Overexpression of HES6 has prognostic value and promotes metastasis via the Wnt/β-catenin signaling pathway in colorectal cancer

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Abstract. HES6 is a member of the hairy-enhancer of the split homolog family, which has been implicated in oncogenesis and cancer progression in a variety of human cancers, including prostate and breast cancer. However, its clinical significance and biological role in colorectal cancer (CRC) remain unclear. In the present study, the expression of HES6 was significantly upregulated in CRC cell lines and CRC tissues at both the mRNA and protein levels. The present study also reported high expression of HES6 in 138/213 (64.8%) paraffin-embedded archived CRC specimens. HES6 expression was significantly correlated with T classification (P<0.001), N classification (P=0.020), and distant metastasis (P<0.001). Patients with higher HES6 expression levels exhibited a reduced overall survival (P<0.001). In addition, a multivariate analysis revealed that the expression of HES6 may be a novel prognostic marker for the survival of patients with CRC. Furthermore, the present study demonstrated that ectopic expression of HES6 enhanced the migration and invasive abilities of CRC cells. These abilities were significantly inhibited upon knockdown of endogenous HES6 expression by specific short hairpin RNAs. Additionally, the present study reported that the effects of HES6 on metastasis may be associated with the activation of the Wnt/β-catenin signaling pathway. Collectively, the findings of the present study revealed that overexpression of HES6 played a key role in the progression of CRC, leading to a poor prognosis and clinical outcome.

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Introduction

Colorectal cancer (CRC) is one of the most common malignant cancers worldwide. In the United States, it has been reported to be the third most common type of cancer with cancer-associated mortality ranking second in men and third in women (1). Various advances have been made in the diagnosis and treatment of CRC, however, the prognosis of patients with CRC remains very poor, with metastasis as the leading cause of cancer-associated mortality among such patients (2,3). Unfortunately, no effective therapeutic strategies for patients with metastasis are currently available, and the underlying molecular mechanism of CRC-associated metastasis remains unclear (4). An improved understanding of the molecular mechanisms that mediate CRC-associated metastasis may contribute to increasing the effectiveness of current therapies for the treatment of CRC.

Metastasis is a multiple-step process. The activation of numerous signaling pathways can lead to metastasis (5-7). The canonical Wnt/β-catenin signaling pathway has been demonstrated to play a key role in the promotion of cancer metastasis (8-10). Binding of the Wnt ligand to its receptor leads to inhibition of the cytoplasmic degradation complex and stabilization of β-catenin. β-catenin then accumulates in the cytoplasm and translocates into the nucleus, resulting in the induction of downstream Wnt genes, including LCF-1, MMP7 and Slug, which are factors for cancer metastasis (10-12).

Hairy and enhancer of split family basic helix-loop-helix transcription factor 6 (HES6; 24 kDa), a member of the HES family of proteins, is located on chromosome 2q37 and the amplification of this region has been reported in prostate, breast and lung cancers (13-15). In addition, the overexpression of HES6 has been detected in various human cancers, including prostate and ovarian cancers, hepatic carcinoma and glioma (16-18). HES6 functions as a basic helix-loop-helix transcription repressor and has been demonstrated to play a vital role in the progression of various tumors (19-21). However, the clinical significance and biological role of HES6 in human CRC, and its association with the canonical Wnt/β-catenin signaling pathway requires further investigation.

In the present study, the expression of HES6 was evaluated within CRC cell lines and tissues. In addition, the present study investigated the association of the expression of HES6 with the clinicopathological characteristics and prognosis of patients with CRC, and the effect of HES6 on the metastasis of CRC, as well as the underlying molecular mechanism.

Materials and methods

Cell linesand treatment. A total of 9 CRC cell lines (HT-29, COLO 205, LoVo, SW480, SW620, HCT116, HCT-15, Caco-2 and LS174T) and a normal colon epithelial cell line (FHC) were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). All CRC cell lines as well as the FHC cell line were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories; GE Healthcare Life Sciences, Logan, UT, USA), 100 μg/ml streptomycin and 100 μg/ml penicillin (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C.

Patients and tissue specimens. A total of 213 paraffin-embedded CRC samples were collected at the Sun Yat-sen University Cancer Center (Guangzhou, China), from patients with a median age of 56 years (ranging from 20 to 85 years) who were histopathologically and clinically diagnosed from January 2005 to December 2007. The present study was approved by the Sun Yat-sen University Cancer Center Institutional Board. Prior patient consent was also obtained. The clinicopathological characteristics of the 213 patients are summarized in Table I. In addition, 15 pairs of primary CRC tissues and the adjacent normal tissues (5 cm away from the cancer lesions) were collected from patients with CRC who were enrolled between July 2015 and December 2016 and underwent colorectal resection at the Third Affiliated Hospital of Guangzhou Medical University. The clinicopathological features of 15 patients are summarized in Table II. Tissue samples were snap-frozen in liquid nitrogen and then stored at -80˚C until further use. The pathological diagnosis and confirmation of tissue specimens were performed by at least two pathologists. The tumor, node and metastasis (TNM) staging system was used according to the National Comprehensive Cancer Network (NCCN) guidelines (22). None of the patients received chemotherapy or radiotherapy prior to sample collection and patients with other malignancies were excluded. The research protocols were approved by the Clinical Research Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University (reference no. 2017, no. 117). All patients provided written informed consent for the analysis of their tissue for research purposes.

Table I. Clinicopathological features and tumor expression of HES6 in 213 patients with colorectal cancer.

Features	No. of cases (%)
Total no. of patients	213
Age (years)	
<56	93 (43.7)
≥56	120 (56.3)
Sex	
Male	123 (57.7)
Female	90 (42.3)
Differentiation	
Well and moderate	174 (81.7)
Poor and undifferentiated	39 (18.3)
Chemotherapy	
Yes	91 (42.7)
No	122 (57.3)
T stage	
1	7 (3.3)
2	15 (7.0)
3	158 (74.2)
4	33 (15.5)
N stage	
0	122 (57.3)
1	66 (31.0)
2	25 (11.7)
Metastasis	
M0	149 (70.0)
M1	64 (30.0)
Prognosis	
Survival	122 (57.3)
Death	91 (42.7)
Expression of HES6	
High	138 (64.8)
Low	75 (35.2)

RNA extraction, reverse transcription and quantitative real-time PCR (RT-qPCR). Total RNA from cultured cells and CRC tissue samples were isolated using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The present study examined the mRNA expression levels of HES6 in CRC cell lines relative to normal colon epithelial cells, as well as in each of the primary CRC tissues relative to the adjacent normal tissues obtained from the same patient, by PCR with published primers (16). Complementary DNA (cDNA) was synthesized from 2 μg total RNA using M-MLV Reverse Transcriptase (Promega Corp., Madison, WI, USA). Quantitation and amplification

Table II. Clinicopathological features of 15 patients with colorectal cancer.

Features	No. of cases (%)
Total no. of patients	15
Age (years)	
<56	6 (40.0)
≥56	9 (60.0)
Sex	
Male	8 (53.3)
Female	7 (46.7)
Differentiation	
Well and moderate	7 (46.7)
Poor and undifferentiated	8 (53.3)
Chemotherapy	
Yes	5 (33.3)
No	10 (66.7)
T Stage	
1	2 (13.3)
2	3 (20.0)
3	7 (46.7)
4	3 (20.0)
N Stage	
0	5 (33.3)
1	7 (46.7)
2	3 (20.0)
Metastasis	
M0	11 (73.3)
M1	4 (26.7)

were performed using SYBR Green I (Roche Diagnostics GmbH, Manheim, Germany) and an ABI 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions were as follows: initial denaturation at 95˚C for 5 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, primer annealing at 60˚C for 30 sec and extension at 72˚C for 1 min. The expression levels of HES6 were normalized to GAPDH. The procedures were performed as previously described and expression levels were analyzed by the $2^{-\Delta\Delta Cq}$ method used for relative quantification (23). Each experiment was performed in triplicate and repeated at least three times. The primer sequences of HES6 and GAPDH are provided in Table III.

