicated that ARHGEF7 may be associated with colorectal adenocarcinoma metastasis by regulating actin cytoskeleton rearrangements.

*High expression of ARHGEF7 correlates with aggressive clinicopathological characteristics and predicts poor prognosis in patients with colorectal adenocarcinoma.* It has been demonstrated that ARHGEF7 is involved in colorectal adenocarcinoma metastasis, which is the main causative factor for poor prognosis in colorectal adenocarcinoma. Therefore, the present study further determined whether the expression of ARHGEF7 was clinically relevant to colorectal adenocarcinoma according



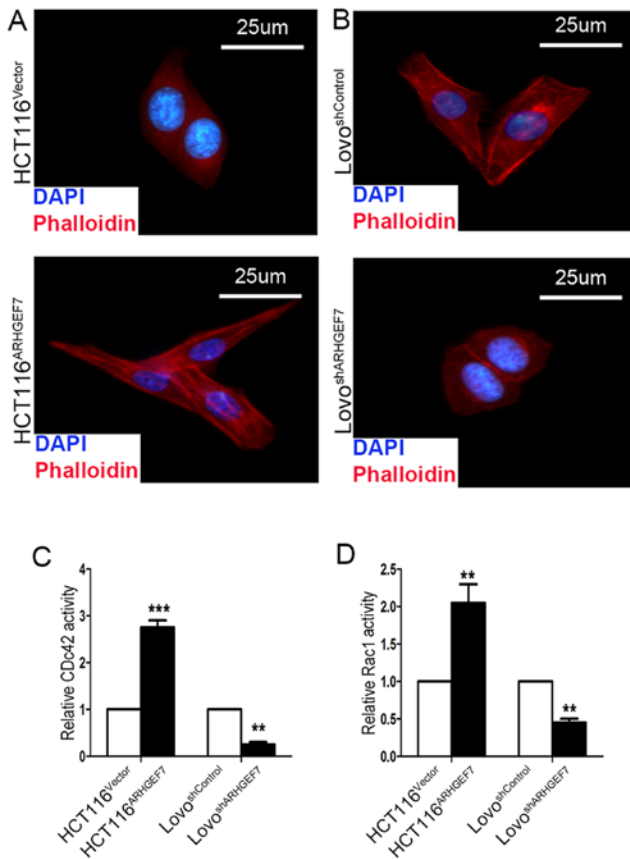


Figure 5. ARHGEF7 promotes colorectal adenocarcinoma metastasis by regulating actin cytoskeleton rearrangements. Representative images of (A) HCT116 and (B) LoVo cell cytoskeletons show that ARHGEF7 affected the polymerization of filamentous actin in the colorectal adenocarcinoma cells. Original magnification,  $\times 40$ . The effects of ARHGEF7 on the GTP/GDP-binding status of (C) Cdc42 and (D) Rac1 and were determined. GTP-Cdc42 and GTP-Rac1 activation was significantly increased in ARHGEF7-overexpressing HCT116 cells. In LoVo cells, basal Rac1 and Cdc42 activity was markedly reduced by ARHGEF7 knockdown. ARHGEF7, Rho guanine nucleotide exchange factor 7; shRNA, short hairpin RNA; Rac1, Ras-related C3 botulinum toxin substrate 1; Cdc42, cell division cycle 42.

to ReMARK guidelines for reporting prognostic biomarkers in cancer (18). An IHC method was used to assay two sets of colorectal adenocarcinoma samples from two independent cohorts, including training and validation cohorts (Fig. 6). The clinicopathologic characteristics of the patients in these two sets are shown in Table II. Based on the training cohort, the results showed that the expression of ARHGEF7 was mainly located in the cytoplasm (Fig. 7A), and the expression level of ARHGEF7 was significantly higher in tumor tissues than in adjacent NCTs (Fig. 7B). An ROC curve for metastasis was utilized to determine the cut-off value according to the results of the IHC staining analysis (Fig. 7C). The highest Youden index was the cut-off value, defined as 4, thus an IS of 4 was selected as a cut-off value for low expression of ARHGEF7. In the training cohort, a high expression of ARHGEF7 was found in 60.0% (108/180) of the tumor tissues, compared with only 20% (36/180) of the adjacent NCTs ( $P < 0.001$ ; Table III). A high expression of ARHGEF7 was significantly correlated with lymph node, mesenteric and distant metastasis (Table IV). Following analysis with the Kaplan-Meier method and log-rank test, patients with colorectal adenocarcinoma with a high

