Snail inhibits metastasis via regulation of E-cadherin and is associated with prognosis in colorectal cancer

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Abstract. The overall survival (OS) rate of patients with colorectal cancer (CRC) remains low due to the lack of clear prognostic markers. Therefore, the identification of valuable prognostic markers is urgently required. Snail and E-Cadherin (E-Cad) are important protein molecules in the EMT process and play a crucial role in tumor invasion and metastasis. The present study investigated the clinical significance of Snail and E-cad expression in CRC. Compared with those in adjacent tissue, the expression levels of Snail and E-cad were significantly increased and decreased, respectively, in CRC. Moreover, low Snail and high E-cad expression were associated with clinicopathological features and longer OS time. Furthermore, Snail and E-cad could predict the prognosis of patients with CRC. Reverse transcription-qPCR, Western blotting, Wound scratch assay, High content cell migration experiment, which showed that low Snail or high E-cad expression inhibited invasion and metastasis of CRC. In conclusion, Snail can promote CRC invasion and metastasis by regulating E-cad. Snail and E-cad expression constitute a novel prognostic marker for CRC, and the present study revealed a greater combined effect of Snail and E-cad as effective prognostic markers in CRC for the first time.

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Introduction

Colorectal cancer (CRC) is a prevalent gastrointestinal malignancy. Recent data show that it has the second highest mortality and third highest incidence worldwide (1). Despite improvements in early diagnosis and treatment techniques, developing countries are still experiencing an increase in the incidence rate and mortality of CRC (2). Additionally, patients with metastatic CRC have a poor prognosis with a median 5-year survival rate of 18.5% in the United States and 27.7% in Europe (1). Therefore, it is imperative to identify effective biomarkers and therapeutic targets to improve patient prognosis.

Epithelial-mesenchymal transition (EMT) is a key process in embryonic development. Studies have shown that it contributes to tumor progression. EMT causes epithelial cells to acquire fibroblast-like characteristics, decreases intercellular adhesion and increases motility (3,4). Snail, a member of the zinc finger transcription factor Snail family, induces EMT by downregulating EMT-associated genes, including E-cadherin (E-cad), claudin, occludin, protein associated with mouse musculus veli-7, membrane-associated guanylate kinase homolog (MAGUK) p55 family member and Pals1-associated tight junction (PATJ) crumbs cell polarity complex component (4,5). E-cad, a cadherin protein family member, is a component of adhesion junctions and the primary organizer of the epithelial phenotype (6,7). Studies have shown that the loss of E-cad expression is associated with tumor progression and metastasis and induced expression of E-cad in cancer cells can prevent tumor progression and invasion (5,8).

The present study investigated the role of Snail and E-cad in CRC and demonstrated that they can individually predict CRC prognosis, with their joint prediction having a greater combined effects that can more accurately predict patient prognosis.

Materials and methods

Patients and cancer tissue samples. Data of 470 patients with CRC were collected from Yixing People's Hospital affiliated

to Yangzhou University in the present study. The patients underwent radical colon cancer surgery at the Department of Oncology of Yixing People's Hospital between January 2006 and December 2010 and were followed up for at least 5 years. The mean age of 470 patients is 63, these are 282 males and 188 females. The clinicopathological features are shown in our previously published article (9). Overall survival (OS) was the primary end point, which was calculated from the date of surgery to the date of death or final follow-up.

The Ethics Committee of Yixing Hospital approved the present study, which was performed according to the principles of the Declaration of Helsinki. The human and animal experiments were approved by the Ethics Committee of Yixing Hospital Affiliated to Yangzhou University (approval no. YXYLL-2015-42). All patients provided written informed consent for use of their tissues.

Construction of tissue microarray (TMA) and immunohistochemistry. Tumor tissues were selected from paraffin blocks and confirmed by hematoxylin and eosin staining. TMA construction was performed using cancer tissues and corresponding adjacent tissue (5 cm from cancer tissue). Each point on the TMA chip had a diameter of 1.5 mm to accommodate both tumor and non-tumor tissue. TMA chips were placed in a 55˚C incubator for 10 min and cooled at room temperature. These chips were placed in a cryostat and 4 μm thick slices were produced. The slices were placed in water at 45˚C for 2 min, baked at 58˚C for 18 h and stored at -20˚C for future use.

The immunostaining was performed as described previously (10). Rabbit monoclonal antibodies, including anti-C-terminus of Hsc70-interacting protein (CHIP; 1:100; no. 1132; Cell Signaling Technology, Inc.) and Gal1 (cat. no. ab108389; 1:100; Abcam), were incubated at 4˚C overnight. The staining score of the tissue controls were pre-evaluated to ensure the quality control of immunostaining for each microarray slide.

Evaluation of immunostaining. Two pathologists who were blinded to the clinical data scored the staining of Snail or E-cad in the tissue. The presence of Snail or E-cad in cancer and adjacent tissue was evaluated using the semi-quantitative immunoreactivity score (IRS) reported previously (11). Intensity of immunostaining was categorized as 0-3 (0, negative; 1, weak; 2, moderate; 3, strong). Proportion of immunoreactive cells was categorized as 1, (0-25%), 2 (26-50%), 3 (51-75%) and 4 (76-100%). The product of these scores was used to calculate IRS ranging from 0 to 12. To determine the optimum cutoff value of Snail or E-cad IRS for 1-, 3- and 5-year OS rate, receiver operator characteristic (ROC) analysis was used. The optimum cutoff point for CHIP IRS was 4 since it had the best predictive value for survival.

Cell lines and animals. HCT 116 and HT 29 CRC cells were obtained from Procell Life Science & Technology Co., Ltd. These cells were cultured in RPMI-1640 medium supplemented with 10% FBS (Beyotime Biotechnology) and 1% penicillin/streptomycin. All cells were incubated at 37˚C with 5% CO_2. These cells were authenticated by short tandem repeat profiling.

