

RAD18 promotes colorectal cancer metastasis by activating the epithelial-mesenchymal transition pathway

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Abstract. RAD18 is an E3 ubiquitin-protein ligase that has a role in carcinogenesis and tumor progression owing to its involvement in error-prone replication. Despite its significance, the function of RAD18 has not been fully examined in colorectal cancer (CRC). In the present research, by collecting clinical samples and conducting immunohistochemical staining, we found that RAD18 expression was significantly increased in the CRC tissue compared with that noted in the adjacent non-cancerous normal tissues and that high expression of RAD18 was associated with lymph node metastasis and poor prognosis in CRC patients. *In vitro*, as determined by cell transfection, scratch, and Transwell experiments, it was also demonstrated that RAD18 increased the invasiveness and migration capacity of CRC cells (HCT116, DLD-1, SW480). The signaling pathway was analyzed by western blotting and the clinical data were analyzed by immunohistochemical staining and RT-PCR, indicating that the process of epithelial-mesenchymal transition (EMT) may be involved in RAD18-mediated migration and invasion of CRC cells. All of the above data indicate that RAD18 is a novel prognostic biomarker that may become a potential therapeutic target for CRC in the future.

Introduction

According to a recent statistical analysis in 2018, colorectal cancer (CRC) ranked third in regards to cancer incidence (10.2% of total cancer numbers) and second in terms of cancer mortality (9.2% of total cancer deaths) (1). Although significant progress has been made both in conventional treatment options, such as surgery, radiotherapy, and chemotherapy, and in targeted drugs for CRC patients, distant metastasis is still the leading cause of CRC-related death (2). Thus, identification of novel prognostic biomarkers associated with metastasis could improve the outcome for patients with CRC.

The E3 ubiquitin-protein ligase RAD18 plays a vital role in DNA damage bypass and post-replication repair (PRR) through the promotion of proliferating cell nuclear antigen (PCNA) mono-ubiquitination at stalled replication forks (3). Recent studies have shown that high expression of RAD18 in cancerous tissues is associated with cancer metastasis and tumor progression in a variety of cancers (4-6). In melanoma, studies demonstrated that RAD18 participates in the regulation of cell proliferation, and its high expression is associated with poor five-year patient survival (7). In glioma, RAD18 was found to suppress apoptosis and accelerate cell proliferation (8). In cervical cancer, RAD18 was found to promote the migration and metastasis of cancer cells through the interleukin (IL)-1 β pathway (9). In esophageal squamous cell carcinoma, RAD18 exhibited the characteristics of an oncogene and promoted tumor metastasis through the JNK-MMP pathway (10). Our previous work demonstrated that RAD18 expression increases the resistance to radiotherapy and chemotherapy in CRC cells (11). However, the association between the expression of RAD18 and metastasis in CRC remains unclear.

In the present research study, we analyzed differences in the expression of RAD18 in CRC tissues and found that overexpression of RAD18 was closely related to the strength of metastatic and invasive tumor phenotype in CRC. However, the possible cellular mechanisms and molecular signal regulation were not well understood. Epithelial-mesenchymal transition (EMT) is undoubtedly one of the important mechanisms of

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tumor metastasis (12). EMT is a process considered to be one of the initial steps in the invasion and metastasis cascade, during which tumor epithelial cells dedifferentiate into mesenchymal cells, separating from the original site to new transfer sites (13). EMT changes the polarity of the cells, deconstructs the cell connections, adjusts the motility of cells, and modifies the cytoskeleton, changes that together may contribute to the promotion of tumor cell metastasis (14). To evaluate whether EMT occurs requires the detection of relevant molecular markers such as E-cadherin, N-cadherin, and vimentin (15). Hence, we further examined the role of EMT-related molecular markers in the context of RAD18-mediated invasion and migration of CRC cells.

Materials and methods

Clinical data and pathological specimen collection. We collected samples from 93 patients with CRC who were treated at the Nanjing Medical University Affiliated Suzhou Hospital from November 2009 to May 2010, and we obtained adjacent normal tissue samples from 87 of them. The mean age \pm standard deviation was 66.76 ± 14.01 years (range, 22-93 years). Among the patients, 52 were males and 41 were females. None of the patients received any treatment (such as chemotherapy, radiotherapy, or biotherapy) before surgery. All specimens were confirmed to be adenocarcinoma by pathology. The Ethics Committee of Nanjing Medical University (Suzhou, Jiangsu, China) approved this research, and all patients signed an informed consent before surgery. The follow-up deadline for all patients was June 6, 2016.

Immunohistochemical staining and pathological evaluation criteria. All tissue samples were immunohistochemically stained using 10% formalin-fixed for >24 h at room temperature, and paraffin-embedded [sections (4- μ m)] using conventional labeling with horseradish peroxidase (HRP). The nuclei were counterstained with hematoxylin. The primary antibodies used were rabbit polyclonal anti-human RAD18 antibody (dilution 1:100, cat. no. ab188235; Abcam Biotechnology), and rabbit polyclonal anti-human E-cadherin (cat. no. ab32741), N-cadherin (cat. no. ab34241), and vimentin (cat. no. ab36067) antibodies (dilution 1:50; MultiSciences Biotech). The primary antibody was incubated at 37°C for 2 h, and horseradish peroxidase (HRP)-conjugated anti-rabbit antibody (cat. no. ab6721; Abcam Biotechnology) was incubated at 37°C for 1 h. The stained sections were observed by a Leica microscope (magnification, $\times 200$, Leica). The results were assessed as follows: The intensity of staining was scored 0, 1, 2, and 3 according to the degree of color (no color, weak, moderate, and strong color, respectively). The area of staining was scored 0, 1, 2, 3, and 4 according to the following: 0-10, 11-25, 26-50, 51-75 and >75%, respectively. The final score was equal to the product of the above two scores. Two pathologists examined all specimens in a blinded manner. When the scoring results differed between the scorers, a final conclusion was reached through discussion. Specimens with a final score of ≤ 6 were taken to have low expression, and scores >6 were deemed to have high expression.

