Original Article

Expression, clinical significance and correlation of RUNX3 and HER2 in colorectal cancer

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Background: The incidence of colorectal cancer is high and on the rise. The genetic and protein expressions of RUNT-associated transcription factor 3 (RUNX3) and human epidermal growth factor receptor 2 (HER2) in colorectal cancer (CRC) and adjacent normal tissues were detected to preliminarily explore their correlation and clinical significance.

Methods: CRC specimens excised during general surgery were selected for localization and quantitative analysis of protein and gene expression by SP (Streptavidin-peroxidase conjugated method) immunohistochemical staining, reverse transcription-polymerase chain reaction (RT-qPCR) and western blot. Combined with the patients' data, the relationship between the expression of the two genes and tumor characteristics was analyzed. Log-rank test was used to analyze the correlation between the two proteins and survival prognosis of CRC patients. The expression of RUNX3 in RKO and HCT-116 was knocked down, and the relative expression of HER2 in the two cell lines was detected.

Results: Immunohistochemical, RT-qPCR and Western blot results showed that the positive expression rate of RUNX3 in CRC was lower than in the normal group, and HER2 in CRC was higher than in the normal group. The positive expression of the two proteins correlated with the pT and pN stages of CRC. A significant negative correlation between the expression of the two genes in CRC. Follow-up results showed that the number of *Runx3*-positive patients was higher than negative ones, while *HER2* positive were fewer than negative ones. In vitro experiments showed that *RUNX3* protein and gene expression decreased, HER2 protein and gene expression increased in *RUNX3* knockdown RKO and HCT-116 cells, respectively ($P<0.05$).

Conclusions: The expression of *RUNX3* and *HER2* in CRC is related to the occurrence and development of CRC, and the two genes have a negative regulating effect.

Keywords: Colorectal cancer (CRC); RUNX3; HER2; molecular markers

Submitted Jun 08, 2021. Accepted for publication Aug 19, 2021.
doi: 10.21037/jgo-21-403
View this article at: https://dx.doi.org/10.21037/jgo-21-403

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Introduction

According to the latest epidemiological report, China has an average of 376,000 new patients with colorectal cancer (CRC) every year, about 191,000 people die of CRC, and there is a trend of gradual growth in cases and younger age, with most patients being diagnosed in the middle and late stages, which carries a poor prognosis (1). The pathogenesis of CRC is complex due to its multi-factor and multi-stage co-pathogenesis. RUNT-associated transcription factor 3 (*RUNX3*) is a tumor suppressor gene that has attracted much attention in CRC research in recent years. Previous studies have shown that its expression is higher in normal tissues than in cancer tissues, especially in digestive tract tumors (2-6). Human epidermal growth factor receptor 2 (*HER2*) is a well-studied oncogene; its expression in breast cancer and gastric cancer is relatively mature, and its expression is related to prognosis and there is consensus on the effectiveness of it as a therapeutic target. However, there are few studies on *HER2* expression in CRC and there are certain differences (Most researchers believe that the high expression of *HER2* in CRC is related to the occurrence, development and prognosis of CRC.A few researchers have suggested that *HER2* is not associated with the prognosis of CRC) (7-12). This study aimed to jointly detect the expression of *RUNX3* and *HER2* in CRC to preliminarily explore their correlation and clinical significance. RUNX3 may lead to CRC by negatively regulating HER2 gene-related pathways and detection of RUNX3 and HER2 in CRC could be used as a guide for targeted therapy thus provide experimental basis and scientific basis molecular mechanisms that occur and for targeted therapy of CRC. We present the following article in accordance with the REMARK reporting checklist (available at https://dx.doi.org/10.21037/jgo-21-403).

Methods

General data

All CRC specimens surgically resected from the First Affiliated Hospital of Hebei North University from May 2015 to January 2016 were collected, a total of 53 cases, and the adjacent normal tissues >10 cm from the tumor were taken as control samples. Part of each sample was frozen in liquid nitrogen to extract mRNA and proteins. The remaining tissue was fixed with 10% formalin solution for sectioning. There were 28 males and 25 females among the CRC patients; 21 were aged from 41 to 61, 32 were aged from 62 to 84 years; 45 of the patients had high or medium differentiation, 8 patients had low differentiation; there were 44 cases of adenocarcinoma and 9 of mucinous adenocarcinoma; 23 cases were located in the colon and 30 in the rectum; 24 patients were pN+, 29 were pN–; 34 patients were pT I + II, and 19 were pT III + IV. All the diagnoses were confirmed by pathology, and none of the patients underwent chemotherapy, radiotherapy or immunotherapy before surgery. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). This study was reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Hebei North University (No.:K2020291). All patients signed informed consent. Human colorectal adenocarcinoma cell lines RKO and HCT-116 were used for si-RNA interference experiments.