Female BALB/c nude mice were obtained from the Comparative Medicine Laboratory Animal Center [license no. scxk (SU) 2012-0004] of Yangzhou University. The mice (age, 6-8 weeks) were kept in specific pathogen-free conditions and cared for according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

A total of ~$2x10^6$ HCT 116 stable cells and control cells (0.2 ml/mouse; 5 mice/group) were implanted subcutaneously into the flank of each mouse. After 21 days, the mice were sacrificed. All nude mice were euthanized by cervical dislocation and all animal experiments were conducted under the animal use license of Yangzhou University (no. SYXK2022-0044).

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from CRC tissue and cells. cDNA was synthesized using a PrimeScript™ RT kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. SYBR Green qPCR analysis (Applied Biosystems) was performed using the Applied Biosystems 7500 real-time PCR system (Roche Applied Science). The method of quantification was $2^{-\Delta\Delta Cq}$ (12). The sequences of the primers were as follows (5'□3'): E-cad forward, CGAGAGCTACACGTTCACGG and reverse, GGGTGTCGAGGGAAAAATAGG; Snail forward, CCTCGCTGCCAATGCTCATCTG and reverse, CTCTGC CACCCTGGGACTCTC and GAPDH forward, ACGGAT TTGGTCGTATTGGG and reverse, CGCTCCTGGAAGATG GTGAT (all Sangon Biotech Co., Ltd.).

Western blotting. Cells or tissues were lysed with cold lysis buffer supplemented with a protease inhibitor (Beyotime Biotechnology) on ice for 30 min. The total protein concentration was measured using the Bicinchoninic Acid Protein assay kit (Thermo Fisher Scientific, Inc.). Protein (80 μg/lane) was separated by SDS-PAGE on 10% gels. Subsequently, protein was transferred to the PVDF membrane, which was incubated with antibodies. The protocol was executed in the aforementioned manner. Rabbit monoclonal anti-E-cad (cat. no. ab40772; 1:1,000;Abcam), rabbit monoclonal anti-Snail (cat. no. ab216347; 1:1,000; Abcam) and mouse monoclonal anti-β-actin (cat. no. AF0003; 1:2,000; Beyotime Biotechnology) were used as the primary antibodies. ImageJ software (v 1.44; National Institutes of Health) was used to normalize expression to the expression of β-actin and the band strength of each protein was semi-quantified.

Wound scratch assay. HCT 116 cells (~$5x10^5$) were added into each well of a marked six-well plate to ensure that the plate was fully covered. After 24 h, a sterile pipette tip was used to scratch a horizontal line perpendicular to the bottom of the plate and the cells were washed with PBS three times. Serum-free RPMI-1640 medium (Beyotime Biotechnology) was added and cells were cultured in a 37˚C 5% CO_2 incubator. The process of tumor cell migration was observed and photographed at 50x magnification 0, 24 and 48 h after wounding.

High content cell migration experiment. The cells were plated in 96-well plates (2,000 cells/well). When the cells grew to

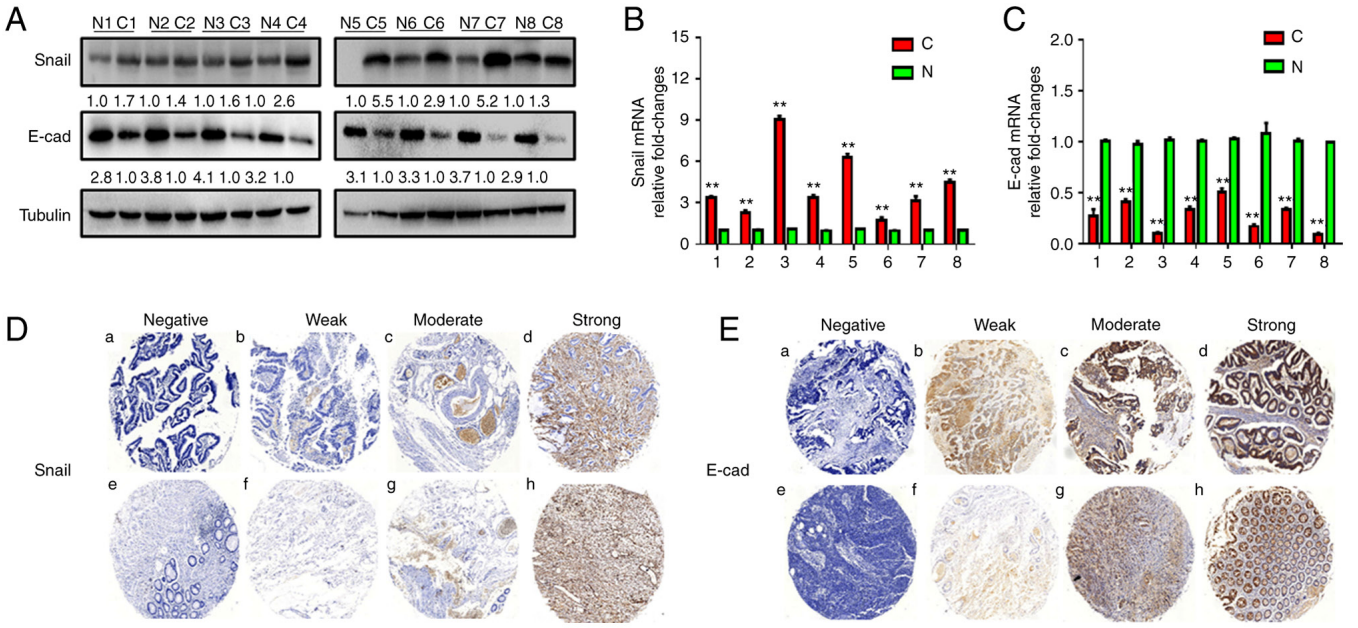

Figure 1. Snail and E-cad expression in colorectal cancer. (A) Ratio of grayscale values of each band: Expression of Snail was increased in C compared with paired N tissue by western blot, whereas expression of E-cad was decreased. The mRNA expression of (B) Snail and (C) E-cad was detected by reverse transcription-quantitative PCR in C compared with paired N tissue, **P<0.01. Representative images of (D) Snail and (E) E-cad immunohistochemical staining in a tissue microarray of (a-d) C and (e-h) adjacent N tissue. 40x magnification. C, cancer; N, normal; E-cad, E-cadherin.