Western blot analysis. The cells were harvested in sampling buffer (62.5 mmol/l Tris-HCl pH 6.8, 10% glycerol, 2% SDS) and then heated at 100˚C for 5 min. Protein concentration was determined via a Bradford assay using Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Equal quantities of protein (30 μg) were electrophoretically separated via 9% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). Following blocking for 1 h in Tris-buffered saline containing 0.1% Tween-20 at room temperature with 5% fat-free milk, the membranes were incubated with anti-HES6 antibody (dilution 1:3,000; cat. no. ab66461; Abcam, Cambridge, UK) overnight at 4˚C, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG antibody (dilution 1:4,000; cat. no. SC-2004; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Finally, the enhanced chemiluminescence (ECL) Prime Western Blotting Detection reagent (GE Healthcare, Chicago, IL, USA) was used to detect the expression of HES6 according to the manufacturer's instructions. An anti-α-tubulin antibody (dilution 1:4,000; cat. no. T9026; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used as a loading control.

Immunohistochemistry (IHC). IHC was used to assess the protein expression profile of HES6 in 213 formalin-fixed, paraffin-embedded CRC tissues. The procedures followed standard protocols as previously described (23). The degree of immunostaining for each sample was reviewed and scored independently by two pathologists. The scores were based on both the proportion of positively-stained tumor cells and the intensity of staining. The proportions of tumor cells were scored as follows: 0, <5% positive tumor cells; 1, 6-10% positive tumor cells; 2, 11-50% positive tumor cells; 3, 51-75% positive tumor cells and 4, >75% positive tumor cells. The grade of staining intensity was as follows: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown) and 3, strong staining (brown). The staining index (SI) was calculated by multiplying the score of the staining intensity by the proportion of positive tumor cells, which ranged from 0-12. Samples with an SI ≥6 were denoted as the high expression group and those with an SI<6 were classified as the low expression group.

Gene set enrichment analysis (GSEA). In the present study, a CRC cohort was downloaded from The Cancer Genome Atlas (https://cancergenome.nih.gov/) and GSEA 2.0.9 (http://www.broadinstitute.org/gsea/) was used. Gene set permutations were performed 1,000 times for each analysis. The pathways enriched in each phenotype were sorted by the nominal P-value and enrichment score (ES).

Overexpression and knockdown experiments. The coding sequence of the human HES6 gene was amplified by PCR and cloned into the pSin-EF2 lentiviral vector. Two short hairpin RNA (shRNA) oligonucleotides targeting HES6 were cloned into the pSuper-retro-puro vector, and the target sequences were as follows: (NM_018645.4) 5'-CCGGGCTGAACT GAGTCAGGCTCCTCTCGAGAGGAGCCTGACTCAGTT CAGCTTTTT-3'. Transfection of plasmids or shRNAs was performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The procedures of retroviral production and infection were conducted as described in a previous study (24). All stable cell lines expressing HES6 or HES6 shRNA were selected with 0.5 μg/ml puromycin for 48 h or 10 days following infection. The TCF4-dn (pLX303) plasmid (cat. no. 42592) was purchased from Addgene (Cambridge, MA, USA). The sequences of the shRNAs are listed in Table III.

Wound healing assay. Cell migration ability was determined using the scratch-wound assay. In brief, cells were cultured in

Table III. Primer and shRNA sequences used in present study.

Name	Sequence (5' to 3')
HES6-F	TGCCGAGCTCCTGAACCATC
HES6-R	TGGTTCAGGAGCTCGGCAGCGACG
GAPDH-F	CGCTGAGTACGTCGTGGAGTC
GAPDH-R	GCTGATGATCTTGAGGCTGTTGTC
β-catenin-F	CATCCTAGCTCGGGATGTTCAC
β-catenin-R	TCCTTGTCCTGAGCAAGTTCAC
HES6-shRNA1-F	GATCCCCCAGCCTGACCACAGCCCAAATTTCA AGAGAATTTGGGCTGTGGTCAGGCTGTTTTTA
HES6-shRNA1-R	AGCTTAAAAACAGCCTGACCACAGCCCAAATT CTCTTGAAATTTGGGCTGTGGTCAGGCTGGGG
HES6-shRNA2-F	GATCCCCCGAGCTCCTGAACCATCTGCTTTCAA GAGAAGCAGATGGTTCAGGAGCTCGTTTTTA
HES6-shRNA2-R	AGCTTAAAAACGAGCTCCTGAACCATCTGCTTC TCTTGAAAGCAGATGGTTCAGGAGCTCGGGG

F, forward; R, reverse.

6-well plates with RPMI-1640 medium and 10% FBS until a monolayer of cells was formed. Then, a straight linear wound was created in the middle of the cell monolayer with a sterile pipette tip. Images of the cells along the wound line were captured at 0 and 24 h following wounding under an inverted Olympus IX50 microscope (Olympus Corp., Tokyo, Japan) with a x10 objective lens.

Transwell matrix invasion assay. Cells ($2x10^5$) were seeded into the upper chamber of polycarbonate Transwell filters coated with Matrigel (BD Biosciences, San Jose, CA, USA), and 20% FBS was added into the lower chamber to induce invasion. After incubation at 37˚C for 24 h, the cells that had invaded to the bottom surface of the membrane were fixed in 1% paraformaldehyde, stained with hematoxylin, and counted in 10 random fields of view/well. All experiments were performed in triplicate.

Three-dimensional (3D) spheroid invasion assay. Cells ($1x10^4$) were seeded into 24-well plates coated with 2% Matrigel (BD Biosciences), and the medium was replaced every other day. Images of the cells were captured at 2-day intervals for 2 weeks under a light microscope with a magnification of x200.

Dual-Luciferase assay. Cells ($2x10^4$) were seeded in triplicate in 24-well plates and allowed to settle for 24 h. Subsequently, 150 ng luciferase reporter plasmids or the control luciferase plasmid plus 5 ng pRL-TK *Renilla* plasmid (Promega Corp.) were transfected into CRC cells using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). At 48 h following transfection, a Dual-Luciferase reporter assay (Promega Corp.) was performed according to the manufacturer's instructions. Three independent experiments were performed and the data are presented as the mean ± standard deviation.

Immunofluorescence analysis. Cells ($2x10^5$) were seeded on coverslips for 48 h. The cells were incubated with a primary antibody against β-catenin (dilution 1:1,000; cat. no. 8480; Cell Signaling Technology, Inc., Danvers, MA, USA), and then incubated with rhodamine-conjugated or FITC-conjugated goat antibodies against rabbit IgG (dilution 1:5,00; cat. no. 4412; Cell Signaling Technology). Coverslips were counter stained with DAPI and visualized under a confocal laser-scanning microscope (Olympus FV1000; Olympus Corp.). Data were processed with FV10-ASW 1.7 Viewer. The sequences of β-catenin are listed in Table III.

Statistical analysis. SPSS 19.0 statistical software (IBM Corp., Armonk, NY, USA) and GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA) were used for all statistical analyses. The χ^2 test was used for comparisons between groups, while Cox regression analysis was performed for univariate and multivariate survival analyses. The Kaplan-Meier method was used to plot the survival curves followed by a log-rank test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HES6 is upregulated in CRC cell lines. The data of the Cancer Genome Atlas (TCGA) revealed that HES6 mRNA expression levels were elevated in CRC tissues compared with normal and matched adjacent non-cancerous tissues, respectively (Fig. 1A and B).