Table II. Clinicopathologic characteristics of patients with colorectal adenocarcinoma in the training cohort and validation cohort.

Clinicopathologic variable	Training cohort (n) (n=180)	Validation cohort (n) (n=150)	P-value
Sex			
Female	75	65	0.760
Male	105	85	
Age (years)			
$\leq 60$	89	70	0.615
$> 60$	91	80	
CEA (ng/ml)			
$\leq 5$	110	98	0.429
$> 5$	70	52	
Tumor differentiation			
I/II	83	67	0.454
III/IV	97	83	
Tumor site			
Colon	78	64	0.903
Rectum	102	86	
Tumor size			
$\leq 5$ cm	94	88	0.241
$> 5$ cm	86	62	
pT stage			
T1/T2	56	34	0.086
T3/T4	124	116	
pN stage			
N0	85	63	0.342
N <sup>+</sup>	95	87	
Lymphatic/microvascular/nerve invasion			
Negative	81	61	0.429
Positive	99	89	
Mesenteric tumor deposit formation			
Negative	114	91	0.619
Positive	66	59	
Distant metastasis			
Negative	98	83	0.872
Positive	82	67	

Distant metastasis was identified during postoperative follow-up. CEA, carcinoembryonic antigen.

expression of ARHGEF7 had either shorter DFS ( $P < 0.001$ ; Fig. 7D) or shorter OS ( $P = 0.003$ ; Fig. 7E), compared with patients with a low expression of ARHGEF7. Finally, to determine whether high expression of ARHGEF7 was an independent prognostic factor for colorectal adenocarcinoma, univariate analysis was first performed followed by multivariate Cox proportional hazards analysis. The data showed a high expression of ARHGEF7 as an independent risk factor for DFS (HR 3.541, 95% CI 1.959-6.400,  $P < 0.001$ ; Table V)

Table III. Immunohistochemical analysis of the protein expression of ARHGEF7 in tumor tissues and NCTs from the training and validation cohort.

Type	Training cohort			Validation cohort		
	ARHGEF7 expression		P-value <sup>a</sup>	ARHGEF7 expression		P-value <sup>a</sup>
	High	Low		High	Low	
Tumor	108	72	<0.001	93	57	<0.001
NCT	36	144		45	105	

<sup>a</sup>Calculated by  $\chi^2$  analysis. ARHGEF7, Rho guanine nucleotide exchange factor 7.