80% confluence, the fresh RPMI-1640 medium supplemented with 10% FBS was refreshed. Under the conditions of 37˚C and 5% CO_2, the migration of cells was observed using an Operetta CLS high connotation cell imaging system (PerkinElmer). The cells were scanned and images were captured every 10 min for 12 h. The cell migration curve was constructed.

Lentiviral (LV) infection. LV vector (Shanghai GenePharma Co.,Ltd.) was used to knockdown or increase with Snail or E-cad expression. LV-Snail, LV-Snail-control (ctrl), LV-Snail-short hairpin RNA (shRNA) and LV-Snail-shRNA-ctrl; LV-E-cad, LV-E-cad-ctrl, LV-E-cad-shRNA and LV-E-cad-shRNA-ctrl were transfected into HT 29 and HCT 116 cells at a MOI of 20 with 10 μg/ml Polybrene (Shanghai GeneChem Co., Ltd.). The cells were maintained with normal RPMI-1640 culture medium at 37˚C with 5% CO_2 for 24 h after lentiviral infection 8 h. After 24 h, the cells were incubated in RPMI-1640 with 2 μg/ml puromycin. The sequences of the shRNAs and shRNA-control were as follows (5'→3'): Snail: CCACTCAGA TGTCAAGAAGTA and ctrl: TTCTCCGAACGTGTCACG TTT. E-cad: CAUGGAUAACCAGAAUAAATT and UUC UCCGAACGUGUCACGUTT.

Statistical analysis. The association between Snail and E-cad expression and clinicopathological data was evaluated using Fisher's exact test. IRS expression of Snail and E-cad in tumor and corresponding non-tumor tissue was compared using Wilcoxon signed-rank test (grouping). Kaplan-Meier (log-rank test) survival analysis was used to determine differences in OS (13). Univariate or multivariate Cox regression analysis was used to estimate the hazard ratio (HR) and 95% CI. STATA software (v10.1; StataCorp LP) was used to analyze all experimental data. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test. Mann-Whitney U was used as non-parametric test to compare unpaired data. P<0.05 was considered to indicate a statistically significant difference. All experiments were repeated in triplicate.

Results

Snail and E-cad expression in CRC vs. non-cancerous tissue. The present study used eight pairs of primary CRC and adjacent normal tissues to detect the protein expression levels of Snail and E-cad by western blotting. The protein expression levels of E-cad were lower in tumor compared with adjacent normal tissues, while expression levels of Snail were higher in tumor tissue (Fig. 1A). RT-qPCR was used to detect the mRNA levels of Snail and E-cad, which were higher and lower, respectively, in tumor compared with corresponding normal tissues (Fig. 1B and C).

To confirm Snail and E-cad expression in CRC tissue, immunohistochemical staining was performed (Figs. 1D and E and 2A and B). Expression levels of Snail were markedly upregulated in cancer compared with adjacent normal tissues. Similarly, the expression levels of E-cad were downregulated in tumor compared with adjacent non-tumor tissue (Fig. 2C and D).

Association between Snail and E-cad expression and clinicopathological data in patients with CRC. The present study analyzed the association between Snail and E-cad expression and clinicopathological characteristics of 465 patients with CRC. Snail expression was significantly associated with the depth of invasion, lymph node metastasis, TNM stage and distal metastasis (Table I; P<0.05). Similarly, expression levels of E-cad were significantly associated with pathological classification, lymph node metastasis, TNM stage and distal metastasis (Table II; P<0.05).

Table I. Association between expression levels of Snail and clinicopathological features of patients with colorectal cancer (n=469).

Characteristic	Low Snail, n=275 (58.6%)	High Snail, n=194 (41.4%)	P-value[a]
Age, years			0.077
≤65	164 (61.7)	102 (38.3)	
>65	111 (54.7)	92 (45.3)	
Sex			0.400
Male	166 (59.3)	114 (40.7)	
Female	109 (57.7)	80 (42.3)	
Pathological classification[b]			0.165
I	2 (50.0)	2 (50.0)	
II	253 (59.8)	170 (40.2)	
III	16 (44.4)	20 (55.6)	
Depth of invasion[b]			<0.001
T1/T2	83 (81.4)	19 (18.6)	
T3/T4	187 (51.7)	175 (48.3)	
Lymph node metastasis[b]			<0.001
N0	210 (76.4)	65 (23.6)	
N1/N2	61 (32.1)	129 (67.9)	
TNM stage[b]			<0.001
I	74 (85.1)	13 (14.9)	
II	131 (73.2)	48 (26.8)	
III	60 (33.3)	120 (66.7)	
IV	5 (29.4)	12 (70.6)	
Tumor diameter, cm[b]			0.254
≤5	224 (59.4)	153 (40.6)	
>5	50 (54.9)	41 (45.1)	
Distant metastasis			0.004
M0	270 (60.0)	180 (40.0)	
M1	5 (26.3)	14 (73.7)	

[a]Two-sided Fisher's exact test. [b]Patient clinical pathological data incomplete.