Patient information was collected: sex, age, site of the tumor, tumor tissue type, degree of differentiation, pT stage, pN stage, and Expression levels of RUNX3 and HER2 genes and proteins.

Reagents

For immunohistochemistry, a rabbit SP kit (SP-9001), DAB kit (ZLI-9018, Beijing Zhongshan Jinqiao Biological Co., Ltd.), rabbit-antihuman RUNX3 (EPR20687, Abcam), and rabbit-antihuman HER2 (ER0106, Huaan Bio) were obtained.

For reverse transcription-polymerase chain reaction (RT-qPCR), a total mRNA extraction kit (TR150-50), reverse transcription 5xABScript (RK20402), and SuperReal Fluorescent quantitative kit (2xSYBR Green) (RK21203, Beijing Tianmo Sci & Tech Development Co., Ltd.) were used. Shanghai Sangon Bio designed the synthetic primers.

For western blot, RIPA (Radio Immunoprecipitation Assay), SDS (sodium dodecyl sulfate) gel reagents, SDS-PAGE (SDS polyacrylamide gel electrophoresis), PVDF (polyvinylidene fluoride) membrane, and ECL (electrochemiluminescence) kit were bought from Beyotime Biotechnology. Rabbit-antihuman RUNX3 (AF5188), rabbit-antihuman HER2 (ER0106), and rabbit-antihuman GAPDH (SA30-01) (house-keeping genes) were bought from Huaan Bio. Si-RUNX3, Si-NC and transfection reagents were synthesized and provided by Shanghai Genepharma Company.

Table 1 RT-qPCR primer sequences

Gene name	Sequence	Amplified fragment size (bp)
RUNX3	Forward 5'-TCAACGACCTTCGCTTCGTG- 3'	119
	Reverse 5'-CACGGTCACCTTGATGGCTC-3'	
HER2	Forward 5'-CACAACCAAGAGGTGACAGCAGAG-3'	80
	Reverse 5'-CCATAGCACACTCGGGCACAG- 3'	
GAPDH	Forward 5'-GAGTCAACGGATTTGGTCGT -3'	131
	Reverse 5'-GACAAGCTTCCCGTTCTCAG - 3'	

RT-qPCR reverse transcription-polymerase chain reaction

Immunohistochemistry

Tissue samples were dehydrated, clarified, paraffin impregnated, and embedded before 4 μm tissue sections were dewaxed, hydrated, antigen repaired, 100 ℃ for 5 min, 50 ℃ for 15 min, and cooled at room temperature. Sections were rinsed with phosphate-buffered saline (PBS) for 15 min before dropping reagent each time. The sections were incubated in 0.5% peroxidase at room temperature for 15 min, incubated in goat serum for 30 min at 37 ℃, and primary antibody (RUNX3 1 μL + PBS 100 μL + bovine serum albumin (BSA) 3 μL; HER2 1 μL + PBS 100 μL + BSA 3 μL) was added, before placing them in 4 ℃ refrigerator overnight. On the second day, secondary antibody was added at 37 ℃ for 30 min, and horseradish peroxidase was added before incubation at room temperature for 15 min. Under the microscope, DAB (1:20) developed color, and when yellow-brown particles were observed, the reaction was terminated. Dyeing with hematoxylin for 3 min, hydrochloric acid and alcohol differentiation was followed by water rinse for 10 min, and the sections were sealed with neutral resin.

The whole section was first observed under low power, then under high power five high-power fields were randomly selected for analysis using ImageJ software. (I) Automated grading of sample staining degree was performed using an IHC Profiler plug-in. According to the average gray value and area percentage (positive cells), 4 scores were evaluated: 3 points (strong positive), 2 points (positive cells), 1 point (weak positive cells), 0 point (negative cells). (II) Counting of positive cells using the Trainable WEKA Segmentation plug-in. According to the percentage of positive cells (accounting for the total number of cells), the number of positive cells <5% was rated as 0, 6–25% as 1, 26–50% as 2, 51–75% as 3, >76% as 4; the product of two scores <3 was rated as negative, and >3 as positive.