The present study examined the expression of HES6 in 9 CRC cell lines (HT-29, COLO-205, LoVo, SW480, SW620, HCT 116, HCT-15, Caco-2 and LS174T) and a normal colon epithelial cell line (FHC). HES6 mRNA expression levels were upregulated at least 2-fold in CRC cell lines compared with FHC (Fig. 1C). Western blotting revealed the levels of HES6 protein expression were significantly higher in CRC

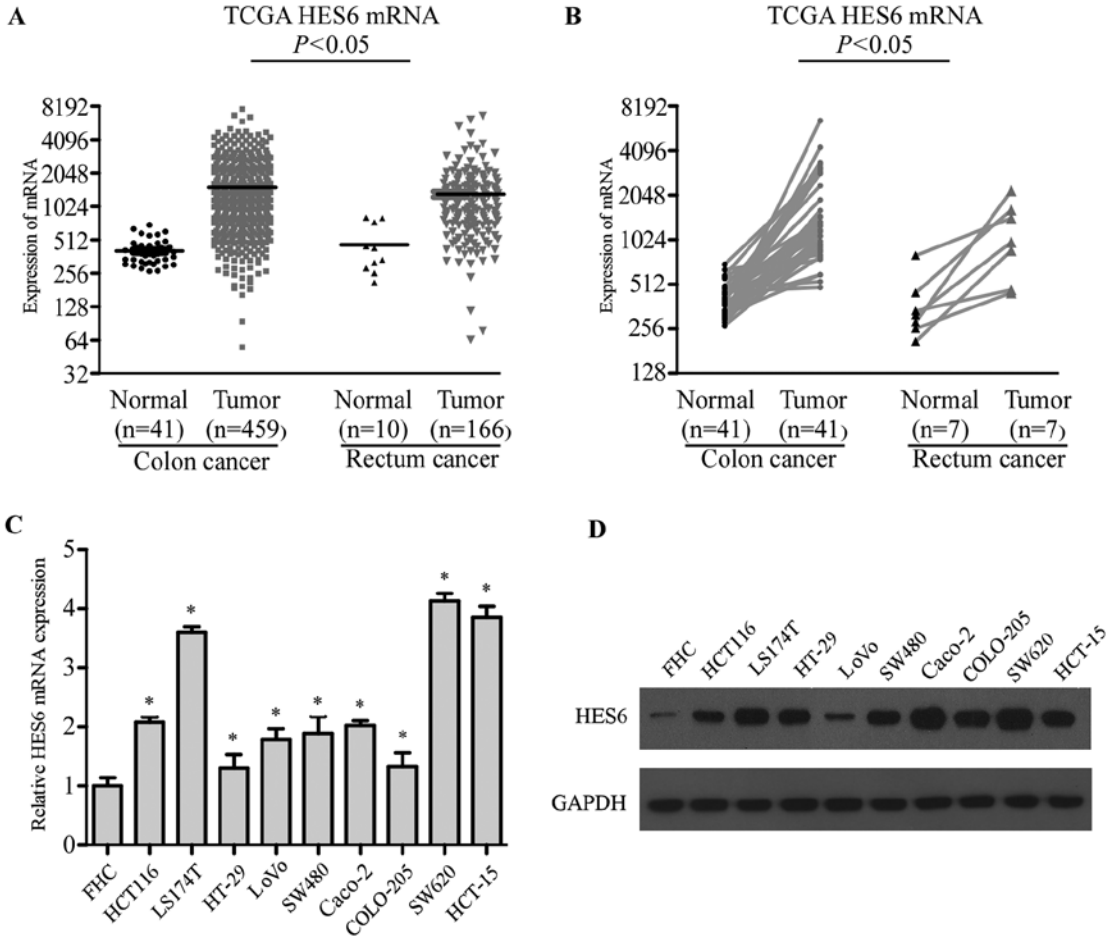

Figure 1. HES6 is upregulated in CRC cell lines. (A and B) mRNA expression profiles of HES6 in The Cancer Genome Atlas. The expression levels of HES6 mRNA and protein in CRC cell lines (HT-29, COLO-205, LoVo, SW480, SW620, HCT116, HCT-15, Caco-2 and LS174T) and the normal colon epithelial cell line (FHC) were determined by (C) quantitative polymerase chain reaction and (D) western blotting. Expression levels were normalized to GAPDH. Values are presented as the mean ± standard deviation of three parallel experiments. *P<0.05. CRC, colorectal cancer; HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6.

cell lines compared with FHC (Fig. 1D). Collectively, these results demonstrated that the expression of HES6 was elevated in CRC cell lines.

HES6 expression is elevated in primary human CRC lesions. Subsequently, the present study investigated the expression of HES6 in 15 primary CRC tissues (T) and matched adjacent non-cancerous tissues (N) from the same patients. HES6 mRNA expression levels in the CRC tissues were at least 3-fold higher than in the paired normal tissues (Fig. 2A). Western blotting revealed that HES6 protein expression levels were significantly higher in the 15 CRC tissues compared with adjacent non-cancerous tissues. Of note, HES6 expression levels were higher in the CRC tissues from patients with distant metastasis compared with primary lesions (Fig. 2B and C). Consistent with these results, IHC analysis demonstrated that the extent of HES6 staining was negative to low in the adjacent normal tissues however, positive staining was observed in the tumor tissues of patients with or without metastasis (Fig. 2D). These results indicated that HES6 expression was elevated in CRC lesions at both the mRNA and protein levels, and higher HES6 expression may be considered as a predictor for the risk of metastasis.

Association between the increased expression of HES6 and the clinical features of CRC. In the present study, the expression level of HES6 in 213 paraffin-embedded, archived CRC tissues was analyzed via IHC. A total of 138 cases (64.8%) exhibited positive staining for HES6 protein in the 213 registered patient samples. The remaining 75 cases (35.2%) demonstrated weak staining for HES6 protein expression. Examples of negative, moderate and strong HES6 staining by IHC in human CRC tissues are presented in Fig. 3A. The samples were divided into low and high HES6 expression groups as summarized in Table I. High HES6 protein expression levels were strongly associated with T and N stages, and distant metastasis (P<0.05). However, no significant association was reported between HES6 protein expression and patient age, sex, histological differentiation grade or chemotherapy in patients with CRC (Table IV).

HES6 is associated with poor prognosis in patients with CRC. Kaplan-Meier survival analysis and a log-rank test were used to evaluate the prognostic value of HES6 protein expression in CRC. A significant difference in survival time was demonstrated between the high and low HES6 protein expression groups via the log-rank test (P<0.001; Fig. 3B). Compared