High Snail and low E-cad expression is associated with shorter survival time in patients with CRC. Kaplan-Meier analysis showed that high Snail expression or low E-cad expression in cancer tissue was significantly associated with poorer 5-year survival rates in patients with CRC (both P<0.001; log-rank test; Fig. 3C and D). Furthermore, univariate and multivariate Cox regression analyses revealed that Snail or E-cad were independent prognostic factors for patients with CRC. The results of the univariate Cox regression analysis demonstrated that Snail and E-cad expression were associated with OS in patients with CRC (Table III). Additionally, multivariate Cox regression analysis revealed that Snail and E-cad expression was an independent prognostic factor in patients with CRC (Table IV; Snail: HR, 0.181; 95% CI, 0.128-0.255; P<0.001; E-cad: HR, 0.212; 95% CI, 0.148-0.303; P<0.001).

Combined Snail and E-cad expression has greater predictive ability of OS in patients with CRC. The survival rate of the Snail low expression and E-cad high expression groups was higher than that of other groups (P<0.001, log-rank test; Fig. 3E). To verify whether Snail combined with E-cad had a great predictive effect on the prognosis of patients with CRC, the clinical risk score (TNM stage, histological type and tumor diameter), Snail expression, E-cad expression and Snail + E-cad expression were used for time-dependent ROC analysis. The results suggested that for patients with CRC, the clinical risk score combined with Snail and E-cad expression had a greater contribution than any of these markers alone (Fig. 3F).

Snail promotes CRC cell migration by decreasing E-cad. Previous studies have shown an association between Snail and E-cad expression and lymph node metastasis, TNM stage and distant metastasis in CRC (4,5). To investigate the effects of Snail and E-cad on CRC cells, lentivirus was used to generate stable cell lines of HCT 116 and HT 29 (Fig. 4A). There following groups were established under normal culture conditions: Overexpression LV-Snail, overexpression LV-E-cad,

Table II. Association between expression levels of E-cad and clinicopathological features in patients with colorectal cancer (n=465).

Characteristic	Low E-cad, n=244 (52.5%)	High E-cad, n=221 (47.5%)	P-value[a]
Age, years			0.111
≤65	131 (49.8)	132 (50.2)	
>65	113 (55.9)	89 (44.1)	
Sex			0.453
Male	147 (52.9)	131 (47.1)	
Female	97 (51.9)	90 (48.1)	
Pathological classification[b]			0.001
I	3 (60.0)	2 (40.0)	
II	210 (50.1)	209 (49.9)	
III	29 (80.6)	7 (19.4)	
Depth of invasion[b]			0.098
T1/T2	47 (46.5)	54 (53.5)	
T3/T4	196 (54.4)	164 (45.6)	
Lymph node metastasis[b]			<0.001
N0	112 (40.9)	162 (59.1)	
N1/N2	131 (69.7)	57 (30.3)	
TNM stage[b]			<0.001
I	37 (43.0)	49 (57.0)	
II	68 (38.0)	111 (62.0)	
III	124 (69.7)	54 (30.3)	
IV	14 (82.4)	3 (17.6)	
Tumor diameter, cm[b]			0.041
≤5	188 (50.3)	186 (49.7)	
>5	55 (61.1)	35 (38.9)	
Distant metastasis			0.004
M0	228 (51.1)	218 (48.9)	
M1	16 (84.2)	3 (15.8)	

[a]Two-sided Fisher's exact test. [b]Patient clinical pathological data incomplete. E-cad, E-cadherin.

Table III. Univariate Cox regression analysis of Snail and E-cad expression predicting survival in patients with colorectal cancer (n=470).

Expression, low vs. high	HR (95% CI)	P-value
Snail	0.143 (0.104-0.197)	<0.001
E-cad	0.166 (0.117-0.235)	<0.001

E-cad, E-cadherin.

knockdown LV-Snail-shRNA, knockdown LV-E-cad-shRNA and corresponding control groups. Wound healing and high content cell migration assay were used to detect changes in cell migration. The migration of LV-Snail cells was significantly increased compared with the corresponding control group, while the migration of LV-Snail-shRNA cells was decreased (Fig. 4B-F).

Subsequently, the present study detected expression levels of Snail and E-cad in each group after lentivirus infection by western blotting. The data showed that E-cad expression was decreased in each group following infection with LV-Snail lentivirus compared with respective control groups. Following infection with LV-Snail-shRNA lentivirus, the expression levels of E-cad were elevated (Fig. 5A and B). The present study altered expression levels of E-cad by secondary lentivirus infection. Cell migration was decreased after re-infection with LV-E-cad lentivirus to increase the expression levels of E-cad in LV-Snail CRC cells. By contrast, LV-E-cad-shRNA cell migration was enhanced following infection with LV-E-cad-shRNA lentivirus (Fig. 5C and D).

Snail promotes CRC cell proliferation in vivo. To study the effect of Snail on the proliferation of CRC cells *in vivo*, stable LV-Snail and LV-Snail-ctrl HCT 116 cells were subcutaneously injected into nude mice. The mice were sacrificed after 21 days. There was much more vascular-rich cancer tissue in LV-Snail group than in the control group (Fig. 6A). The