Nucleic acid extraction and quantitative RT-PCR. TRIzol (Invitrogen Life Technologies; Thermo Fisher Scientific,

Inc.) was used to extract total RNA from frozen CRC tissue samples according to the manufacturer's instructions. The RNA concentration was determined using a NanoDrop2000 (NanoDrop; Thermo Fisher Scientific, Inc.). Total RNA (1 μ g) was reverse transcribed to cDNA, which was then used as a template for RT-qPCR to determine the cycle threshold (Cq) of each tissue. The experimental data were analyzed using the $2^{-\Delta\Delta Cq}$ method (16) to calculate mRNA expression of RAD18, E-cadherin, N-cadherin, vimentin, and GAPDH. All tests were repeated three times and normalized to GAPDH. The primers for RT-PCR were RAD18 F (forward), 5'-GTCCTTTCATCC TCTACTCTCGT-3' and R (reverse), 5'-TAGCCTTCTCTA TGTTGTCTATCCC-3'; E-cadherin F, 5'-CGAGAGCTACAC GTTCACGG-3' and R, 5'-GGGTGTCGAGGGAAAAAT AGG-3'; N-cadherin F, 5'-TGCGGTACAGTGTAACTGGG-3' and R, 5'-GAAACCGGGCTATCTGCTCG-3'; vimentin F, 5'-CCAGGCAAAGCAGGAGTC-3' and R, 5'-GGGTATCAA CCAGAGGGAGT-3'; GAPDH F, 5'-CGACCACTTTGTCAA GCTCA-3' and R, 5'-AGGGGAGATTTCAGTGTGGTG-3'. The PCR reactions were performed in duplicate at 95°C for 2 min and subjected to 40 cycles of 95°C for 5 sec and 60°C for 35 sec.

Cell culture and pharmaceutical reagents. Human CRC cell lines HCT116, DLD-1, and SW480 were purchased from the Shanghai Cell Bank (Shanghai, China) and identified using short tandem repeat profiling. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone. All cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin, and streptomycin in a humidified atmosphere, with 5% CO₂ at 37°C in an incubator. Subculturing was carried out when the cells reached 85% confluence or more, and experiments were carried out in cells that were passaged three times.

Establishment of stably transfected cell strains with upregulated and downregulated expression of RAD18. HCT116, DLD-1, and SW480 cells were seeded in 24-well plates at 5.0×10^4 cells/well, and confluency was allowed to reach 70-80% by the next day. A lentivirus-based short hairpin RNA (shRNA) vector targeting the RAD18 gene and a lentivirus-based cDNA (GenePharm) were added separately to the above cells using Lipofectamine 3000 (Invitrogen) according to the instructions of the manufacturer. The final stably transfected lines, which were selected with puromycin for 7 days and genotyped by PCR, were HCT116 LV3, HCT116 RAD18sh, DLD-1 LV3, DLD-1 RAD18sh, SW480 LV5, and SW480 RAD18.

Extraction of proteins and western blot analysis of protein expression. The cells were trypsinized, harvested, centrifuged, washed twice with PBS, and dissolved in RIPA buffer (Beyotime Biotechnology) on ice, followed by centrifugation at $15,000 \times g$ for 15 min. The supernatant was collected, and the protein concentration was measured with the Bicinchoninic Acid (BCA) Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.). Equal aliquots (20 μ g) from the samples were loaded and run into each lane of 10% SDS-PAGE gels (Amresco), and then transferred to a PVDF membrane (Millipore). After blocking with 5% non-fat milk

in Tween-20 (TBST) in Tris-buffered saline for 1 h at room temperature, the membrane was incubated overnight at 4°C with the appropriate concentration of primary antibody. We used the following antibodies: Rabbit polyclonal anti-human RAD18 antibody (dilution 1:1,000, cat. no. ab188235; Abcam Biotechnology); E-cadherin (cat. no. ab32741), N-cadherin (cat. no. ab34241), vimentin (cat. no. ab36067) polyclonal rabbit anti-human antibodies (dilution 1:500; MultiSciences Biotech); and β -actin monoclonal mice anti-human antibody (dilution 1:1,000, cat. no. sc-47778; Santa Cruz Biotechnology). β -actin antibody was used as a loading control to ensure equal protein loading. After washing three times with TBST, the membrane was incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibody for 2 h. The protein was visualized by enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology). The western blotting results were quantified by ImageJ software version 1.52p [National Institutes of Health (NIH)].

Wound-healing assay and Matrigel Transwell chamber experiment. Cell migration was examined using the wound-healing assay. CRC cells were seeded in a 6-well culture plate at 5.0×10^5 cells/well. In cell cultures that had grown to confluence, typically 24–48 h later, scratches were made with a 200- μ l pipette tip. The detached cells were washed three times with PBS, and the remaining cells were incubated in culture medium without serum. Images were captured at 0, 24, 48, 72 and 96 h with an optical microscope (magnification, $\times 200$) used to assess the distance covered by the movement of the cells.

Cell invasion was determined by the Matrigel Transwell chamber experiment following previous descriptions (17,18). The Transwell chamber (Corning, Inc.) was pre-coated with 60 μ l Matrigel (1:6 dilution; BD Biosciences); 200 ml serum-free medium was added to the upper chamber, and 600 ml of 10% serum medium was added to the lower chamber, as a chemical attractant. The upper chamber, which was separated from the lower one by an 8.0- μ m polycarbonate membrane, was inoculated with the same number of CRC cells (HCT116 and DLD-1, 1×10^5 ; SW480, 5×10^4), cultured for 48 h, fixed with 3.7% paraformaldehyde, and stained with Giemsa for 5 min at room temperature. The cells in the upper chamber were wiped with a cotton swab, and cells that had passed through the membrane were photographed under a light microscope (magnification, $\times 200$).

Rescue and recovery experiments. Mutated RAD18-encoding plasmids obtained from Ribobio were used in the rescue experiment. Transient plasmid transfection was performed using Lipofectamine 3000 DNA transfection reagents (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Western blot analysis, wound-healing assay and Matrigel Transwell chamber experiments were performed after transfection with the above-described methods.

Statistical methods and data analysis. We used the SPSS v.18.0 software (SPSS Inc.) for statistical evaluation of the data, Graphpad PRISM v.5.0 (GraphPad Software, Inc.) for graphing, and ImageJ software v.1.52p [National Institutes of Health (NIH)] for analyzing western blot data and for counting the

numbers of Transwell cells. All experiments were repeated at least three times and are expressed as mean \pm standard error. A Chi-square test was used to analyze the correlation between RAD18 expression and the clinicopathologic data of patients. The overall survival rate of patients was calculated using the Kaplan-Meier method. Univariate and multivariate Cox proportional hazard regression analysis was used to calculate the hazard ratio (HR) of each variable to the 95% confidence interval (CI) for overall patient survival. Student's t-test was used to compare the western blotting protein expression levels, RT-PCR gene expression levels, open wound areas, and Transwell cell numbers between the two groups. For comparison of the three sets of data in the rescue experiment, we used Newman-Keuls method in one-way ANOVA. Pearson's correlation was used to analyze the correlation between the expression of two genes. A two-sided test with a significance level of $\alpha=0.05$ ($P<0.05$) was used.