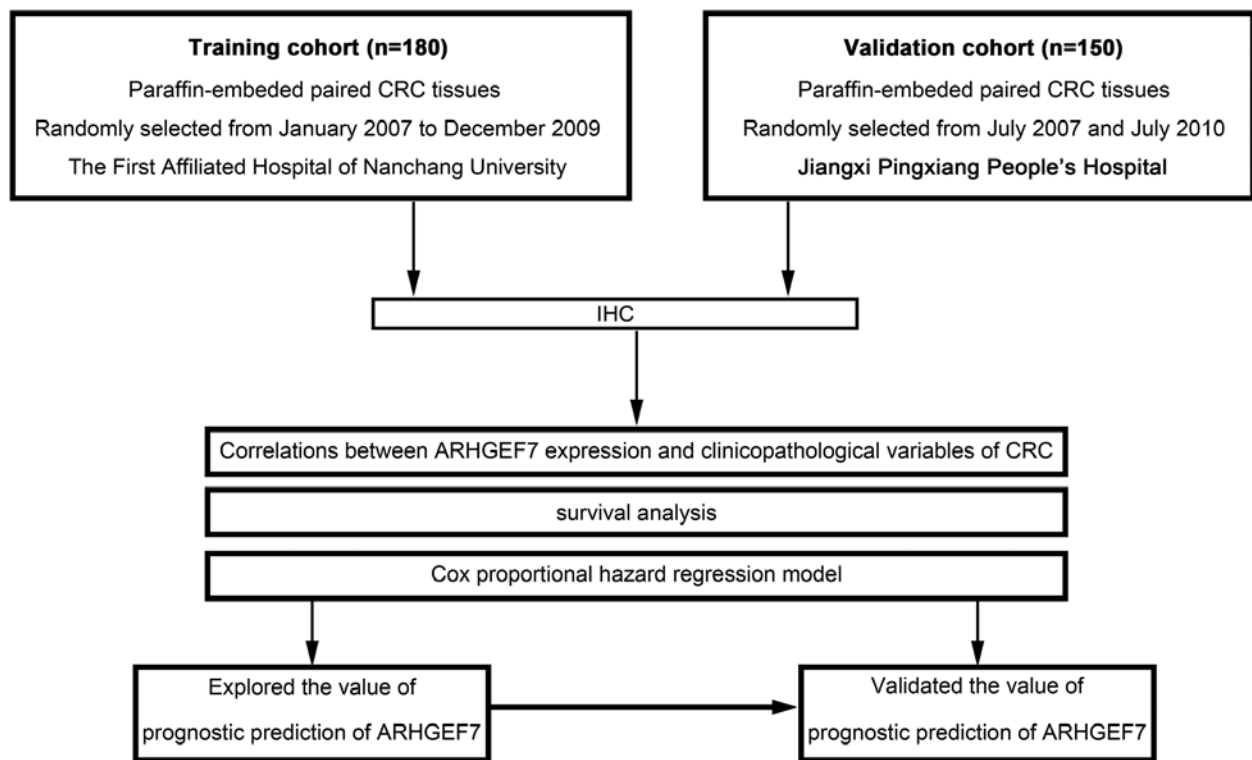


Figure 6. Flowchart of clinical experimental design in two independent cohorts of patients with CRC. ARHGEF7, Rho guanine nucleotide exchange factor 7; CRC, colorectal adenocarcinoma; IHC, immunohistochemistry.

and OS (HR 2.050, 95% CI 1.157-3.633,  $P=0.012$ ; Table V) in colorectal adenocarcinoma.

To validate the clinical significance of ARHGEF7 in colorectal adenocarcinoma, IHC assays of another cohort of CRC samples from the validation cohort showed that the expression level of ARHGEF7 was significantly higher in the tumor tissues than in the adjacent NCTs (Fig. 7B). A high expression of ARHGEF7 was found in 62.0% (93/150) of the tumor tissues, compared with only 30% (45/150) of the NCTs ( $P<0.001$ ; Table III) from the validation cohort. A high expression of ARHGEF7 was significantly correlated with lymph node, mesenteric and distant metastasis (Table VI). Analysis with the Kaplan-Meier method and log-rank test showed that patients with colorectal adenocarcinoma with a high expression of ARHGEF7 had either shorter DFS ( $P=0.003$ ; Fig. 7F) or shorter OS ( $P=0.002$ ; Fig. 7H),

compared with those with a low expression of ARHGEF7. Finally, Cox proportional hazards analysis showed that a high expression of ARHGEF7 was an independent risk factor for DFS (HR 3.128, 95% CI 2.536-3.858,  $P=0.011$ ; Table VII) and OS (HR 2.801, 95% CI 1.503-5.219,  $P=0.015$ ; Table VII) in colorectal adenocarcinoma. Collectively, these data suggested that ARHGEF7 was a potent prognosticator in addition to promoting colorectal adenocarcinoma metastasis.