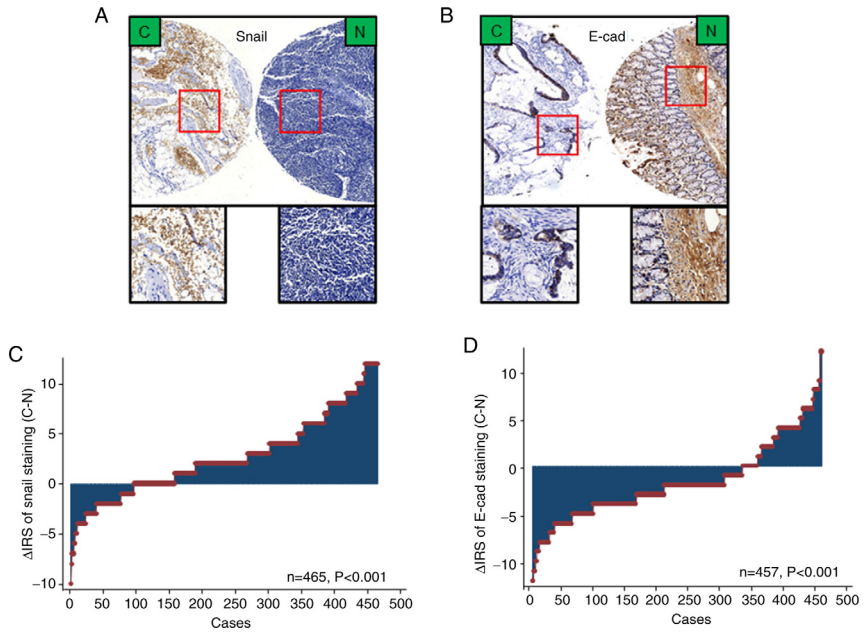

Figure 2. Snail and E-cad expression in a CRC tissue chip array. (A) Snail and (B) E-cad staining in CRC and paired N tissues. Top panel, 40x magnification; bottom panel, 200x magnification. Difference in (C) Snail and (D) E-cad staining in CRC tissue chip array compared with paired N tissue. CRC, colorectal cancer; E-cad, E-cadherin; C, cancer; N, normal; IRS, immunoreactivity score.

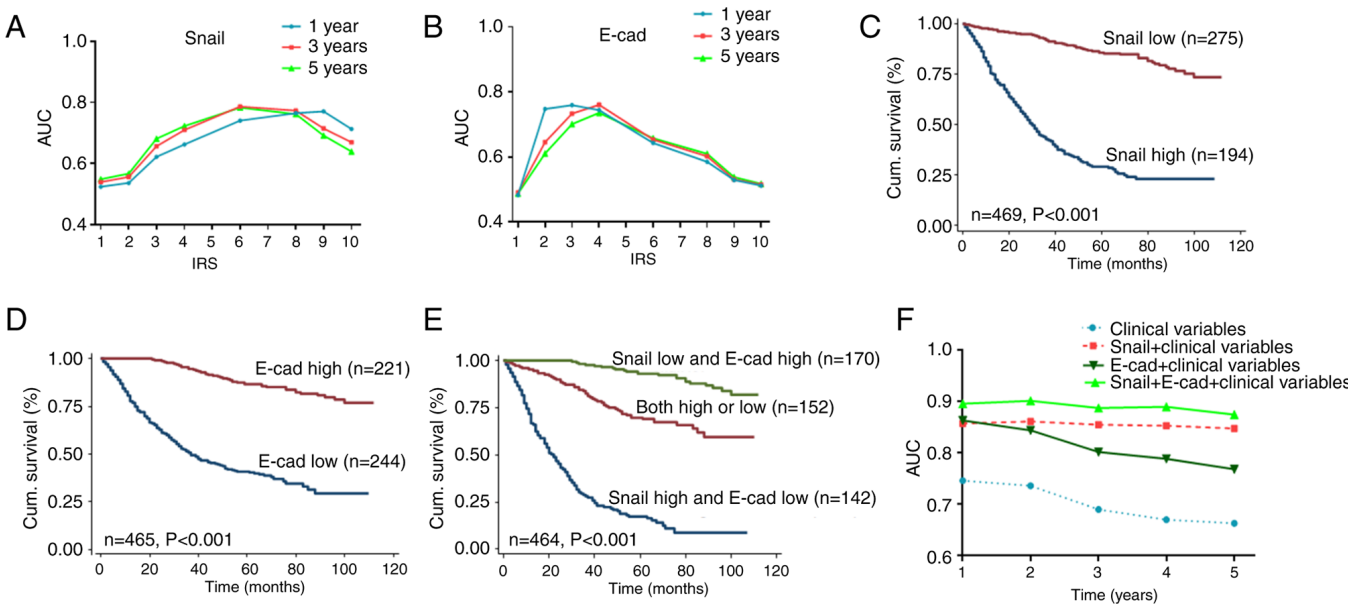

Figure 3. Snail and E-cad expression predict prognosis of CRC. AUC for (A) Snail or (B) E-cad plotted against cut-off values for IRS for 1-, 3- and 5-year OS. Kaplan-Meier curves of (C) Snail, (D) E-cad and (E) Snail + E-cad expression in training cohort for OS. (F) Time-dependent receiver operating characteristic analysis for clinical risk score (TNM stage, histological type and tumor diameter), alone or in combination with Snail, E-cad or Snail + E-cad. AUC, area under the curve; IRS, immunoreactivity score; OS, overall survival; E-cad, E-cadherin; CRC, colorectal cancer; cum, cumulative.

tumor volume of each group was measured every week. The diameter of the largest tumor tissue was ~6 mm. The tumor volume was larger in LV-Snail group than in the control group (Fig. 6B; P<0.01). Expression levels of CD31 were detected in tissue by IHC. The data revealed that the expression levels of CD31 in the transplanted tumors in the LV-Snail group were higher than those in the control group (Fig. 6C and D; P<0.01), which suggested that Snail served a notable role in promoting the angiogenesis of CRC cells *in vivo*.

Discussion

CRC is one of the most common types of cancer of the digestive system. In 2020, there were ~1.9 million new cases of CRC worldwide, resulting in >900,000 deaths (1). Although the incidence and mortality rate of CRC have decreased steadily in previous years, there is an upward trend in the incidence and mortality rates of individuals <50 years old (14-17). Tumor metastasis is a complicated process involving tumor cells

Table IV. Multivariate Cox regression analysis of Snail, E-cad, Snail + E-cad expression and clinicopathological variables predicting survival in patients with colorectal cancer.