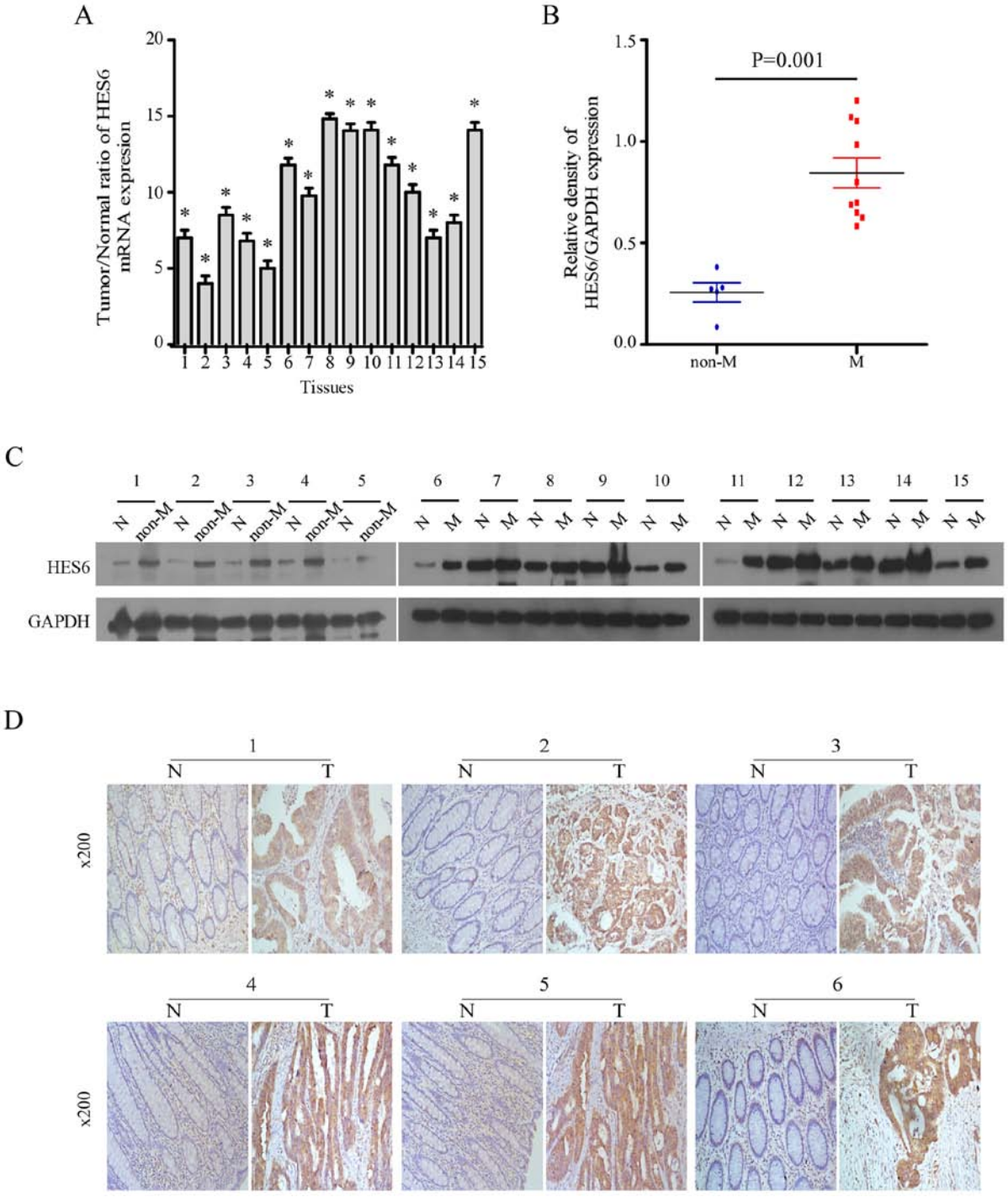

Figure 2. Expression of HES6 in primary human CRC lesions. (A) Average Tumor/Normal ratios of HES6 mRNA expression in paired CRC tissues and adjacent non-cancerous tissues were determined by quantitative polymerase chain reaction and normalized against GAPDH. The error bars represent the standard deviation of the mean calculated from three parallel experiments. (B and C) Western blotting of the expression of HES6 in the adjacent non-cancerous tissues and CRC tissues from primary CRC of 15 patients with or without distant metastasis. GAPDH served as the loading control. (D) The expression of HES6 protein in 6 pairs of matched CRC tissues and adjacent non-cancerous tissues by immunohistochemical analysis. *P<0.05. CRC, colorectal cancer; HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6; T, colorectal cancer tissue; N, adjacent non-cancerous tissue; non-M, colorectal cancer tissue without distant metastasis; M, colorectal cancer tissue with distant metastasis.

with the low HES6 expression group, patients with higher HES6 expression levels exhibited significantly shorter overall survival. In addition, the cumulative overall survival was only 47.8% in the high HES6 expression group compared to 74.7% in the low HES6 expression group.

The prognostic value of HES6 in the T and N stages and distant metastasis subgroups were also investigated in the present study. As presented in Fig. 4, the expression of HES6 was significantly associated with the overall survival of patients without metastasis (log-rank test, P=0.004), without deep stromal invasion (log-rank test, P=0.024), with deep stromal invasion (log-rank test, P=0.017) and with lymph node metastasis (log-rank test, P=0.003). Univariate Cox regression and multivariate analysis revealed that higher HES6 expression

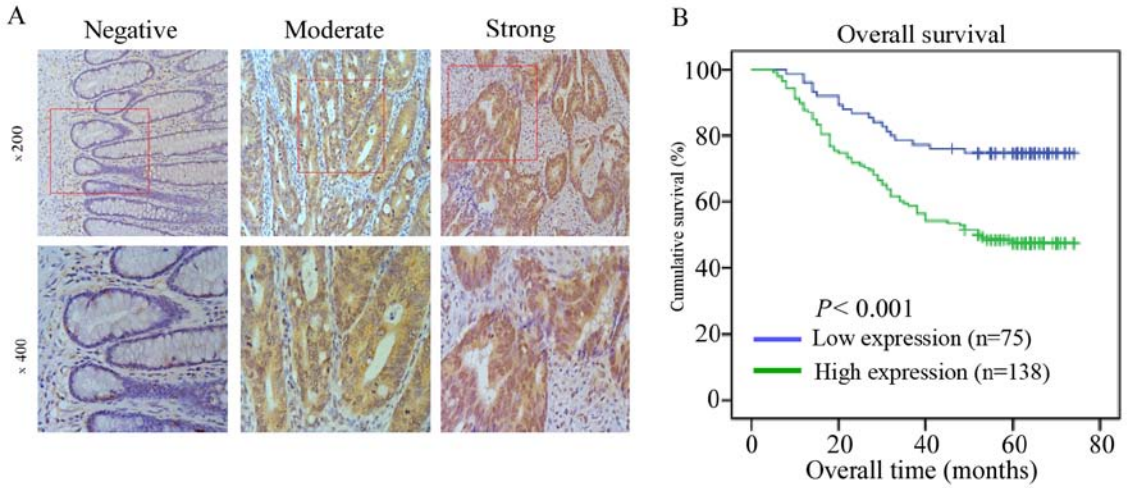

Figure 3. Representative images of HES6 IHC analysis of CRC tissues. (A) IHC staining of the expression of HES6 in human CRC tissues. (B) Kaplan-Meier overall survival curves for the 213 CRC patients stratified by low (n=75) and high (n=138) expression of HES6 (P<0.001). CRC, colorectal cancer; HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6; IHC, immunohistochemistry.

Table IV. Relation between the expression of HES6 and the clinicopathological features of colorectal carcinoma.

Features	Total	HES6 weak expression (%)	HES6 strong expression (%)	P-value (Chi-squared test)	P-value (Fisher's exact test)
Age (years)				0.279	0.313
<56	93	29 (13.6)	64 (30.0)		
≥56	120	46 (21.6)	74 (34.7)		
Sex				0.841	0.885
Male	123	44 (20.7)	79 (37.1)		
Female	90	31 (14.6)	59 (27.7)		
T Stage				<0.001	<0.001
T1-T2	27	19 (8.9)	8 (3.8)		
T3-T4	186	56 (26.3)	130 (61.0)		
N Stage				0.020	0.021
N0	122	51 (23.9)	71 (33.3)		
N1-N2	91	24 (11.3)	67 (31.5)		
Metastasis				<0.001	<0.001
M0	149	65 (30.5)	84 (39.4)		
M1	64	10 (4.7)	54 (25.4)		
Differentiation				0.638	0.711
Well/moderate	174	60 (28.2)	114 (53.5)		
Poor/undifferentiated	39	15 (7.0)	24 (11.3)		
Chemotherapy				0.781	0.885
Yes	91	33 (15.5)	58 (27.2)		
No	122	42 (19.7)	80 (37.6)		
Prognosis				<0.001	<0.001
Survival	122	56 (26.3)	66 (31.0)		
Death	91	19 (8.9)	72 (33.8)		

levels (P=0.027), N stage (P=0.015) and metastasis (P=0.025) may be predictors of poor clinical outcomes in patients with CRC (Table V). These data indicated that high HES6 protein expression levels may be a novel prognostic indicator for CRC.