## Discussion

Colorectal adenocarcinoma is a frequently life-threatening disease with heterogeneous outcomes (27). Surgical resection remains the most important therapy used for patients with colorectal adenocarcinoma (3). However, cancer metastasis

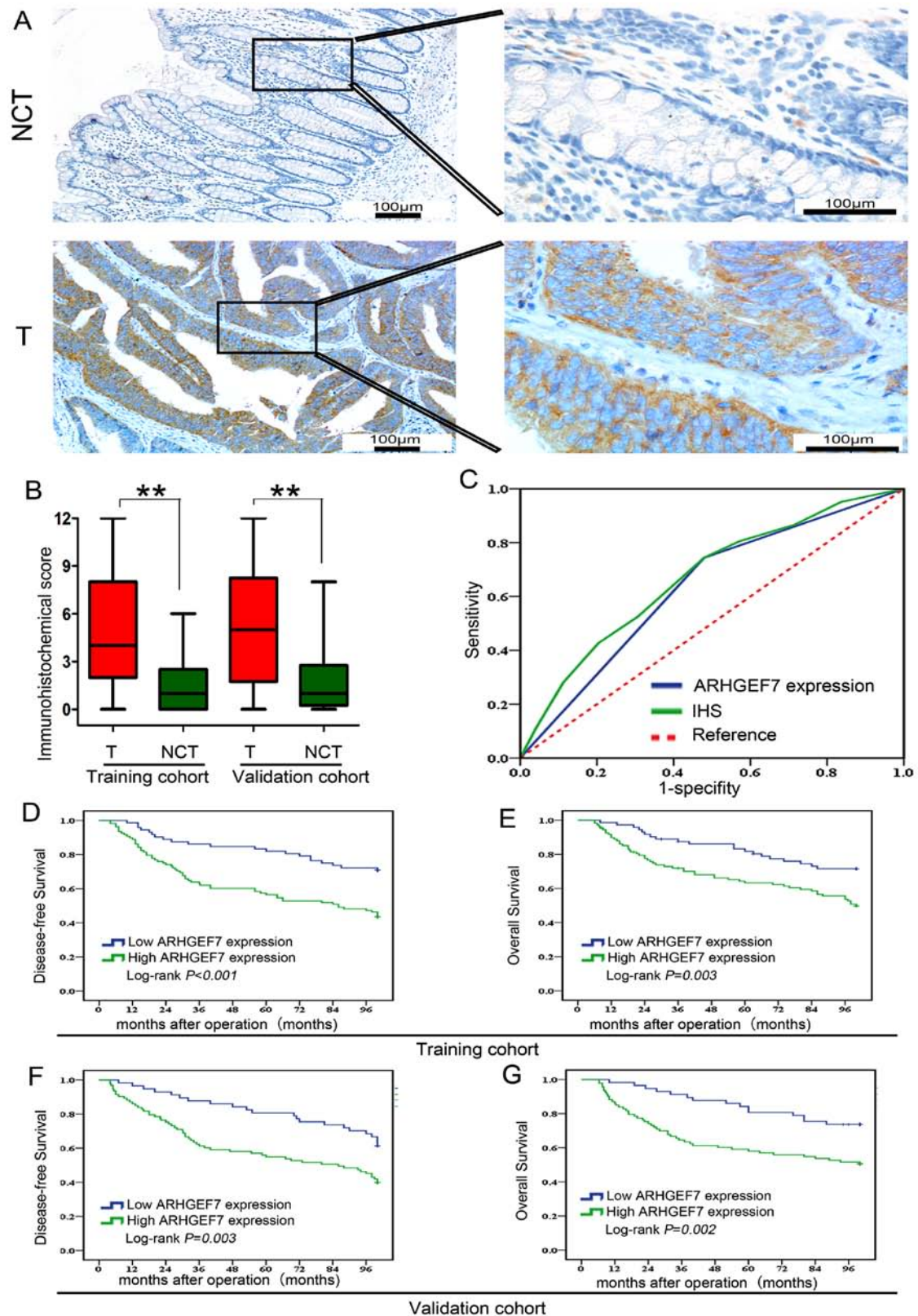


Figure 7. ARHGEF7 is a potential prognosticator for colorectal adenocarcinoma clinical outcome following radical surgery. (A) Representative IHC staining of ARHGEF7 protein in tumor tissues and NCTs from the training and validation cohort. (B) Tumor tissues exhibited higher protein expression of ARHGEF7 than corresponding NCTs. A box-plot was used to show the differential expression and a Mann-Whitney U test was used to analyze the difference between tumor tissues and NCTs from the training and validation cohort. (C) ROC curve analysis. The sensitivity and 1-specificity of IS in colorectal adenocarcinoma tumor tissues was plotted (green line). The AUC and the P-value, as calculated by ROC curve analysis, were AUC=0.668 and  $P < 0.001$  respectively. The highest Youden index cut-off value was defined as 4; an IS of 4 was selected as a cut-off value for low expression of ARHGEF7. The sensitivity and 1-specificity of ARHGEF7 expression in colorectal adenocarcinoma tissues were plotted (blue line). The AUC and the P-value as calculated by ROC curve analysis were AUC=0.632 and  $P = 0.002$ , respectively. Kaplan-Meier analysis of the correlation between the expression of ARHGEF7 and (D) DFS and (E) OS of patients with colorectal adenocarcinoma in the training cohort. Kaplan-Meier analysis of the correlation between the