A, Snail

Variable	HR (95% CI)	P-value
Age, ≤65 vs. >65 years	1.707 (1.278-2.279)	<0.001
Sex, male vs. female	0.887 (0.665-1.183)	0.413
Pathological classification, I/II vs. III	2.130 (1.331-3.411)	0.002
TNM stage, I/II vs. III/IV	1.797 (1.309-2.466)	<0.001
Tumor diameter, ≤5 vs. >5 cm	1.041 (0.727-1.493)	0.825
Expression, low vs. high	0.181 (0.128-0.255)	<0.001

B, E-cad

Variable	HR (95% CI)	P-value
Age, ≤65 vs. >65 years	1.734 (1.304-2.307)	<0.001
Sex, male vs. female	0.995 (0.745-1.328)	0.972
Pathological classification, I/II vs. III	1.620 (1.018-2.578)	0.042
TNM stage, I/II vs. III/IV	2.487 (1.839-3.363)	<0.001
Tumor diameter, ≤5 vs. >5 cm	1.033 (0.719-1.483)	0.861
Expression, low vs. high	0.212 (0.148-0.303)	<0.001

C, Snail/E-cad

Variable	HR (95% CI)	P-value
Age, ≤65 vs. >65 years	1.781 (1.120-2.831)	<0.001
Sex, male vs. female	1.056 (0.663-1.683)	0.818
Pathological classification, I/II vs. III	0.900 (0.324-2.501)	0.840
TNM stage, I/II vs. III/IV	1.862 (1.148-3.020)	0.012
Tumor diameter, ≤5 vs. >5 cm	1.077 (0.596-1.947)	0.806
Expression		
Both low vs. one low	0.272 (0.163-0.455)	<0.001
Both low vs. both high	0.226 (0.172-0.298)	<0.001

E-cad, E-cadherin.

invading the microenvironment, entering the blood, migration, angiogenesis and proliferation. Postoperative recurrence and metastasis are the primary reasons for the low survival rate of patients with CRC (18). Therefore, it is imperative to find molecular markers that can predict the prognosis of patients with CRC.

EMT is a process in which epithelial cells lose epithelioid features and switch to invasive mesenchymal cells, manifested by decreased expression levels of epithelial genes, such as E-cad and occludin, and increased expression of mesenchymal genes, such as N-cad and Vimentin (19). EMT is mainly involved in embryo development, wound healing, cancer cell metastasis and drug resistance (20,21).

E-cad protein primarily exists in epithelial cells and regulates cell adhesion in tissue. Reduction of E-cad expression usually indicates the beginning of EMT. Studies have demonstrated that expression of E-cad can inhibit tumor progression and invasion, and thus E-cad is considered a classical tumor inhibitor (4,5,8).

Snail is a member of the zinc finger transcription factor Snail family. This family encodes transcriptional inhibitors and shares a conserved C-terminal domain containing 4-6 C_2H_2 type zinc fingers that bind to the E-box motif (5'-CANNTG-3') of the target gene promoter (22). Snail is a primary inducer of EMT and has been associated with recurrence, metastasis and poor prognosis of breast cancer (23,24). Additionally, Snail is involved in acquisition of tumor stem cell features and inhibits estrogen receptor signaling (25,26), thus decreasing recurrence-free survival in patients with low-grade breast cancer (27). To the best of our knowledge, however, research on the role of Snail in CRC is currently lacking.