Results

Immunohistochemistry shows high expression of RAD18 in cancer tissues. The immunohistochemical staining data of 93 cancer tissues and 87 corresponding adjacent tissues with the RAD18 antibody are shown in Fig. 1A. Most of the tumor tissues displayed deep brown staining, according to the criteria described in Materials and methods. RAD18 was highly expressed in 44 tissue specimens. Most of the adjacent normal tissues were stained lightly or appeared unstained, with only 16 tissue samples showing a high expression of RAD18. High expression of RAD18 was found in 47.31% (44/93) of tumor tissues and 18.39% (16/87) of normal adjacent tissues. A histogram of RAD18 expression in tumor and normal tissues (Fig. 1B) shows that RAD18 expression in tumor tissues was significantly higher than that noted in the normal tissues ($P<0.001$).

Clinical data of patients reveals that RAD18 is associated with pathological stage and lymphatic metastasis. The association of RAD18 with clinical data and pathological characteristics of 93 CRC patients is shown in Table I. Through the Chi-square test, we demonstrated that the degree of tumor differentiation, lymph node metastasis, tumor stage, and expression of MSH2 and RAD18 were positively correlated, and the results were statistically significant ($P<0.05$). RAD18 had no significant correlation with other clinical and pathological factors ($P>0.05$). The specific details are shown in Table I. The lymph nodes and pathological staging findings suggest that RAD18 is closely related to tumor metastasis.

Overall survival analysis illustrates that RAD18 is an independent prognostic factor. The results of univariate and multivariate Cox regression analyses are shown in Table II. Lymph node metastasis, tumor stage, and RAD18 expression were statistically significant in the univariate survival analysis ($P<0.01$). Tumor staging and expression of RAD18 were still statistically significant ($P<0.05$) when all significant factors were analyzed by multivariate analysis. We have reasons to believe that RAD18 is an independent prognostic factor. As shown in Fig. 2, we performed Kaplan-Meier analysis of patients with high and low expression of RAD18. The results

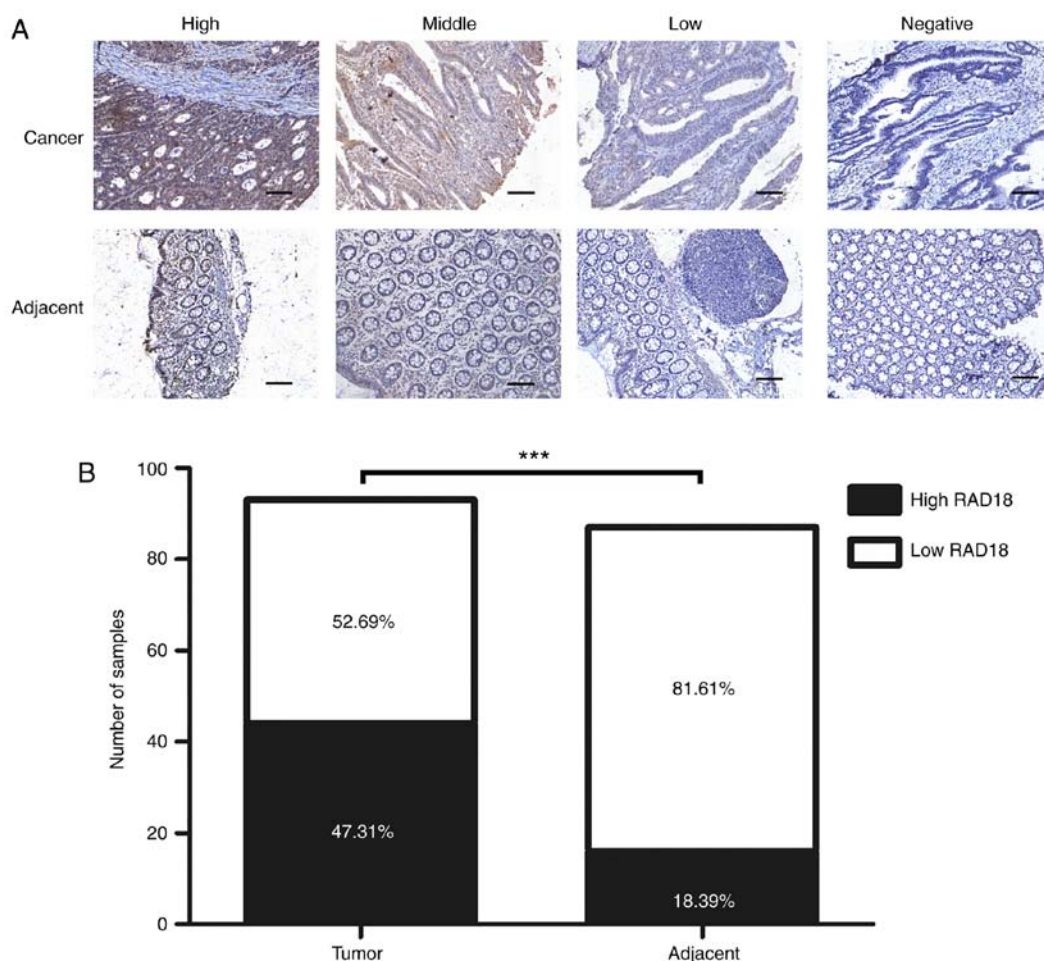


Figure 1. RAD18 expression in CRC tissues and adjacent normal tissues (Scale bar, 100 μ m; magnification, x200). (A) Immunohistochemical analysis of RAD18 in tumor tissue samples. (B) RAD18 expression was examined by IHC in 93 CRC tissue samples and 87 matched adjacent normal colorectal tissue samples. RAD18 expression was significantly increased in tumor tissues compared with that in adjacent colorectal tissues. *** $P < 0.001$, Chi-square test. RAD18, E3 ubiquitin protein ligase; CRC, colorectal cancer; IHC, immunohistochemistry.

showed that the survival curves were clearly separated and that the difference was significantly different ($P = 0.0082$).