expression of ARHGEF7 and (F) DFS and (G) OS of patients with colorectal adenocarcinoma in the validation cohort. ARHGEF7, Rho guanine nucleotide exchange factor 7; T, tumor; NCTs, nontumorous tissues; ROC, receiver operating characteristic; AUC, area under the curve; DFS, disease-free survival; OS, overall survival; IHC, immunohistochemistry; IS, immunostaining score.



Table IV. Correlations between expression of ARHGEF7 in CRC tissues and clinicopathologic variables of patients with CRC in the training cohort.

Clinicopathologic variable	ARHGEF7 expression			P-value
	n	Low (n=72)	High (n=108)	
Sex				
Female	75	35	40	0.123
Male	105	37	68	
Age (years)				
≤60	89	38	51	0.465
>60	91	34	57	
CEA (ng/ml)				
≤5	110	49	61	0.119
>5	70	23	47	
Tumor differentiation				
I/II	83	43	40	0.003
III/IV	97	29	68	
Tumor site				
Colon	78	31	47	0.951
Rectum	102	41	61	
Tumor size				
≤5 cm	94	40	54	0.465
>5 cm	86	32	54	
pT stage				
T1/T2	56	33	23	0.001
T3/T4	124	39	85	
pN stage				
N0	85	47	38	<0.001
N <sup>+</sup>	95	25	70	
Lymphatic/microvascular/ nerve invasion				
Negative	81	42	39	0.003
Positive	99	30	69	
Mesenteric tumor deposit formation				
Negative	114	54	60	0.008
Positive	66	18	48	
Distant metastasis				
Negative	98	51	47	<0.001
Positive	82	21	61	

CRC, colorectal cancer; CEA, carcinoembryonic antigen; ARHGEF7, Rho guanine nucleotide exchange factor 7.

adenocarcinoma tissues compared with that in NCTs. Increased expression of ARHGEF7 was found to be positively correlated with the metastatic potential of colorectal adenocarcinoma cells, suggesting that ARHGEF7 was involved in colorectal adenocarcinoma metastasis.