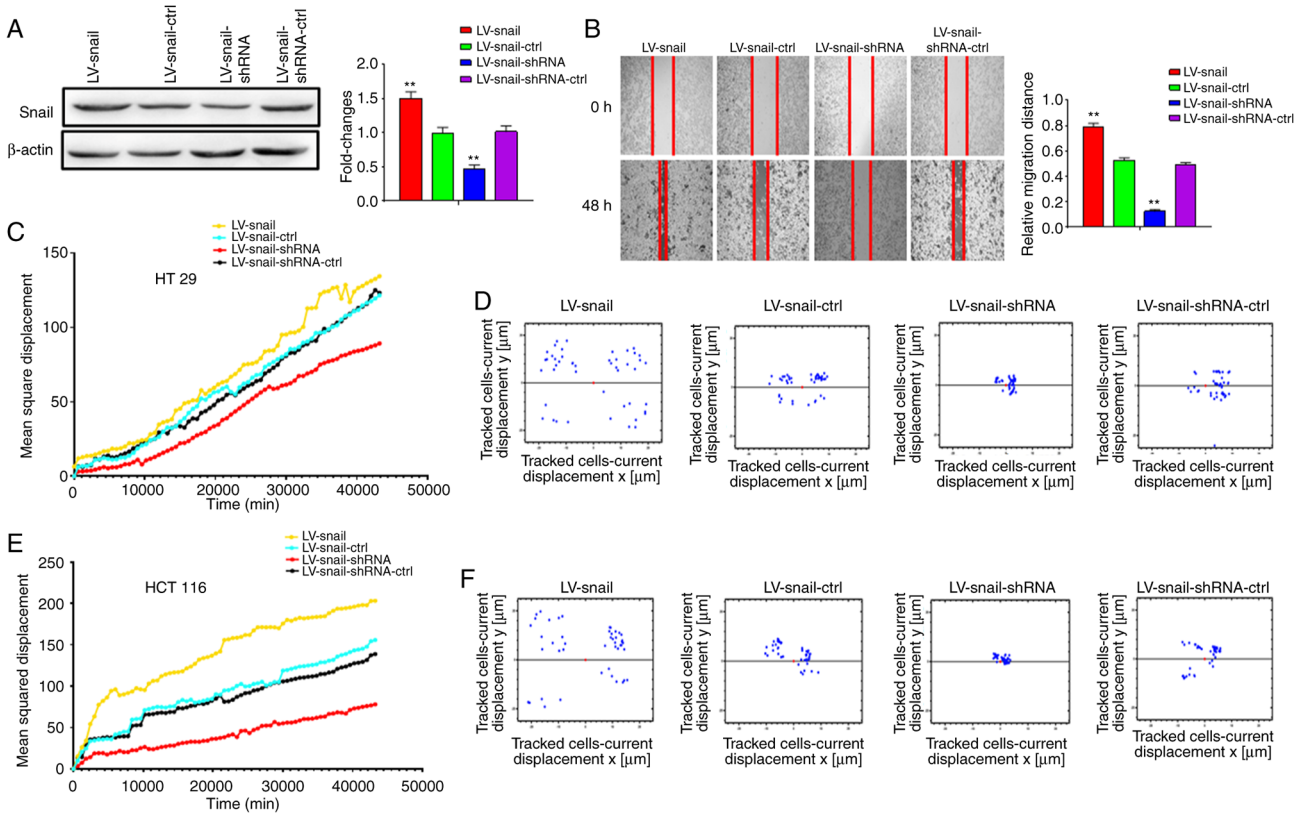

Figure 4. Snail inhibits CRC cell migration *in vitro*. (A) Western blot was used to validate the expression of Snail following viral transfection. (B) Migratory ability of HCT116 cells with different Snail expression levels was analyzed by wound healing assay at 50x magnification. Snail enhanced CRC cell migration by high-content imaging system analysis. (C and E) Mean time square displacement for each group; (D and F) Tracked cells-current displacement; compared with respective control groups (C,D: HT 29 cells; E,F: HCT 116 cells), **P<0.01. CRC, colorectal cancer; LV, lentiviral; shRNA, short hairpin RNA; ctrl, control.

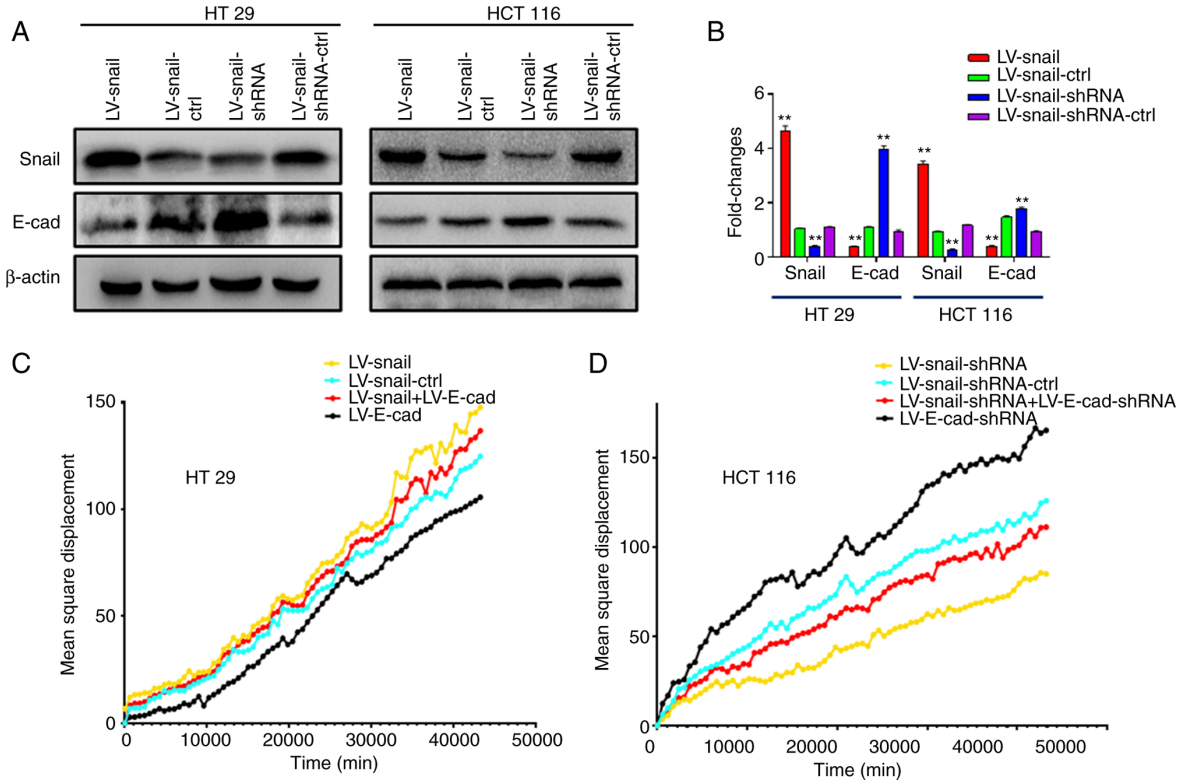

Figure 5. Snail inhibits CRC cell migration *in vitro* via regulating E-cad. (A and B) Western blot analysis showed that Snail protein negatively regulates E-cad protein, compared with respective control groups, **P<0.01. Snail enhanced CRC cell migration by regulating E-cad in (C) HT 29 and (D) HCT 116 cells. CRC, colorectal cancer; LV, lentiviral; shRNA, short hairpin RNA; E-cad, E-cadherin; ctrl, control.