HES6 promotes the migration and invasion of CRC cells. Since the phenotypes of migration and invasion are key characteristics of cancer metastasis, the present study investigated whether HES6 mediates the migration and invasion

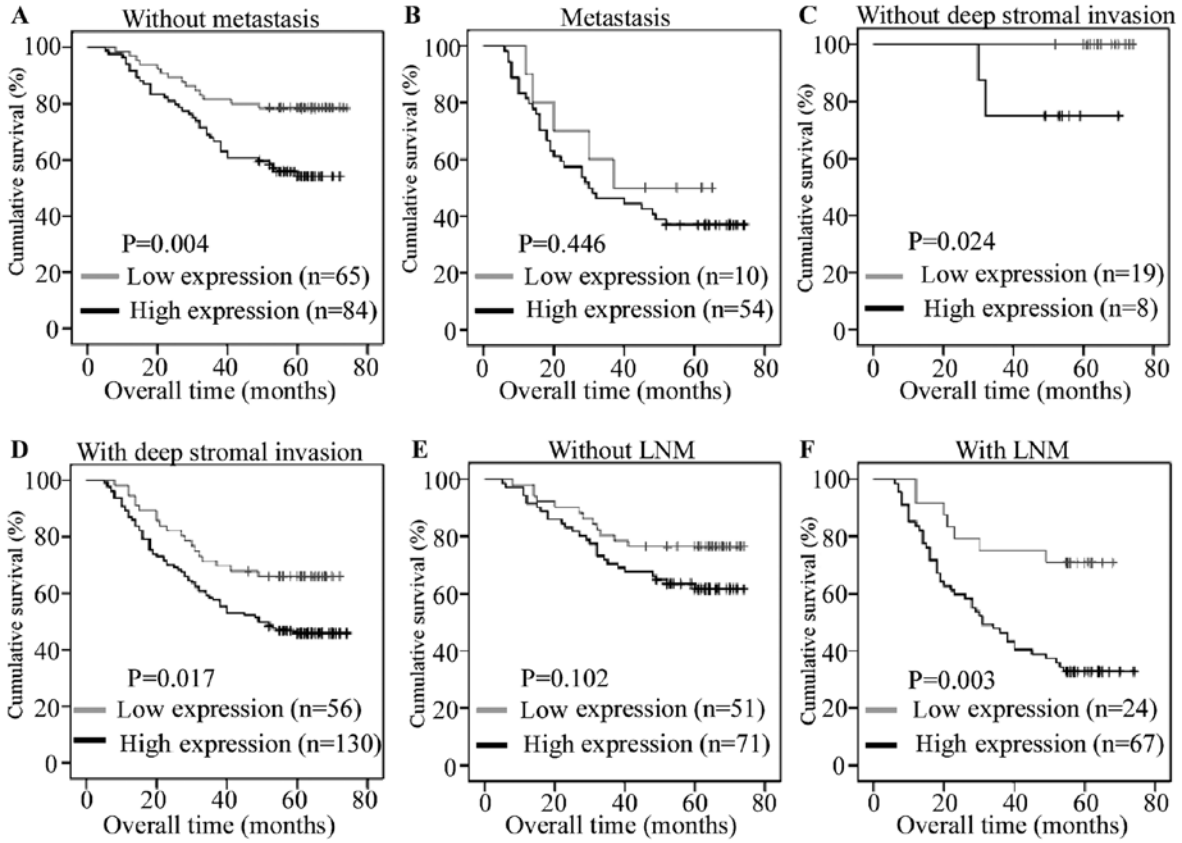

Figure 4. Kaplan-Meier curves (univariate log-rank test) of the high and low HES6 expression groups. (A) Kaplan-Meier curves in the high vs. low HES6 expression groups of patients without distant metastasis, (B) with metastasis, (C) without deep stromal invasion, (D) with deep stromal invasion, (E) without LNM and (F) with LNM. CRC, colorectal cancer; HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6; LNM, lymph node metastasis.

Table V. Univariate and multivariate analyses of prognostic factors in colorectal cancer using a Cox-regression model.

	Univariate analysis			Multivariate analysis		
Factors	Number of patients	P-value	Regression coefficient (SE)	P-value	Relative risk	95% confidence interval
HES6 expression		<0.001	2.478 (0.258)	0.027	1.803	1.069-3.039
Low	75					
High	138					
T Stage		0.003	8.446 (0.715)	0.052	4.184	0.989-17.696
T1-T2	27					
T3-T4	186					
N Stage		<0.001	2.228 (0.212)	0.015	1.703	1.108-2.617
N0	122					
N1-N2	91					
Metastasis		<0.001	2.316 (0.213)	0.025	1.641	1.063-2.535
M0	149					
M1	64					
Differentiation		0.045	1.648 (0.249)	0.146	1.446	0.879-2.377
Well/moderate	174					
Poor/undifferentiated	39					
Chemotherapy		0.010	1.716 (0.210)	0.056	1.514	0.989-2.317
Yes	91					
No	122					