To define the role of ARHGEF7 in metastasis, a series of *in vitro* assays showed that overexpression of ARHGEF7 in colorectal adenocarcinoma cells significantly enhanced cell migration and invasion, whereas the knockdown of ARHGEF7 in colorectal adenocarcinoma cells significantly decreased cell migration and invasion. Furthermore, the *in vivo* assays showed that the overexpression of ARHGEF7 in colorectal adenocarcinoma cells facilitated tumor metastasis, whereas the knockdown of ARHGEF7 in colorectal adenocarcinoma cells significantly inhibited tumor metastasis. Although the effect of ARHGEF7 on the proliferation of colorectal adenocarcinoma cells was not observed, the results were consistent with the involvement of ARHGEF7 in cell migration and metastasis in other malignancies (14,29), suggesting that ARHGEF7 possesses oncogenic properties in promoting metastasis in colorectal adenocarcinoma.

Cancer metastasis occurs via a process involving abnormal cell migration (30). Cell migration, a dynamic physical process, is controlled by the cytoskeletal system, which includes the dynamics of actin organization (30). A previous study showed that ARHGEF7 had a direct regulatory role in promoting dense actin networks to direct cell migration (29). In the present study, the immunofluorescence analysis showed that ARHGEF7 promoted the polymerization of F-actin in colorectal adenocarcinoma cells and facilitated the formation of filopodia and lamellipodia. The results further showed that the activation of GTP-Cdc42 and GTP-Rac1 was significantly increased in the ARHGEF7-overexpressing colorectal adenocarcinoma cells. By contrast, basal Rac1 and Cdc42 activity was markedly reduced by ARHGEF7 knockdown in colorectal adenocarcinoma cells, supporting the hypothesis that ARHGEF7 serves as a GEF protein with activity towards Rac1 and Cdc42 in cell migration (26).

The prognosis for patients with metastatic disease remains poor (31), therefore, the identification of more potent molecular biomarkers for identifying patient subgroups with high-risk metastasis is urgently required. The present study also suggested that ARHGEF7 may be a promising prognostic biomarkers associated with survival rates. The prognostic significance of the expression of ARHGEF7 was validated in two independent cohorts according to ReMARK guidelines for reporting prognostic biomarkers in cancer (18). It was found that a high expression of ARHGEF7 was significantly correlated with metastasis, and shorter DFS or shorter OS. A high expression of ARHGEF7 was found to be an independent prognostic factor in colorectal adenocarcinoma. However, complex pathways contribute to colorectal adenocarcinoma progression, and the prediction of efficacy of ARHGEF7 and outcome in colorectal adenocarcinoma requires validation for clinical use.

In conclusion, the present study identified the frequently increased expression of ARHGEF7 in colorectal adenocarcinoma tissues and this expression pattern was associated with

presents an obstacle in improving the clinical outcome, which affects up to 60% of patients with colorectal adenocarcinoma (28). The detailed molecular mechanism of metastasis remains to be fully elucidated. The present study revealed that the expression of ARHGEF7 was increased in colorectal

Table V. Cox proportional hazard regression analyses for DFS and OS in the training cohort.