RT-qPCR

To investigate the relative expression levels, 5 mg samples of CRC and adjacent normal tissues were taken, 300 μL of lysate were added, the samples were cut into pieces, homogenized, and the supernatant was extracted by centrifugation. Equal volume of anhydrous ethanol was added to the supernatant, centrifuged, 400 μL RNA washing solution was added, and centrifuged again. The supernatant was discarded. Next, 40 μL DNAsel1 reaction solution (DNAsel1 5 μL + DNA digestion solution 35 μL) was added to the precipitate, and incubated at room temperature for 15 min. Pre-wash solution, washing solution and ethanol were added, and then centrifuged and the supernatant was discarded (according to kit instructions). The purity and concentration of total mRNA were determined by UV spectrophotometry after adding 15 μL RNase-free H_2O. A260/280 was between 1.8 and 2.1, and the concentration was between 600 and 1,000 ng/μL. For the reverse transcription system, total mRNA 4 μL + 5× ABscript2RT MIX 4 μL + 12 μL RNase-free H_2O was processed at 25 ℃ for 5 min, 42 ℃ for 15 min, 85 ℃ for 5 s, and 12 ℃ for 30 min. GAPDH was used as reference gene for relative quantitative analysis. Primer sequences are shown in *Table 1*. For the RT-qPCR reaction system: cDNA 2 μL (200 ng) + 2× SYBR Green Fast qPCR Mix 10 μL + 0.4 μL forward primer + 0.4 μL reverse primer + nuclease-free H_2O 7.2 μL were used. Reaction conditions were: 1 cycle at 95 ℃ for 1 min,;5 cycles of 94 ℃ for 20 s, 55 ℃ for 30 s, 72 ℃ for 30 s, and 40 cycles of 94 ℃ for 15 s, 60 ℃ for 30 s, Collect the fluorescent. Three samples were taken from each tissue type, and each sample was replicated in three wells. The

Table 2 si-RUNX3 sequences

Gene name	Forward	Reverse
si-RUNX3-1	5'-UGACGAGAACUACUCCGCUTT-3'	5'-AGCGGAGUAGUUCUCGUCATT-3'
si-RUNX3-2	5'-CCCUGACCAUCACUGUGUUTT-3'	5'-AACACAGUGAUGGUCAGGGTT-3'
si-RUNX3-3	5'-CCUCGGAACUGAACCCAUUTT-3'	5'-AAUGGGUUCAGUUCCGAGGTT-3'
si-NC	5'-UUCUCCGAACGUGUCACGUTT-3'	5'-ACGUGACACGUUCGGAGAATT-3'

RT-qPCR reverse transcription-polymerase chain reaction.

dissolution curves were all unimodal, without bimodal and with good peak type (peak width <7 grids, peak >800). The obtained CT (cycle threshold) values were usable. The analytical method was $2^{-\Delta\Delta Ct}$.

Western blot

For the relative expressions of proteins, 20 mg samples of CRC and adjacent normal tissues were taken, 200 μL RIPA lysate was added, and the samples were shredded, homogenized, and centrifuged. The supernatant was taken, and the total proteins of CRC and adjacent normal tissues were obtained. The BCA (Bicinchoninic Acid Assay) method was used to determine its concentration. Equal volume of buffer was added, the protein was denatured at 100 ℃ for 5 min, and stored at 4 ℃. Preparation of SDS/PAGE separation glue 8% and 10%, concentrated glue 5%. The proteins were separated by electrophoresis and transferred onto the PVDF membrane, which was sealed with TRIS hydrochloric acid buffer (containing 5% BSA) and slowly shaken at room temperature for 2 h. Shaken slowly the PVDF in primary antibody (GAPDH 1:10,000, RUNX3 1:1,000, HER2 1:1,000) was incubated at 4 ℃ for 12 h, washed with 1× TBST membrane wash solution three times for 10 min each time. The incubation with dropwise add secondary antibody (1:10,000) was slowly shaken at room temperature for 2 h and washed with 1× TBST membrane wash solution three times for 10 min each time. For the ECL method, AI600 was used to obtain the blots, and all samples were repeated for three times. Images were quantitatively analyzed by Image J, and the relative expression of the target protein was represented by the mean value of the target band gray value [IOD (integrated optical density)].

Survival follow-up

Patients were actively followed up by telephone or reexamination at the outpatient department. The last visit was in January 2021. The endpoint was tumor-related death.