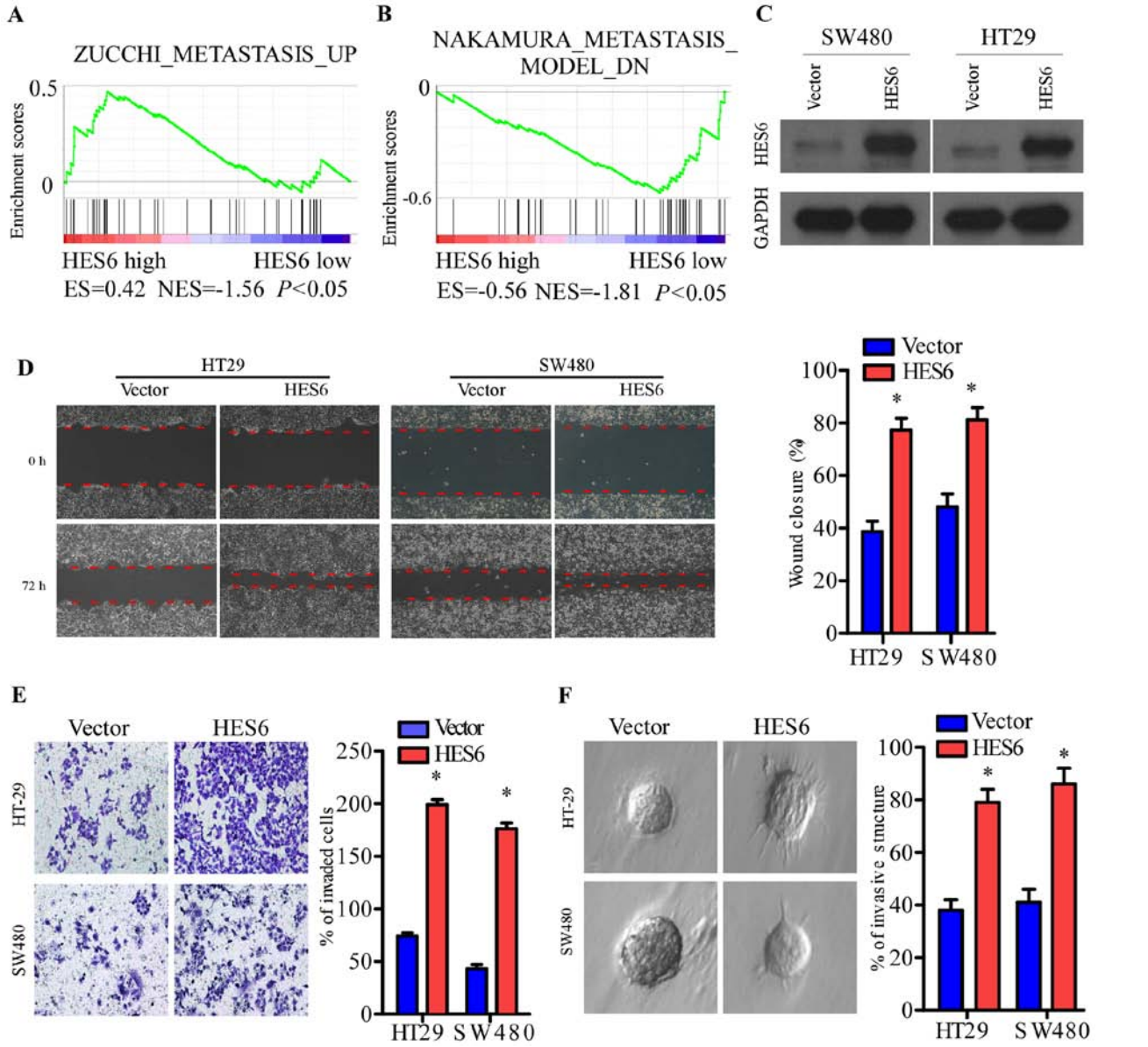

Figure 5. HES6 promotes the migration and invasion of colorectal cancer cells. (A and B) Gene set enrichment analysis plot revealed that HES6 expression was positively correlated with two metastasis gene signatures (ZUCCHI_METASTASIS_UP, NAKAMURA_METASTASIS_MODEL_DN). (C) Western blotting demonstrated overexpression of HES6 in the HT29 and SW480 cell lines. (D) Wound closure by HES6-overexpressing cells in the wound-healing assay. (E) Representative images (left) and quantification (right) of the invasiveness of HES6-overexpressing cells compared with vector control cells in the Transwell matrix invasion assay. (F) Representative images of the invasive structures of HT29 and SW480 cells transduced with vector and HES6 in the three-dimensional spheroid invasion assay. Error bars represent the mean ± standard deviation from three independent experiments. *P<0.05. HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6.

of CRC cells. Firstly, TCGA profiles were analyzed by gene set enrichment analysis (GSEA) (25,26), which revealed that HES6 expression levels were related with cancer metastasis (Fig. 5A and B). In the present study, stably-expressing HES6 cell lines were established using the human CRC cell lines, HT-29 and SW480 (Fig. 5C). The wound healing and Transwell assays revealed that HES6 overexpression promoted the migration and invasion of CRC cells compared with control cells (Fig. 5D and E). However, no significant increase in cell numbers was observed following HES6 overexpression. In the 3D spheroid invasion assay, a better model of tumor invasion *in vivo*, HES6 expression promoted the invasive phenotype, as indicated by an increased number of outward projections from a single cell (Fig. 5F). These results demonstrated that elevated HES6 expression levels may promote the migration and invasion of CRC cells, which supports the association between HES6 expression and T, N, and M classification observed in a large cohort of clinical specimens.

Knockdown of HES6 inhibits the migration and invasion of CRC cells. The role of HES6 in CRC metastasis was investigated in the present study by silencing endogenous HES6 expression with specific shRNAs (Fig. 6A). In the wound-healing, Transwell and 3D spheroid invasion assays, cell migration and invasion were significantly reduced in the SW480 and HT29 cell lines following knockdown of HES6 (Fig. 6B-E). These results revealed that downregulation of HES6 inhibited the migration and invasion of CRC cells *in vitro*.

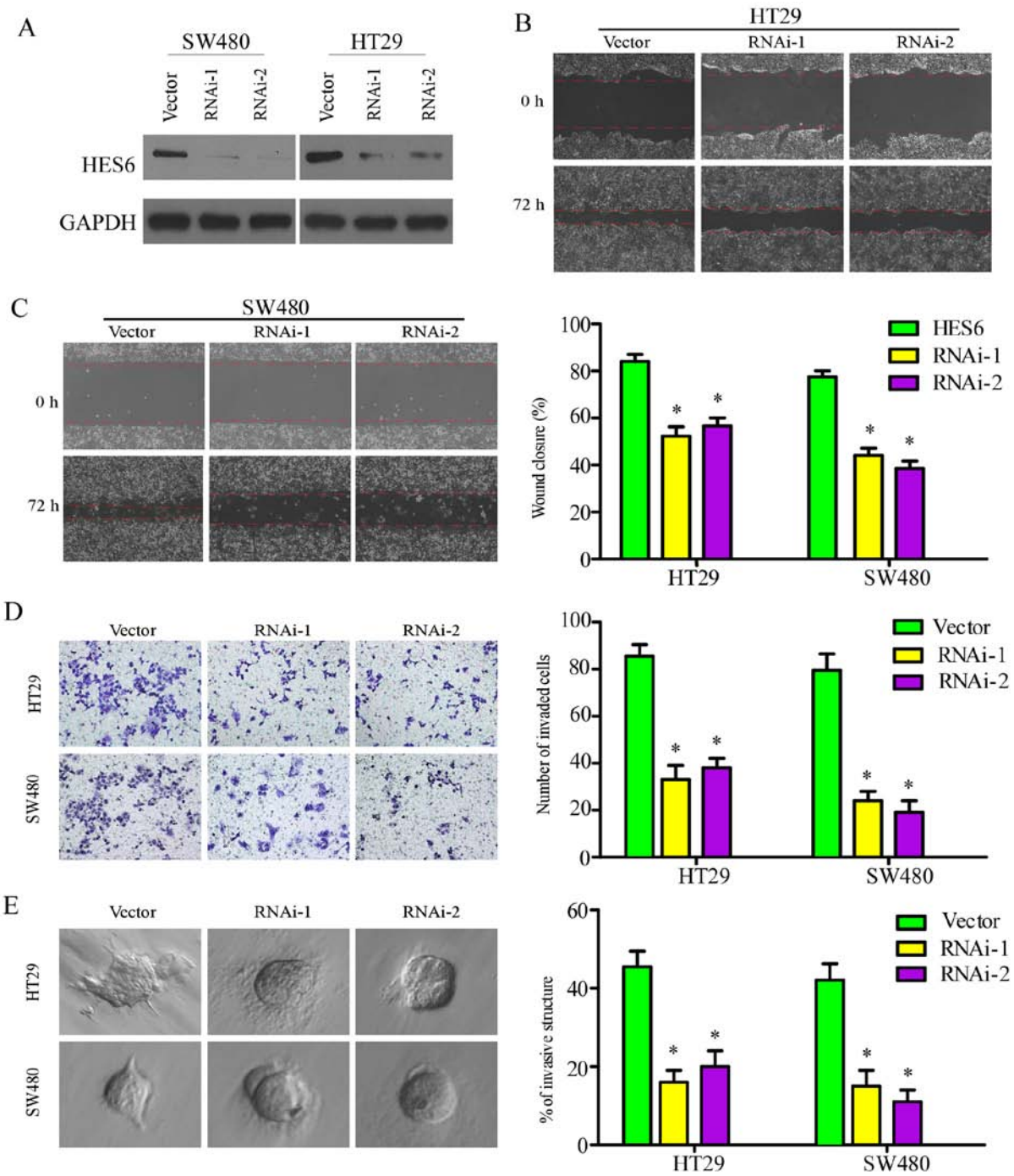

Figure 6. Silencing of endogenous HES6 inhibits the aggressive phenotype of colorectal cancer cells. (A) Western blotting demonstrated knockdown of HES6 expression following incubation with two shRNA oligonucleotides (RNA interference) targeting HES6 in the HT29 and SW480 cell lines. Silencing of HES6 reduced CRC cell migration and invasion as presented by (B and C) wound healing assay and (D) a Transwell matrix invasion assay. (E) Representative images of the indicated cells cultured in the three-dimensional spheroid invasion assay. *P<0.05. HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6.