Variable	n	DFS			OS		
		Univariate analysis		Multivariate analysis	Univariate analysis		Multivariate analysis
		HR (95% CI)	P-value		HR (95% CI)	P-value	
Sex							
Female	75	Reference			Reference		
Male	105	1.051 (0.609-1.814)	0.298	NA	1.176 (0.739-2.833)	0.271	NA
Age (years)							
≤60	89	Reference			Reference		NA
>60	91	1.192 (0.715-1.987)	0.180	NA	1.207 (0.572-2.137)	0.170	NA
CEA (ng/ml)							
≤5	110	Reference			Reference		
>5	70	2.782 (1.948-4.083)	0.003	1.615 (1.271-2.052)	2.064 (1.356-3.142)	0.008	1.432 (1.083-2.348)
Tumor differentiation							
I/II	83	Reference			Reference		
III/IV	97	1.521 (1.034-2.416)	0.045	1.007(0.634-1.629)	1.773 (1.349-3.082)	0.039	1.275 (0.802-2.027)
Tumor site							
Colon	78	Reference			Reference		
Rectum	102	1.206 (0.803-1.811)	0.127	NA	1.216 (0.835-2.238)	0.103	NA
Tumor size							
≤5 cm	94	Reference			Reference		
>5 cm	86	2.033 (1.080-3.824)	0.028	1.594 (0.914-2.780)	2.078 (1.123-3.845)	0.026	1.602 (0.923-2.781)
pT stage							
T1/T2	56	Reference			Reference		
T3/T4	124	2.175 (1.532-4.782)	0.012	1.876 (1.068-3.296)	2.179 (1.723-3.835)	0.010	1.832 (1.214-2.907)
pN stage							
N0	85	Reference			Reference		
N+	95	4.309 (2.908-6.385)	<0.001	2.524 (1.715-3.715)	4.204 (2.841-6.221)	<0.001	1.918 (1.534-3.235)
Lymphatic/microvascular/nerve invasion							
Negative	81	Reference			Reference		
Positive	99	3.034 (1.790-5.145)	0.005	2.175 (1.532-4.782)	4.914 (3.009-8.025)	<0.001	3.190 (1.209-3.967)

Table V. Continued.

Variable	DFS				OS			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	n	HR (95% CI)	P-value	HR (95% CI)	n	HR (95% CI)	P-value	HR (95% CI)
Mesenteric tumor deposit formation								
Negative	114	Reference		Reference		Reference		
Positive	66	4.057 (2.485-6.623)	<0.001	2.323 (1.219-4.427)		4.309 (2.908-6.385)	<0.001	2.875 (1.733-4.769)
ARHGEF7 expression								
Low	72	Reference		Reference		Reference		
High	108	5.854 (3.193-10.733)	<0.001	3.541 (1.959-6.400)		3.377 (2.135-5.342)	0.003	2.050 (1.157-3.633)

DFS, disease-free survival; OS, overall survival; CEA, carcinoembryonic antigen; ARHGEF7, Rho guanine nucleotide exchange factor 7.

Table VI. Correlations between the expression of ARHGEF7 in CRC tissues and clinicopathologic variables of patients with CRC in the validation cohort.

Clinicopathologic variable	n	ARHGEF7 expression		P-value
		Low (n=57)	High (n=93)	
Sex				
Female	65	29	36	0.144
Male	85	28	57	
Age (years)				
≤60	70	30	40	0.139
>60	80	27	53	
CEA (ng/ml)				
≤5	98	38	60	0.788
>5	52	19	33	
Tumor differentiation				
I/II	67	37	30	<0.001
III/IV	83	20	63	
Tumor site				
Colon	64	23	41	0.653
Rectum	86	34	52	
Tumor size				
≤5 cm	88	34	54	0.848
>5 cm	62	23	39	
pT stage				
T1/T2	34	24	10	<0.001
T3/T4	116	33	83	
pN stage				
N0	63	31	32	0.016
N+	87	26	61	
Lymphatic/microvascular/nerve invasion				
Negative	61	40	21	<0.001
Positive	89	17	72	
Mesenteric tumor deposit formation				
Negative	91	35	56	0.885
Positive	59	22	37	
Distant metastasis				
Negative	83	39	44	0.012
Positive	67	18	49	

CRC, colorectal cancer; ARHGEF7, Rho guanine nucleotide exchange factor 7; CEA, carcinoembryonic antigen.

colorectal adenocarcinoma metastasis. Furthermore, it was demonstrated that ARHGEF7 promoted metastasis by regulating actin cytoskeleton rearrangements. Finally, it was shown that a high expression of ARHGEF7 was clinically relevant, with aggressive clinicopathological characteristics and poor



Table VII. Cox proportional hazard regression analyses for DFS and OS in the validation cohort.