In vitro experiments

The RKO cell lines were maintained with Dulbecco modified Eagle medium (DMEM) with the addition of 12% fetal bovine serum (FBS), and the HCT-116 cell lines were maintained with McCoy 5A medium and 12% FBS. The cells were cultured in an incubator at 37 ℃ with 5% CO_2. Trypsin digested the cells in the logarithmic growth phase, the cell density was diluted to 2×10^5 cells/mL, and the cells were seeded into 6-well plates with 2 mL/well before being incubated overnight. Next, 5 μL si-Runx3, si-NC (sequence shown in *Table 2*) and transfection reagents were added with 200 μL DMEM and McCoy 5A without FBS, mixed and held for 5 min. The transfection reagents were added into si-RUNX3 and si-NC respectively and mixed, and then stood for 20 min. The medium in the 6-well plate was removed, the plate was washed twice with PBS, 1.5 mL DMEM or McCoy 5A medium without FBS was added to each well, diluent of the mixture was dropped in, and mixed thoroughly. After 6 h of culture, transfection efficiency was observed. The medium containing FBS was changed. After 24 h, lytic solution was added to collect the cells. cDNA and protein was extracted and the relative expression of RUNX3 in the RKO and HCT-116 cells was detected by RT-qPCR and Western blot. HER2 expression was measured by cDNA and protein with knockdown efficiency ≥50% (knockdown efficiency = 1-si-Runx3 relative expression/si-NC relative expression × 100%).

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical software. Measurement data are expressed as mean ± standard deviation, and pairwise comparison was performed by *t* test. Enumeration data are expressed as percentage,