Experiments with transfected cell lines confirm that RAD18 promotes the migration and metastasis of CRC cells. We established stably transfected clones with low expression of RAD18 by introducing small interfering RNA into CRC cell lines HCT116 and DLD-1. At the same time, stably transfected SW480 strains with high expression of RAD18 were obtained by transfection with a lentivirus vector containing a RAD18 cDNA. The expression of the protein was verified by western blot analysis (Fig. 3A and B) and RT-PCR (Fig. 3C). Next, we carried out a scratch test and Matrigel Transwell experiment on the three pairs of transgenic cell lines (HCT116, DLD-1, and SW480) knocked down for RAD18 or overexpressing RAD18. Wound-healing assays demonstrated the migratory ability of the cells (Fig. 4). We found that the mobility of the cell lines with low expression of RAD18 was markedly reduced and the mobility of the cell lines showing high expression of RAD18 was increased, and the results were statistically significant ($P < 0.01$ and $P < 0.05$). The Matrigel Transwell experiment demonstrated the invasiveness of the cells (Fig. 5). Knockdown of RAD18 significantly reduced the invasive ability of the HCT116 ($P < 0.001$) and DLD-1 cells ($P < 0.05$; Fig. 5A, B, D and E). In contrast, overexpression

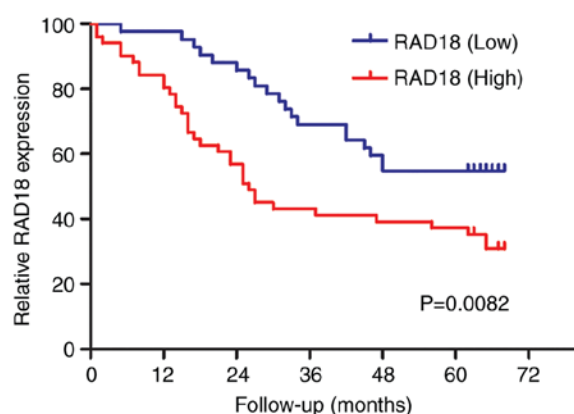


Figure 2. RAD18 expression correlates with poor prognosis in human CRC patients. Kaplan-Meier overall survival curves for patients with Low RAD18 and High RAD18. RAD18, E3 ubiquitin protein ligase; CRC, colorectal cancer.

of RAD18 significantly ($P < 0.01$) increased the invasive ability of the SW480 cells (Fig. 5C and F).

Clinical specimens and cell experiments indicate that RAD18 promotes CRC metastasis through the EMT signaling pathway.

Table I. Association of RAD18 expression with clinicopathological characteristics of the CRC patients (N=93).

| Variables | N | RAD18 expression, n (%) | | χ^2 | P-value |
|---------------------------|----|-------------------------|-----------|----------|--------------------|
| | | Low | High | | |
| Sex | | | | 0.591 | 0.442 |
| Male | 52 | 25 (48.1) | 27 (51.9) | | |
| Female | 41 | 23 (56.1) | 18 (43.9) | | |
| Age (years) | | | | 0.056 | 0.813 |
| ≤60 | 34 | 17 (50.0) | 17 (50.0) | | |
| >60 | 59 | 31 (52.5) | 28 (47.5) | | |
| Degree of differentiation | | | | 5.551 | 0.018 ^a |
| Well/Moderate | 12 | 10 (83.3) | 2 (16.7) | | |
| Poor | 81 | 43 (53.1) | 38 (46.9) | | |
| pT | | | | 0.253 | 0.615 |
| T1-3 | 69 | 34 (55.1) | 35 (44.9) | | |
| T4 | 16 | 9 (62.5) | 7 (37.5) | | |
| pN | | | | 4.669 | 0.031 ^a |
| N0 | 56 | 34 (60.7) | 22 (39.3) | | |
| N1-3 | 37 | 23 (62.2) | 14 (37.8) | | |
| TNM stage | | | | 4.652 | 0.031 ^a |
| II | 54 | 33 (61.1) | 21 (38.9) | | |
| III-IV | 39 | 15 (38.5) | 24 (61.5) | | |
| MSH2 | | | | 4.321 | 0.038 ^a |
| Low | 56 | 24 (42.9) | 32 (57.1) | | |
| High | 37 | 24 (64.9) | 13 (35.1) | | |
| MSH6 | | | | 0.014 | 0.905 |
| Low | 81 | 42 (51.9) | 39 (48.1) | | |
| High | 12 | 6 (50.0) | 6 (50.0) | | |

^aP<0.05, Fisher's exact test was performed. CRC, colorectal cancer; N, number; p, pathological staging; TNM, Tumor-Node-Metastasis; MSH2, mutS homolog 2; MSH6, mutS homolog 6; RAD18, E3 ubiquitin protein ligase.

Table II. Univariate and multivariate Cox regression analysis for OS in CRC patients.

| Variates | Categories | Univariate analysis | | Multivariate analysis | |
|------------------|------------------------|---------------------|---------------------|-----------------------|--------------------|
| | | HR (95% CI) | P-value | HR (95% CI) | P-value |
| Sex | Female vs. Male | 1.212 (0.706-2.081) | 0.485 | | |
| Age | >60 vs. ≤60 years | 1.504 (0.826-2.736) | 0.182 | 1.810 (0.954-3.435) | 0.069 |
| Differentiation | Poor vs. Well/Moderate | 1.904 (0.758-4.787) | 0.171 | 1.150 (0.433-3.053) | 0.779 |
| pT | T4 vs. T1-3 | 1.557 (0.899-2.697) | 0.114 | 1.489 (0.823-2.694) | 0.189 |
| pN | N1-3 vs. N0 | 2.782 (1.610-4.809) | <0.001 ^b | 0.300 (0.061-1.463) | 0.136 |
| TNM stage | III-IV vs. II | 3.092 (1.776-5.383) | <0.001 ^b | 9.067 (1.781-46.152) | 0.008 ^b |
| MSH2 expression | High vs. Low | 0.635 (0.359-1.122) | 0.118 | 0.760 (0.412-1.402) | 0.760 |
| MSH6 expression | High vs. Low | 0.554 (0.220-1.392) | 0.209 | | |
| RAD18 expression | High vs. Low | 2.365 (1.361-4.110) | 0.002 ^b | 1.809 (1.005-3.256) | 0.048 ^a |

^aP<0.05, ^bP<0.01; Fisher's exact test was performed. OS, overall survival; HR, hazard ratio; CI, confidence interval; p, pathological staging; TNM, Tumor-Node-Metastasis; MSH2, mutS homolog 2; MSH6, mutS homolog 6; RAD18, E3 ubiquitin protein ligase.