HES6 activates the Wnt/β-catenin signaling pathway in CRC. The GSEA revealed that the HES6 expression profiles in TCGA were associated with the Wnt/β-catenin signaling pathway (Fig. 7A and B). In addition, whether HES6 enhances cell invasion and migration in CRC via the activation of the Wnt/β-catenin signaling pathway was investigated in the present study. The luciferase reporter assay demonstrated that overexpression of HES6 enhanced, while HES6 silencing reduced, the ratio of TOP flash to FOP flash activity in the indicated cells (Fig. 7C). Western blotting revealed that overexpression of HES6 upregulated the expression of β-catenin levels in the nuclei (Fig. 7D). The immunofluorescence staining assays demonstrated that overexpression of HES6 led to the nuclear accumulation of β-catenin, while the opposite results were observed following the silencing of HES6 (Fig. 7E).

To confirm the effects of HES6 on the Wnt/β-catenin signaling pathway in CRC, RT-qPCR was used to examine

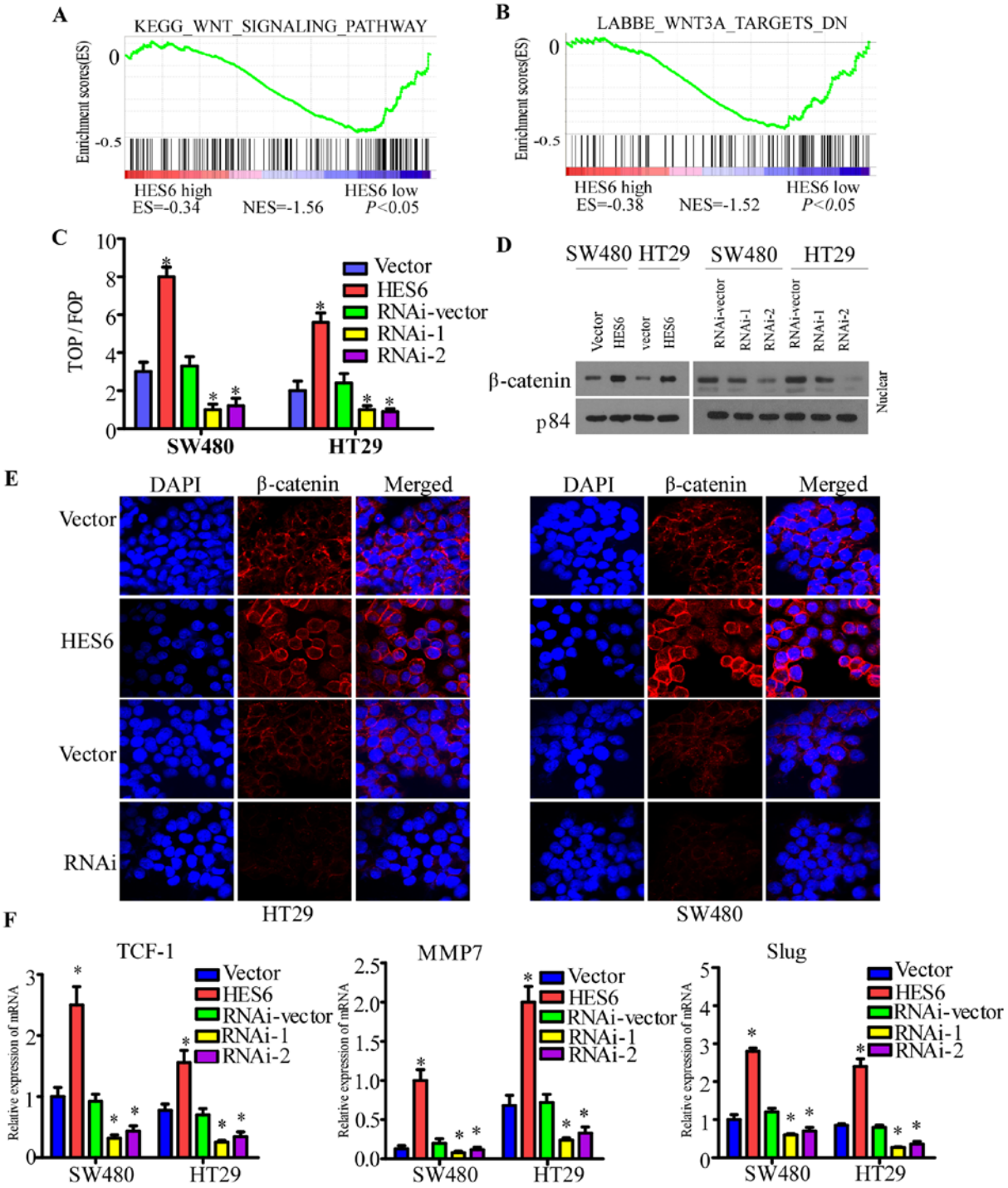

Figure 7. HES6 activates the Wnt/β-catenin signaling pathway in colorectal cancer. (A and B) Gene set enrichment analysis plot revealed that HES6 expression was positively correlated with two metastasis gene signatures (KEGG_WNT_SIGNAILING_PATHWAY, LABBE_WNT3A_TARGETS_DN). (C) Dual-Luciferase assay of TCF/LEF transcriptional activity in indicated cells with overexpression or knockdown of HES6. (D) Altered nuclear translocation of β-catenin in response to overexpression or knockdown of HES6; p84 was used as loading control. (E) Immunofluorescence staining demonstrated the localization of β-catenin in indicated cells. (F) Wnt target gene expression was analyzed by quantitative polymerase chain reaction following HES6 overexpression or knockdown in the indicated cells. GAPDH was used as the loading control. *P<0.05. by the Student's t-test. HES6, hairy and enhancer of split family basic helix-loop-helix transcription factor 6; TCF/LEF, T-cell factor/lymphoid enhancer factor.

the expression of several Wnt target genes. Overexpression of HES6 upregulated, while HES6 silencing downregulated, the expression levels of TCF-1, MMP7 and Slug in the indicated cells (Fig. 7F). The Wnt/β-catenin signaling pathway was inhibited by silencing of TCF-4 expression, as previously described (10). The migration and invasive abilities of the indicated cells were attenuated (Fig. 8A and B). Collectively, these results revealed that HES6 promoted migration and invasion by activating the Wnt/β-catenin pathway in CRC.