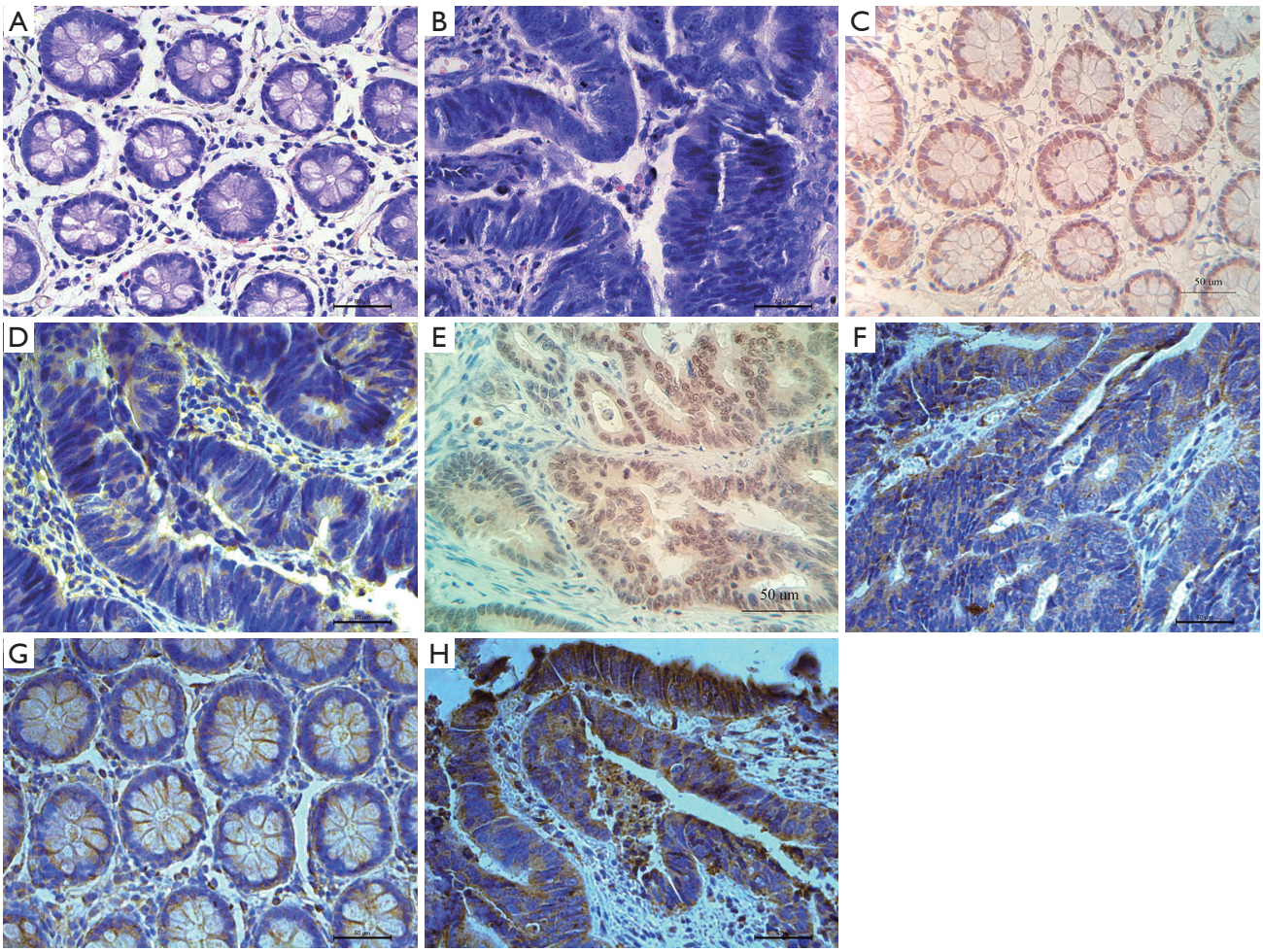

Figure 1 Hematoxylin-eosin staining of (A) normal tissue, (B) cancer tissue. Immunohistochemical SP staining of (C) RUNX3 staining in normal tissues; (D) low RUNX3 expression in cancer tissue; (E) high RUNX3 expression in cancer tissue; (F) low HER2 expression in cancer tissue; (G) HER2 staining in normal tissue; (H) high HER2 expression in cancer tissue.

and pairwise comparison was performed by chi-square test. The disease-free survival (DFS) rate was analyzed by Kaplan-Meier, and the difference was analyzed by log-rank. GraphPad 9.0 was used to create graphs, and Pearson analysis was used to analyze the correlation. $P<0.05$ was considered statistically significant.

Results

Immunohistochemical results

Normal intestinal adipocyte showed uniform size, regular arrangement and clear structure. The adenocarcinoma cells were uneven in size and disordered in arrangement, and the normal tissue structure disappeared (*Figure 1A,1B*). A total of 18 cases (33.9%) of the 53 cancer tissues were positive for RUNX3, while 43 cases (81.1%) of the normal tissues were positive for RUNX3. The positive expression rate of RUNX3 in CRC was lower than in the adjacent normal tissues, and the difference was statistically significant ($P<0.01$). RUNX3 was located in the nucleus, and was brown in normal tissue, but negative or dislocation nucleus expressed in CRC (*Figure 1C-1E*). Among the 53 cases of CRC, 29 (54.7%) were HER2 positive, and in the normal tissue 4 cases (7.5%) were HER2 positive. The positive expression rate of HER2 in CRC was higher than that in the control group, and the difference was statistically