The proteins from the three CRC cell lines were subjected to western blot assay to examine the expression of RAD18 and the

EMT-related proteins E-cadherin, N-cadherin, and vimentin. We found that RAD18 expression was negatively correlated

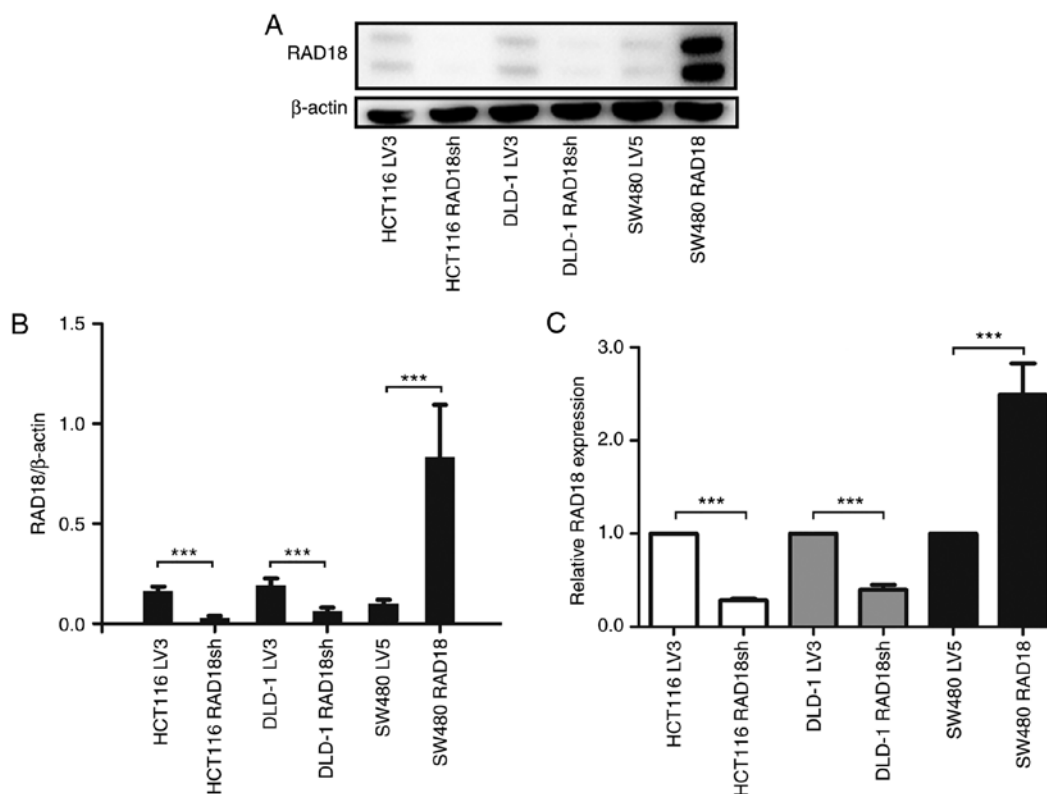


Figure 3. Establishment of stably transfected strains of HCT116, DLD-1, SW480 cell lines. (A) Western blot analysis of RAD18 expression in the three stably transfected strains. (B) Results of the quantitative analysis of the western blotting by ImageJ. (C) Results of RT-PCR of RAD18 in the three stably transfected strains. ***P<0.001, Student's t-test. RAD18sh, RAD18-knockdown cells; SW480 RAD18, RAD18-overexpressing SW480 cells. RAD18, E3 ubiquitin protein ligase.