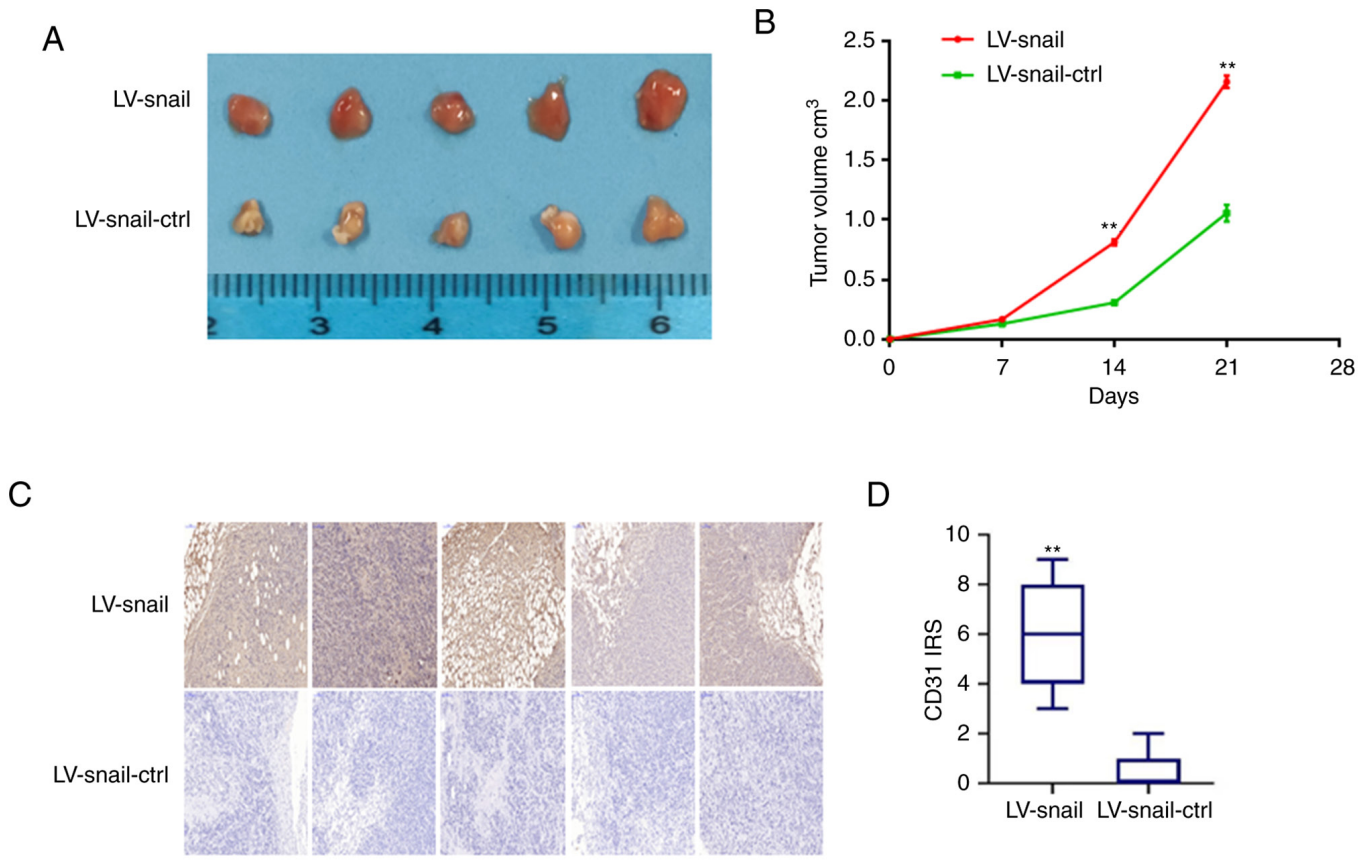

Figure 6. Snail inhibits angiogenesis *in vivo*. (A) Representative cancer tissue in mice: cancer tissue in LV-Snail or LV-Snail-ctrl group. (B) Tumor volume was measured every week. (C) Specific anti-CD31 antibody for blood vessels was stained by immunohistochemical method at 20x magnification. (D) IRS of mean CD31 of the LV-Snail compared with the LV-control mice, **P<0.01. IRS, immunoreactivity score; LV, lentiviral; ctrl, control.

The present study suggested that Snail could promote the proliferation and migration of CRC cells *in vitro*. Furthermore, Snail was found to be a poor prognostic marker for patients with CRC. Based on our CRC database analysis, it was concluded that Snail and E-cad were independent prognostic markers. Next, the present study attempted to analyze whether these two indicators had a greater combined effect in predicting CRC prognosis. Notably, Snail and E-cad had a greater combined effect based on Kaplan-Meier survival and ROC curve analysis of clinical variables.

The present study was only a retrospective study of a single center; a multi-center study should be performed in the future to expand the sample size. In future, prospective studies should be performed and database analysis conclusions should be verified using cell phenotype experiments and animal models of transplanted and metastatic tumors *in vivo*.

In conclusion, the present study demonstrated that Snail and E-cad were prognostic molecular biomarkers in patients with CRC. Snail promoted proliferation of CRC cells *in vivo* and *in vitro*. Notably, the present study identified a greater combined effect of Snail and E-cad in predicting prognosis. Further research into the role of these proteins may improve the survival of patients with CRC.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the SUB13034094 repository, [https://www.ncbi.nlm.nih.gov/biosample/34085159 to https://www.ncbi.nlm.nih.gov/biosample/34085290.

Authors' contributions

WMW, YZ and JS conceived and designed the study and methodology. WMW, JJ, ZZ, YFW and KM performed the experiments and acquired the data. JJ, ZZ, YFW, KM, JPJ and XZ analyzed and interpreted the data. WMW, JJ, ZZ and JPJ wrote, reviewed and revised the manuscript. YZ and JS provided study supervision. All authors have read and approved the final manuscript. WMW and YZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The Ethics Committee of Yixing Hospital approved the present study, which was performed according to the principles of the Declaration of Helsinki. The human and animal experiments were approved by the Ethics Committee of Yixing Hospital Affiliated to Yangzhou University (approval no. YXYLL-2015-42).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

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