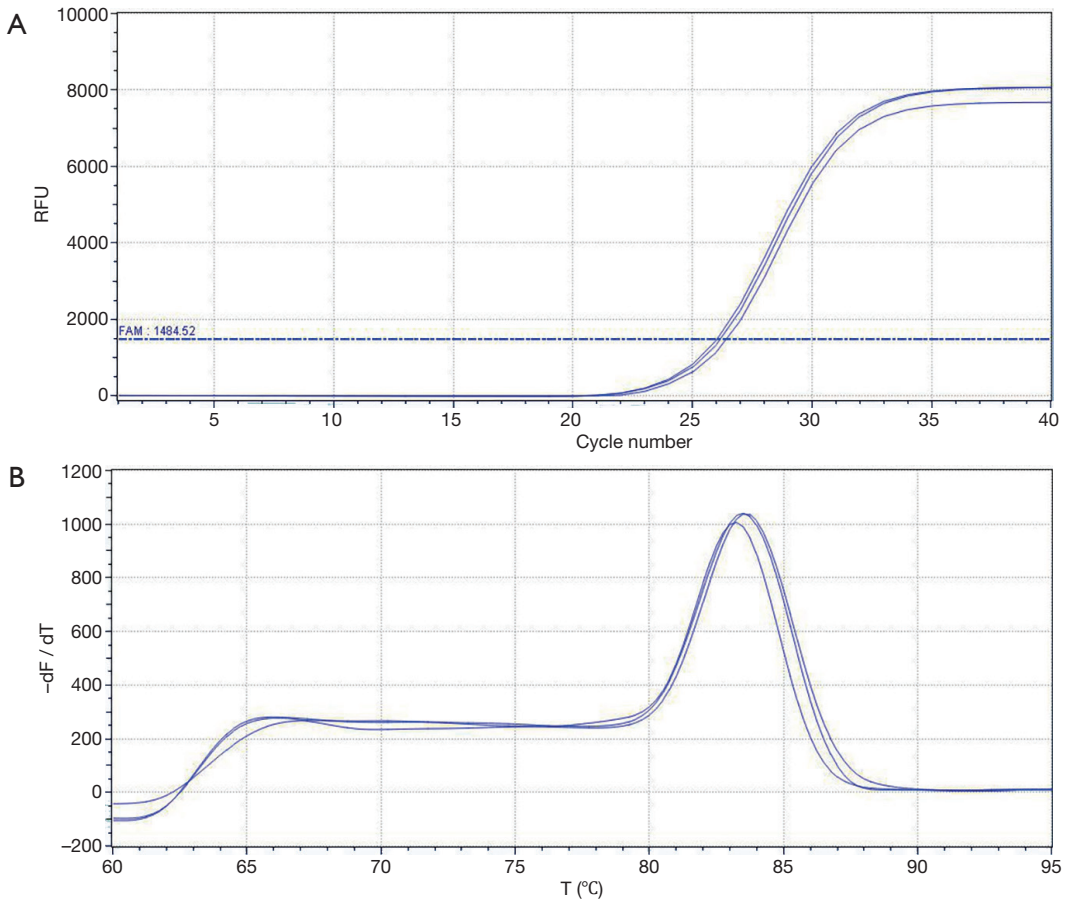

Figure 2 RT-qPCR amplification and dissolution curves. (A) RT-qPCR amplification curve; (B) RT-qPCR dissolution curve. RT-qPCR, reverse transcription-polymerase chain reaction.

significant (P<0.01). HER2 mainly localized in the cell membrane and can be secreted out of the cell. CRC tissues were stained brown, while normal tissues were pale yellow or negatively expressed (*Figure 1F-1H*).

RT-qPCR results

The amplification curve and dissolution curve were good. The dissolution curve was unimodal, the peak width was <7 cells, and the peak height was >1,000 (*Figure 2A,2B*). The relative expression level of *RUNX3* in cancer tissues (2.554±0.8429) was lower than that in adjacent normal tissues (8.864±2.4632), with s statistically significant difference (t=3.481, P=0.005) (*Figure 3A*). The relative expression level of *HER2* in cancer tissues (2.783±0.7444) was higher than that in adjacent normal tissues (1.323±0.2381), with a statistically significant difference (t=–2.656, P=0.017) (*Figure 3B*).

Western blot results

The relative expression level of RUNX3 protein in the three CRC tissue samples (14,575.667±314.766) was lower than that in the adjacent normal tissues (63,896.667±467.255), with a statistically significant difference (t=131.988, P=0.000). The relative expression level of HER2 protein in cancer tissues (59,142.000±800.989) was higher than that in adjacent normal tissues (4,057.667±648.519), with a statistically significant differences (t=–59.720, P=0.000) (*Figure 4*).

Relationship between positive expression of RUNX3 and HER2 and clinical features of tumors

The expressions of RUNX3 and HER2 in CRC did not significantly correlate with sex, age, lesion site, type, etc., but closely correlated with pT and pN staging, as shown in *Table 3*.

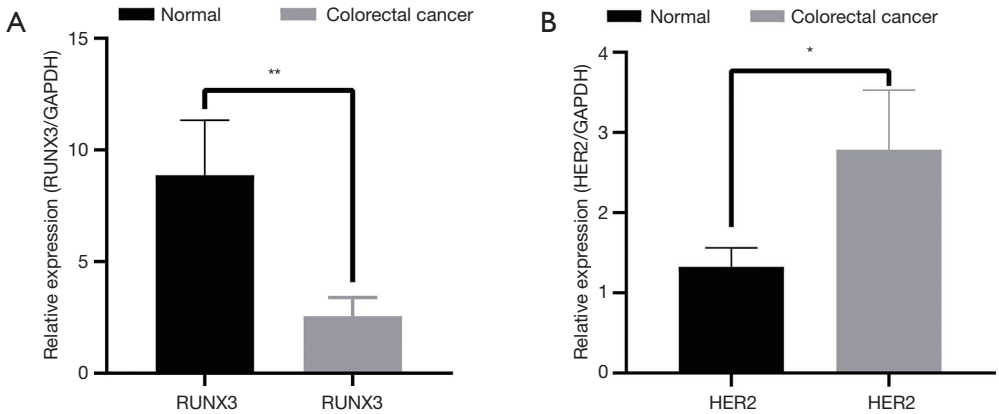

Figure 3 *RUNX3* and *HER2* mRNA relative expression (A) *RUNX3* mRNA relative expression; (B) *HER2* mRNA relative expression. *, P<0.05; **, P<0.01.