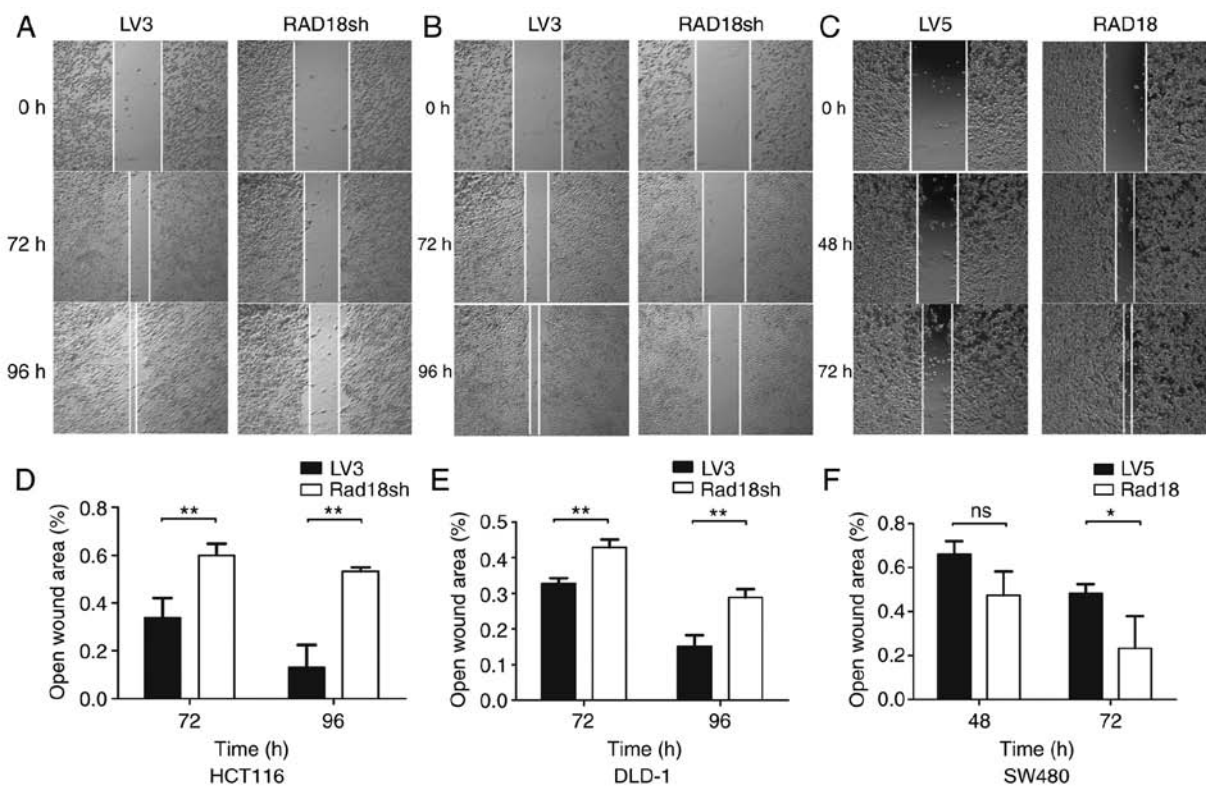


Figure 4. Effect of RAD18 on cell migration *in vitro*. Wound-healing assay was performed to examine cell migration. The ability of cell migration was suppressed after RAD18 knockdown in both (A) HCT116 and (B) DLD-1 cells, whereas (C) overexpression of RAD18 enhanced the cell migration of SW480 cells. (D-F) The open wound area was normalized to the area at the initial time (0 h) and the percentage of the filled wound area was calculated and represented as mean \pm SD relative to the control. *P<0.05, **P<0.01, Student's t-test. NS, not significant. RAD18, E3 ubiquitin protein ligase. RAD18sh, RAD18-knockdown cells; RAD18, RAD18-overexpressing cells.

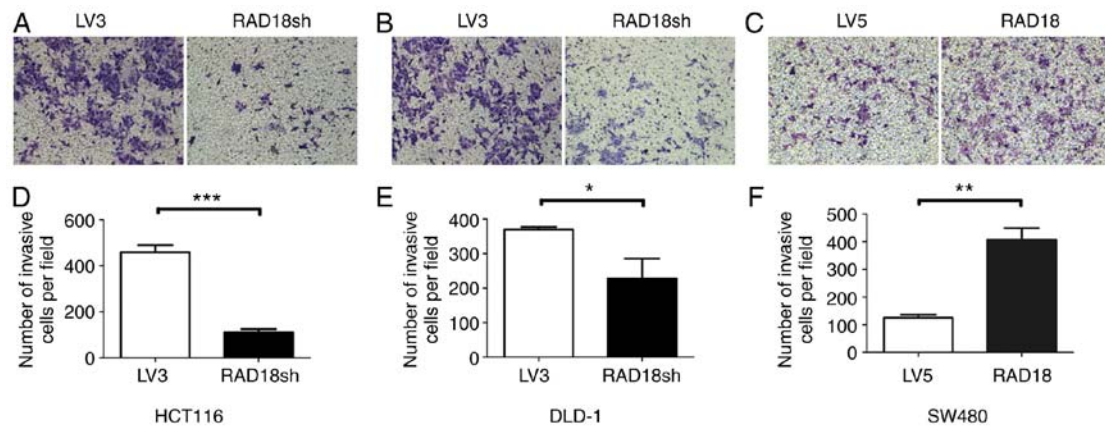


Figure 5. RAD18 promotes CRC cell invasion *in vitro*. The Matrigel Transwell assay was performed to determine cell invasion potential. The number of cells migrated to the bottom was calculated manually. RAD18 knockdown suppressed the cell invasion in HCT116 and DLD-1 cells, whereas overexpression of RAD18 enhanced the cell invasion of SW480 cells. (A-C) Representative images of invasive cells. (D-F) Quantification analysis of the invasive cell number per field. Data represent mean \pm SD of duplicates from three fields of view. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t-test. RAD18sh, RAD18-knockdown cells; RAD18, RAD18-overexpressing cells. RAD18, E3 ubiquitin protein ligase; CRC, colorectal cancer.

with E-cadherin and positively correlated with N-cadherin and vimentin (Fig. 6A). These findings were confirmed by the analysis of the protein levels in clinical samples using immunohistochemical staining. Images of representative tissue samples stained for the above EMT-related proteins and RAD18 are shown in Fig. 6B. Interestingly, the results of the tissue samples were strikingly consistent with those obtained with the cell lines. To further explore the association of EMT-related proteins with RAD18 expression in CRC tissues at the organizational level, we used RT-PCR to detect the expression levels of E-cadherin, N-cadherin, vimentin, and *RAD18* mRNAs in 93 CRC clinical samples. The correlation between the RAD18 and EMT-related protein levels is shown in Fig. 6C. High expression of RAD18 was accompanied by a high expression of N-cadherin and vimentin ($P < 0.001$) and low expression of E-cadherin ($P < 0.001$). These findings indicate that the activation of EMT could be a vital pathway by which RAD18 promotes migration and metastasis of CRC cells.

Rescue experiments suggest that the mutant RAD18 reverses the genetic phenotype of the RAD18-knockdown cells. After RAD18-silenced DLD-1 RAD18sh cells were retransfected with the mutated RAD18 gene, the protein expression of RAD18 was restored, and the expression of EMT-related proteins was correspondingly reversed (Fig. 7A and B). Transwell experiment found that the invasion ability of DLD-1 RAD18sh cells was restored (Fig. 7C and D), and the migration ability of DLD-1 RAD18sh cells was also restored in the scratch experiment (Fig. 7E and F).

Discussion

Metastasis is one of the main characteristics of tumors, and most patients with colorectal cancer (CRC) succumb to the disease due to distant metastasis (19). The transfer of tumor cells from the primary tumor site to a non-adjacent organ to form a secondary tumor is a complex multi-step process (20). The first step in tumor cell metastasis is the infiltration of normal tissues surrounding the tumor (20). Cancer migration and invasion are the major factors that

determine metastasis (21). Therefore, effective inhibition of migration and invasion of tumor cells is crucial to the control of metastasis in CRC. Although a series of intracellular and extracellular protein biomarkers for CRC have been identified as potential prognostic and predictive markers by various methods (22-29), the conversion of the growing differential proteome data into a database that could be used as a clinical tool to predict the patient prognosis is still lacking (30). Thus, it is imperative to identify more effective biomarkers that can be used for the reliable prediction of metastasis in CRC.