Discussion

In the present study, it was reported that HES6 expression was elevated in primary human CRC lesions at the protein

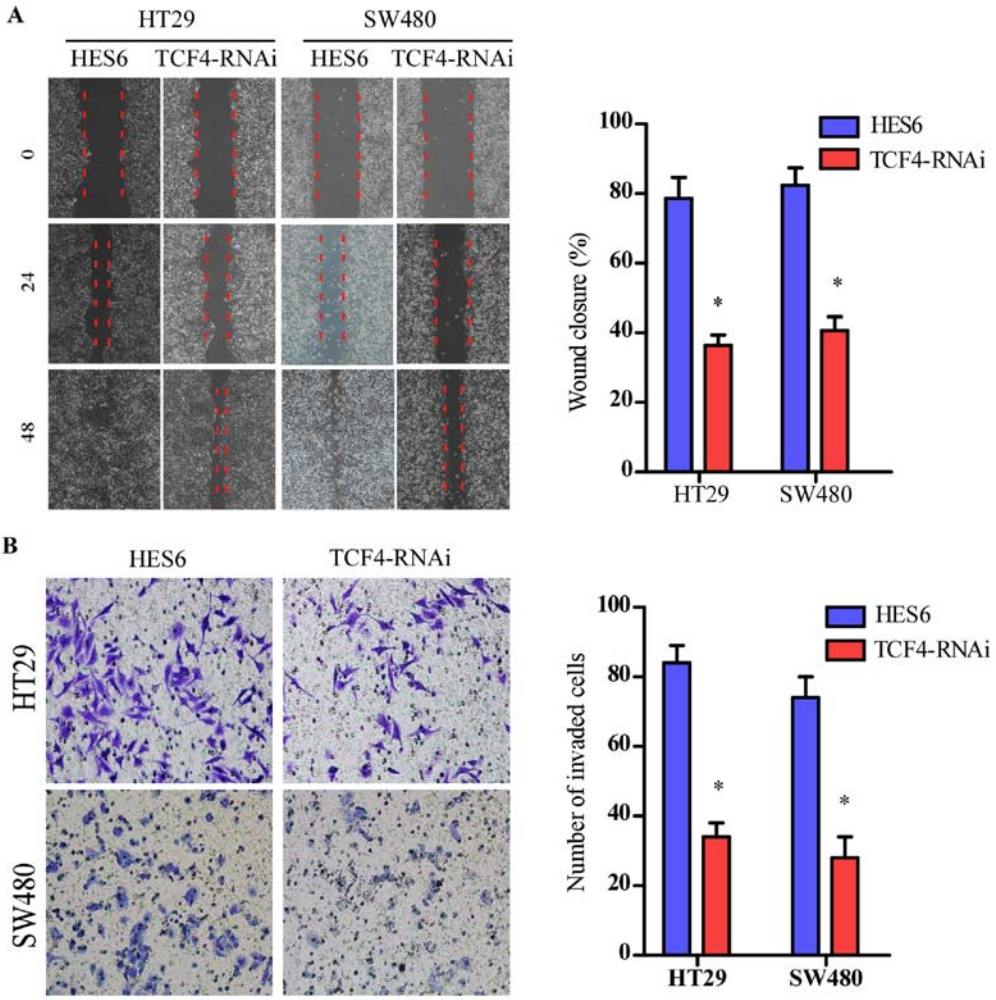

Figure 8. (A) Migration and (B) invasion assays of the indicated cells following treatment with an inhibitor of the Wnt signaling pathway (TCF4-RNA interference). Error bars represent the mean ± standard deviation from three independent experiments. *P<0.05 by the Student's t-test.

and mRNA levels. HES6 protein expression was associated with T stage, lymph node metastasis and distant metastasis in patients with CRC. CRC patients with elevated levels of HES6 expression exhibited a poor prognosis. In addition, the multivariate analysis conducted in the present study demonstrated that HES6 may be a potential novel prognostic indicator for survival in patients with CRC. Finally, ectopic HES6 expression was proposed to enhance the migration and invasion of CRC cells by activating the Wnt/β-catenin signaling pathway in the present study.

The oncogenic role of HES6, a helix-loop-helix transcriptional suppressor, has been previously reported in several studies: for example, HES6 was demonstrated to regulate the differentiation of numerous cell types during myogenesis (27). Nam *et al* (28) proposed that HES6 binds to HES1, suppressing the function of HES1 and promoting the differentiation of neural stem cells. Additionally, elevated HES6 expression was reported to lead to a significant increase in the invasive phenotype of prostate cancer and glioma (14,29). The depletion of HES6 decreased cell migration in alveolar rhabdomyosarcoma, as determined by scratch-wound assays (30). In addition, high HES6 expression levels have been correlated with poor survival in patients with prostate, breast and ovarian cancers (16,17,31). However, to the best of our knowledge, this is the first study that has demonstrated the association between HES6 and the pathogenesis of CRC.

Swearingen *et al* (15) previously reported that HES6 was upregulated only at the transcriptional level in a xenograft model of metastatic CRC. The results of the present study, however, demonstrated that HES6 was upregulated at both the protein and mRNA levels in CRC cell lines and primary CRC lesions. Furthermore, analysis of TCGA data revealed that HES6 was upregulated in primary lesions of patients with CRC. These differing results may be reflective of the small sample size employed in the study of Swearingen *et al* (15), as only three isogenic lung tumor metastases were analyzed.

Based on the IHC analyses of the present study, high HES6 expression levels were significantly correlated with T stage, lymph node metastasis, distant metastasis and survival status in patients with CRC. These results indicated that patients with CRC possessing high HES6 expression levels tended to have a poorer prognosis. In addition, univariate and multivariate analyses in the present study indicated that HES6 expression was a significant independent predictor of poor prognosis in CRC patients, as previously described for other types of cancer (16,17,31). Furthermore, an association was observed between poor overall survival and high HES6 protein expression in patients with CRC without metastasis, with and without

deep stromal invasion, or lymph node metastasis, which indicated that HES6 may be a novel biomarker for the prediction of overall survival in these subgroups. However, no correlation between HES6 expression and survival was observed in patients with metastasis. This may be due to the small number of patients in this subgroup (n=64). Therefore, the results of the present study should be further verified with a larger cohort of patients in the future.

At present, the main therapy applied to patients with CRC is surgical resection. However, patients may experience various effects following resection even in the same TNM stage (32). It has been reported that ~50% of patients will experience a recurrence within the first 3 years following surgery (33). Therefore, the results of the present study indicated that additional radiotherapy and chemotherapy to reduce malignancy or metastasis, may be beneficial for patients with high HES6 expression levels.

The Wnt/β-catenin signaling pathway is crucial for the progression of CRC (34,35). In the present study, HES6 was reported to enhance cell migration and invasion in CRC by activating the Wnt/β-catenin signaling pathway, and upregulating the expression of downstream target genes, including Slug and MMP7, which have been associated with cancer metastasis (36,37). The *in vitro* investigations conducted in the present study, in which HES6 was overexpressed in CRC cells, demonstrated that HES6 upregulation increased cell migration and invasive abilities. This suggested that HES6 may be involved in epithelial-mesenchymal transition, whereby cells lose their cell-cell adhesion, and gain migratory and invasive properties. Thus, the results of the present study revealed a novel pathological mechanism underlying CRC, in which the Wnt/β-catenin signaling pathway is activated via the overexpression of HES6. Therefore, targeting the activation of the HES6-mediated Wnt/β-catenin signaling pathway may represent a novel anti-metastasis therapy for CRC.

In the present study, the clinical significance of HES6 expression in patients with CRC was investigated and revealed that HES6 may enhance the migration and invasive abilities of CRC cell lines by activating the Wnt/β-catenin signaling pathway. However, these findings require further validation in a larger cohort of samples. In addition, the precise mechanism underlying the HES6-associated activation of the Wnt/β-catenin signaling pathway requires further study. In conclusion, HES6 protein levels may be considered as a novel predictor of clinical outcome and metastasis in CRC patients.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article. Still, more details about the datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YX and XX conceived and designed the study. YX, XL, HZ and ZZ performed the experiments. YX and XL wrote the manuscript. YX, LS and XX reviewed and edited the manuscript. XianW, XiaoW, SL and HZ performed data acquisition and curation. Supervision throughout this manuscript was done by LS and XX. 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

Ethical approval of the study was obtained by the Clinical Research Ethics Committee of the Third Affiliated Hospital of Guangzhou Medical University and the Sun Yat-sen University Cancer Center Institutional Board. Written informed consent was obtained from each patient.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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