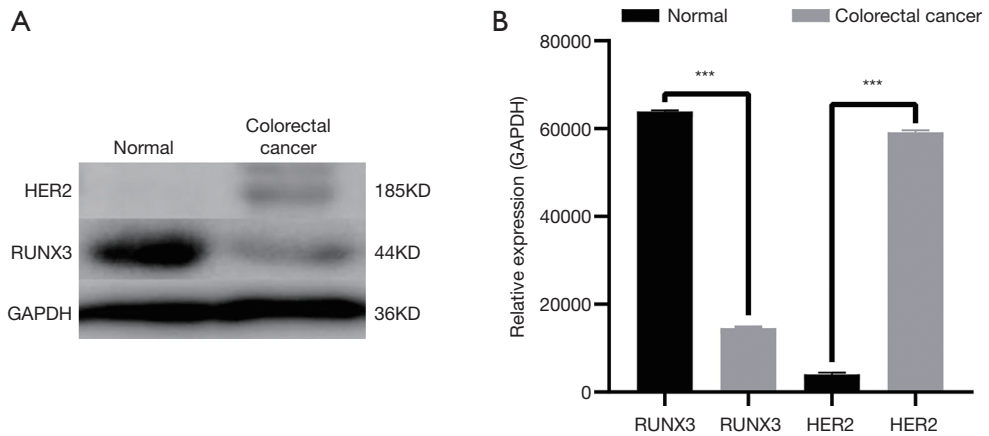

Figure 4 RUNX3 HER2 protein relative expressions. ***, P<0.001.

Survival analysis

Kaplan-Meier method was used to draw the survival curve of CRC patients, and the survival rate of RUNX3 and HER2 expression was analyzed. The results showed that there were more patients with positive RUNX3 expression in 5-year DFS than those with negative RUNX3 expression (75% *vs.* 46.15%, P=0.036) (*Figure 5A*). The patients with HER2 positive expression in DFS at 5 years were fewer than those with HER2 negative expression (53.57% *vs.* 82.46%, P=0.023) (*Figure 5B*).

Correlation analysis

The expression of *RUNX3* negatively correlated with the expression of *HER2* in CRC (r=–0.826, P=0.000) (*Figure 6*).

In vitro experiments

When *RUNX3* was knocked down in RKO and HCT-116, *HER2* expression increased. The transfection efficiency of si-RUNX3 is shown in *Figure* 7. The knocking out efficiency of si-RUNX3-1 was 84.7% (P=0.001), that of si-RUNX3-2 was 45.1% (P=0.004), and that of si-RUNX3-3 was 56.1% (P=0.003) (*Figure 8*). We selected si-RUNX3-1 for subsequent experiments. In RKO and HCT-116 cells knocked down by si-RUNX3, RUNX3 protein expression decreased by 59.3% (P=0.001) and 84.1% (P=0.000),*RUNX3* gene expression decreased by 77.4% (P=0.000) and 70.0% (P=0.003), respectively, while HER2 protein expression increased by 3.9% (P=0.000) and 31.7% (P=0.000),*HER2* gene expression increased by 6.3% (P=0.041) and 7.2% (P=0.024), respectively (*Figure 9*).

Table 3 Correlation between the expressions of RUNX3 and HER2 in CRC and CRC pathological features

Variable	n	RUNX3			HER2		
		Positive (%)	χ^2	P	Positive (%)	χ^2	P
Sex			0.081	0.776		0.862	0.353
Male	28	10 (35.71)			17 (60.71)		
Female	25	8 (32.00)			12 (48.00)		
Age (years)			0.451	0.502		0.083	0.774
41–61	21	6 (28.57)			12 (57.14)		
62–84	32	12 (37.50)			17 (53.13)		
Location			0.225	0.635		0.621	0.431
Colon cancer	23	7 (30.43)			14 (60.87)		
Rectal cancer	30	11 (36.67)			15 (50.00)		
Type			0.000	1.000		1.096	0.295
Adenocarcinoma	44	15 (34.09)			26 (59.09)		
Mucinous adenocarcinoma	9	3 (33.33)			3 (33.33)		
Differentiation			0.031	0.860		0.009	0.925
High and medium	45	16 (35.56)			24 (53.33)		
Low	8	2 (25.00)			5 (62.50)		
pN stage			4.526	0.033		10.582	0.001
N+	24	4 (16.67)			19 (79.17)		
N–	29	14 (48.28)			10 (34.48)		
pT stage			5.716	0.017		5.576	0.018
I + II	34	16 (47.06)			14 (41.18)		
III + IV	19	2 (10.53)			15 (78.95)		

CRC, colorectal cancer.