Variable	n	DFS			OS		
		Univariate analysis		Multivariate analysis	Univariate analysis		Multivariate analysis
		HR (95% CI)	P-value		HR (95% CI)	P-value	
Sex							
Female	65	Reference			Reference		
Male	85	1.316 (0.782-1.894)	0.141	NA	1.617 (0.883-2.308)	0.190	NA
Age (years)							
≤60	70	Reference			Reference		
>60	80	1.196 (0.616-2.322)	0.342	NA	1.104 (0.804-1.623)	0.239	NA
CEA (ng/ml)							
≤5	98	Reference			Reference		
>5	52	1.702 (1.301-2.227)	0.010	1.201 (1.009-1.430)	1.875 (1.290-2.725)	0.008	1.287 (1.100-1.505)
Tumor differentiation							
I/II	67	Reference			Reference		
III/IV	83	1.349 (1.072-1.698)	0.037	1.064 (0.804-1.801)	1.381 (1.012-2.783)	0.044	1.109 (0.803-1.532)
Tumor site							
Colon	64	Reference			Reference		
Rectum	86	1.332 (0.680-2.609)	0.108	NA	1.206 (0.660-2.153)	0.204	NA
Tumor size							
≤5 cm	88	Reference			Reference		
>5 cm	62	1.603 (1.108-2.319)	0.042	1.180 (0.904-1.540)	1.506 (1.041-2.198)	0.036	1.145 (0.937-1.399)
pT stage							
T1/T2	34	Reference			Reference		
T3/ T4	116	3.054 (1.625-5.154)	0.007	1.795 (1.187-2.753)	3.634 (2.327-5.675)	0.004	1.837 (1.351-2.985)
pN stage							
N0	63	Reference			Reference		
N <sup>+</sup>	87	4.725 (3.051-7.317)	<0.001	3.314 (2.191-5.013)	4.982 (3.087-8.040)	<0.001	3.526 (2.354-5.282)
Lymphatic/microvascular/nerve invasion							
Negative	61	Reference			Reference		
Positive	89	3.004 (2.172-7.624)	0.007	1.934 (1.072-3.489)	3.582 (2.723-4.712)	0.009	2.036 (1.288-4.732)

Table VII. Continued.

Variable	n	DFS			OS		
		Univariate analysis		Multivariate analysis	Univariate analysis		Multivariate analysis
		HR (95% CI)	P-value		HR (95% CI)	P-value	
Mesenteric tumor deposit formation							
Negative	91	Reference		Reference	Reference	Reference	
Positive	59	3.904 (2.605-5.851)	<0.001	2.524 (1.715-3.715)	0.019	3.972 (1.433-6.587)	<0.001
ARHGEF7							
Low	57	Reference		Reference		Reference	
High	93	3.834 (1.972-7.624)	0.003	3.128 (2.536-3.858)	0.011	3.854 (2.009-7.393)	0.002
</							

DFS, disease-free survival; OS, overall survival; CEA, carcinoembryonic antigen; ARHGEF7, Rho guanine nucleotide exchange factor 7; NA, not applicable.

prognosis in patients with colorectal adenocarcinoma, when consulting ReMARK guidelines for reporting prognostic biomarkers in cancer. Collectively, the data indicated that ARHGEF7 was important in colorectal adenocarcinoma metastasis and may be an independent potential prognostic marker for predicting clinical outcome in colorectal adenocarcinoma.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

TL, XL and BX designed the experiments. XL, LD, JL, ZW, DL, SL, HD, GH, CT and CX performed experiments and analyzed data. TL, BX, XL and LD provided patient samples and collected data. TL, XL, JL, ZW wrote and revised the paper.

### Ethics approval and consent to participate

The study was approved by the Ethics Committee of the Institutional Review Boards of the First Affiliated Hospital of Nanchang University and Jiangxi Pingxiang People's Hospital, and was performed in accordance with the Declaration of Helsinki and current ethical guidelines. Prior informed consent was obtained from all participants.

### Patient consent for publication

Patients provided written informed consent for publication.

### Competing interests

The authors confirm that they have no competing interests.

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