RAD18 is an E3 ubiquitin ligase that plays a key role in promoting PCNA mono-ubiquitination. It was reported to be involved in carcinogenesis and tumor progression owing to its role in error-prone DNA synthesis. High expression of RAD18 promotes melanoma cell proliferation (7). Low expression of RAD18 inhibits glioblastoma development (8). Previously, our data demonstrated that RAD18 is a cancer-promoting gene for metastatic esophageal squamous cell cancer (10). In the present study, we found that RAD18 expression levels were significantly increased in CRC tissues compared with that noted in the adjacent non-cancerous normal tissues. The level of RAD18 expression was also found to be positively associated with lymph node metastasis and poor prognosis in patients with CRC. Consistent with these findings, we additionally found that RAD18 promotes mobility and invasiveness of CRC cells in well-established cell model systems. However, to elucidate whether this result is due to RAD18, we further carried out a rescue experiment. The experimental results showed that the invasion and migration ability of DLD-1 cells were weakened after downregulation, and the invasion and migration ability of the cells were restored after the *RAD18-c* plasmid was again transfected into the cells. Meanwhile, the expected synchronous changes were also found in EMT-related proteins. Hence, RAD18 may play a crucial role in the migration and invasion of CRC cells.

Epithelial-mesenchymal transition (EMT) is generally considered to be the first step in cancer metastasis because it promotes the migration of the tumor cells from the original site to the tumor stroma. One of the hallmarks of EMT, which

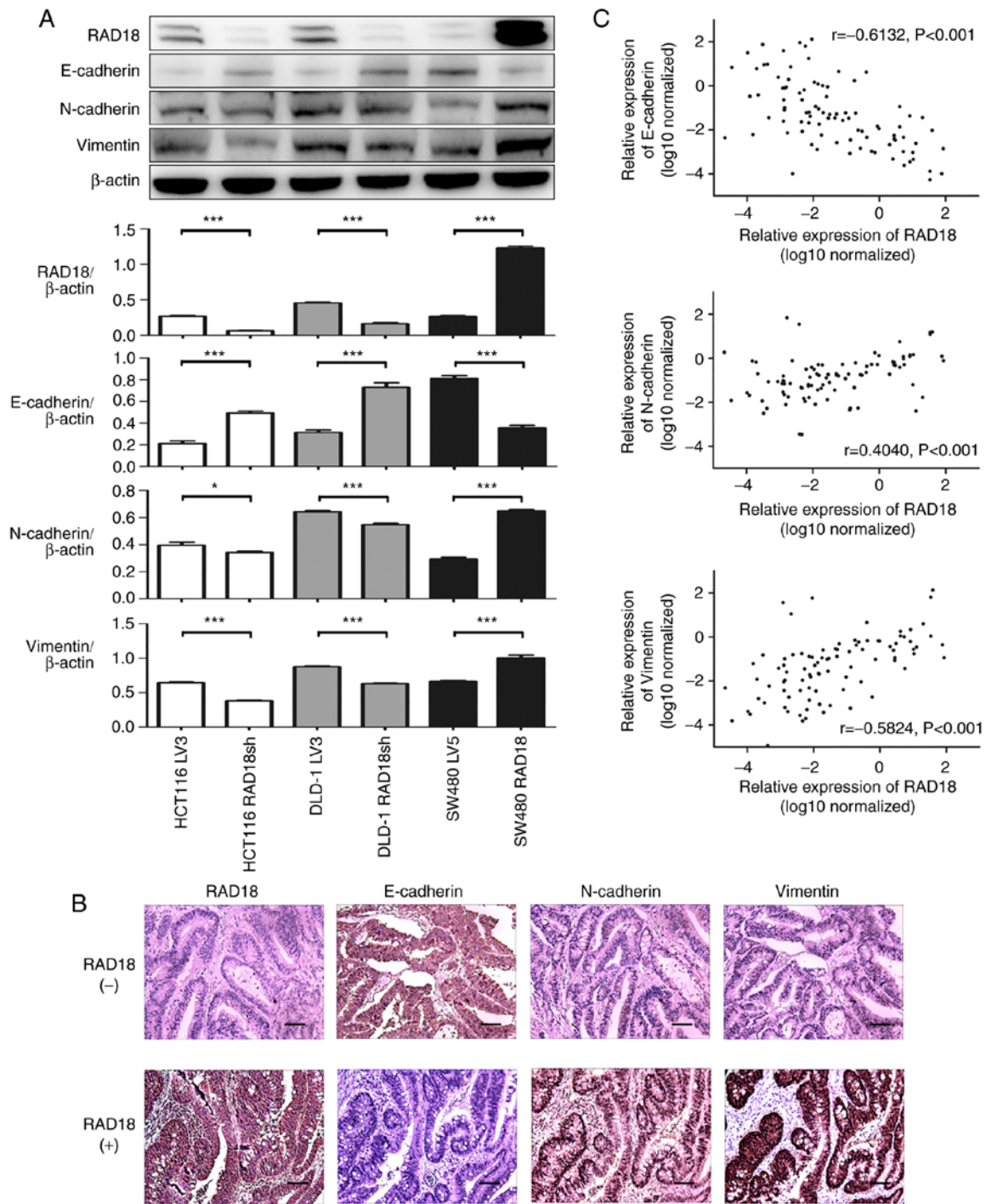


Figure 6. Overexpression of RAD18 increases the metastatic potential of CRC cells via the EMT pathway. (A) EMT biomarkers, including E-cadherin, N-cadherin, vimentin and RAD18 were detected by western blot analysis in HCT116 LV3 cells, HCT116 RAD18sh (RAD18 knockdown), DLD-1 LV3, DLD-1 RAD18sh (RAD18 knockdown), SW480 LV5 and SW480 RAD18 (RAD18-overexpressing) cell lines. All the experiments were repeated three to four times with similar findings. Band intensity was quantified by ImageJ software and are shown by a histogram. * $P < 0.01$, *** $P < 0.001$, Student's t-test. (B) Immunohistochemical analysis of RAD18, E-cadherin, N-cadherin and vimentin in CRC tissues. Representative patients, RAD18 (+) and RAD18 (-) were selected from 93 patients with CRC (Scale bar, 100 μ m; magnification, x200). (C) E-cadherin, N-cadherin, vimentin and RAD18 expression was detected in 93 CRC tissues by PCR. RAD18 expression was positively correlated with N-cadherin and vimentin expression ($P < 0.001$), but negatively correlated with E-cadherin expression ($P < 0.001$). EMT, epithelial-mesenchymal transition; RAD18, E3 ubiquitin protein ligase; CRC, colorectal cancer.

is essential for this process to occur, is the cadherin switch, where E-cadherin is downregulated, and N-cadherin is upregulated (31-33). Current studies have shown that EMT in CRC cells is a key factor in distant metastasis of colorectal cancer (12). In the CRC cell line, we observed that an increase in RAD18 expression was associated with reduced E-cadherin

expression and increased N-cadherin and vimentin expression. Consistent with this observation, our clinical data also demonstrated that the expression of RAD18 affected the expression of the EMT markers. Therefore, the EMT signaling pathway could be the molecular mechanism by which RAD18-mediated CRC cell metastasis takes place.

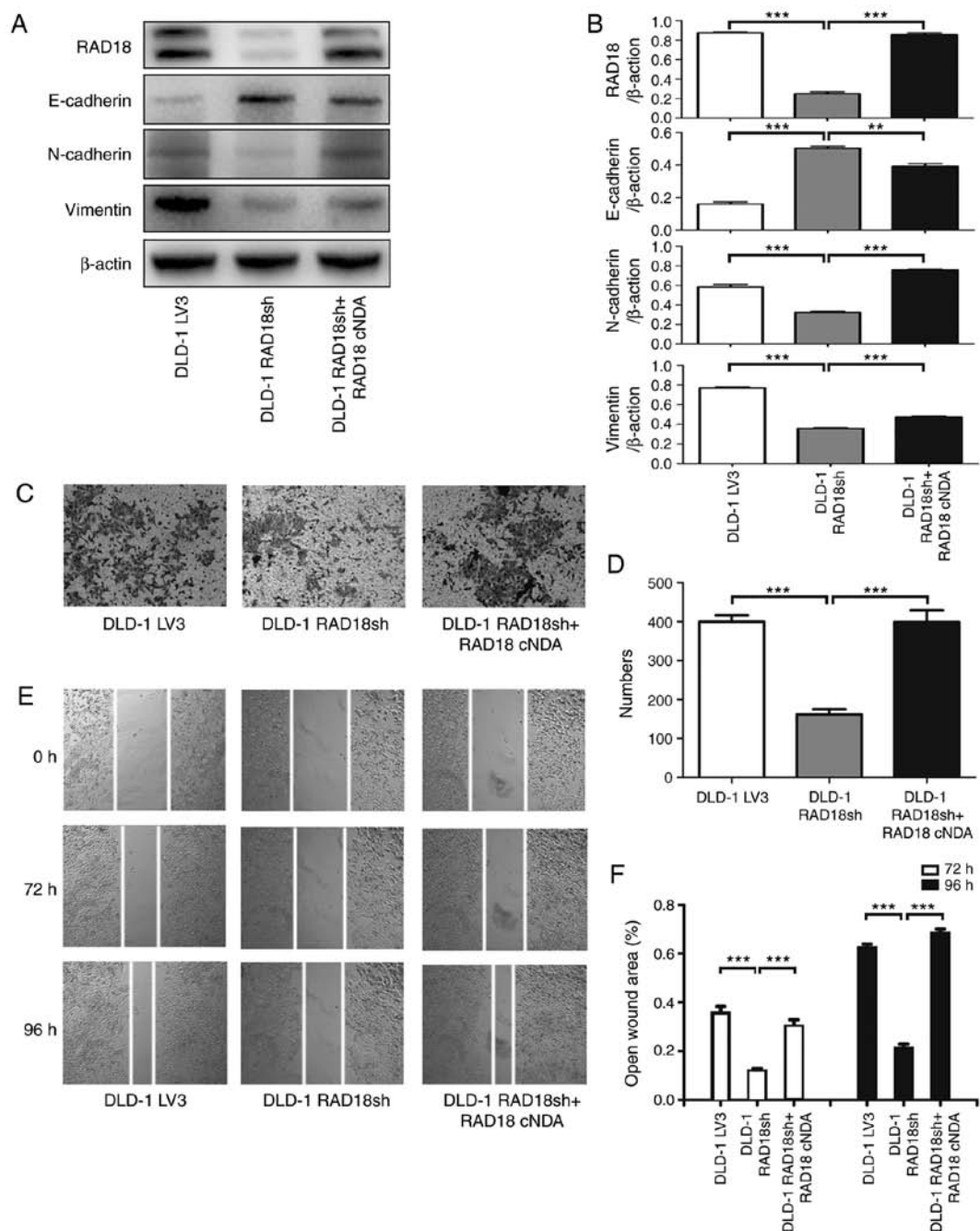


Figure 7. The mutant RAD18 reverses the genetic phenotype of RAD18-knockdown cells. DLD-1 RAD18sh (RAD18 knockdown) cells were also transfected with the RAD18-encoding plasmid. (A and B) EMT biomarkers, including E-cadherin, N-cadherin, vimentin and RAD18 were detected by western blot analysis, and quantified (right panel). (C and D) Transwell assays and (E and F) wound-healing assays were performed to confirm the RAD18-mediated invasion and migration ability of DLD-1 cells, and the results were quantified and shown in histograms (right panels). ** $P < 0.01$, *** $P < 0.001$, Newman-Keuls. EMT, epithelial-mesenchymal transition; RAD18, E3 ubiquitin protein ligase.

RAD18 has been found to actively promote migration and invasion of CRC cells by activating the EMT signaling pathway, but the exact mechanism remains unclear. Therefore, in future studies, we will further establish a nude mouse model of CRC metastasis through caudal vein injection and demonstrate that RAD18 promotes metastasis of colorectal cancer cells to liver, lung and other organs. At the same time, the collected blood and tissue from mice will be used to explore and identify the molecular mechanisms linking RAD18 and the EMT pathway to other signaling pathways that may contribute to the migration and invasion of CRC cells. Based on available data, we

believe that RAD18 could play a crucial role in a subset of patients with metastatic tumors and advanced stage disease. RAD18 may also be a potential therapeutic target for treating CRC patients.

This research is the first to report that RAD18 promotes the metastasis of CRC. We also demonstrated that the EMT signaling pathway plays a vital role in RAD18-mediated metastasis. These conclusions suggest that RAD18 is an essential biomarker for distant metastasis of CRC, and further studies should aim at exploring its use for the diagnosis and treatment of metastatic CRC.

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Availability of data and materials

The data used to support the results of this study are included in this article.

Authors' contributions

PL conducted most of the experiments and drafted the manuscript. CH and AG provided guidance and assistance with conduction of the experiments. XY analyzed the data and plotted the charts. XX performed the statistical analysis. JZ and JW designed the research, and reviewed the manuscript. All authors read and approved the manuscript 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 study was approved by the Ethics Committee of Nanjing Medical University, and all patients signed an informed consent before surgery. The research was conducted following the Helsinki Declaration of the World Medical Association.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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