Discussion

RUNX3 is a tumor suppressor gene that has attracted much attention in recent years. Located on human chromosome 1p36.1, the gene is 67 kb and the protein has a molecular weight of ≈44 kD. *RUNX3* has two promoters, P1 and P2, and the P1 promoter contains a large number of T cell binding sites. P2 has cytosine-phosphate-guanine islands and a GC (guanine cytosine) content up to 64%. It is characterized by a GC promoter and is more prone to methylation. RUNX3 protein has 415 amino acid residues, which constitute two subunits (A and B). A binds to the target DNA, and B enhances the binding force of A to maintain the normal regulatory function. The carboxyl terminus is rich in proline and serine, which play an important role in transcriptional regulation (5,10). The human transforming signal transduction molecule Smad protein is the main mediator of the TGF-β(transforming growth factor-β) signal pathway in the cell. Smad's entry into a specific position must be guided by RUNX3 to jointly activate target genes and regulate cell differentiation, cell cycle, apoptosis and transformation. RUNX3 binds TCF4 and β-catenin in the Wnt pathway, competing for its binding to target DNA and inhibiting carcinogenesis. RUNX3 can regulate the process of epithelial-mesenchymal transformation and claudin-1 protein expression (2,4,13). RUNX3 inhibits gastrointestinal tumor invasion and metastasis by regulating vascular endothelial growth factor

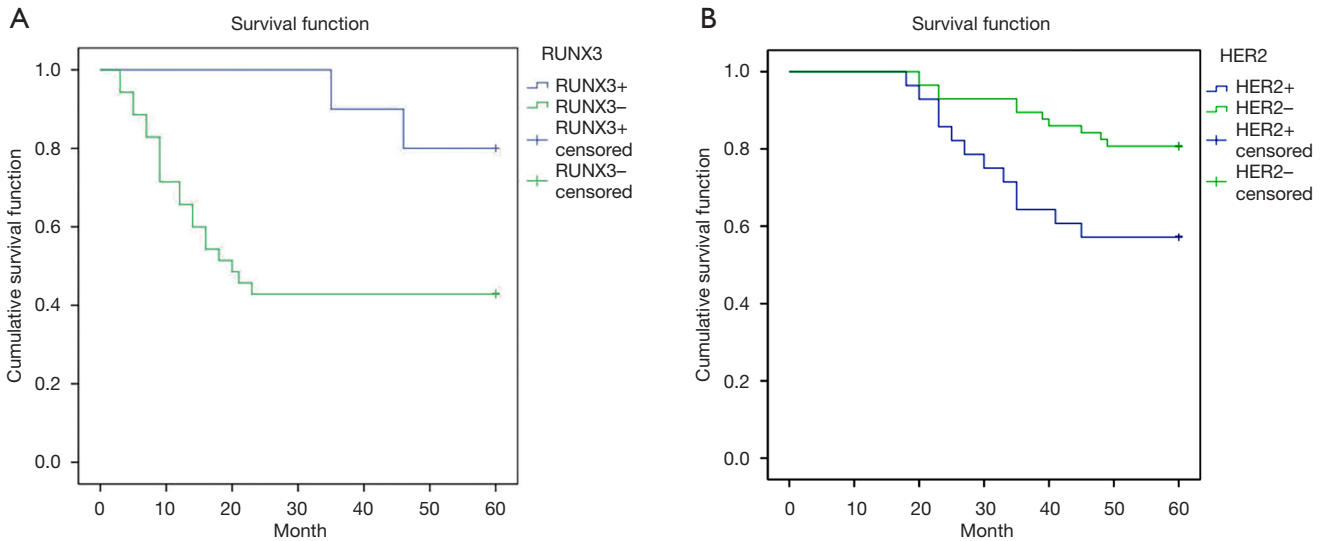

Figure 5 Relationship of (A) RUNX3 expression and (B) HER2 expression with DFS. DFS, disease-free survival.

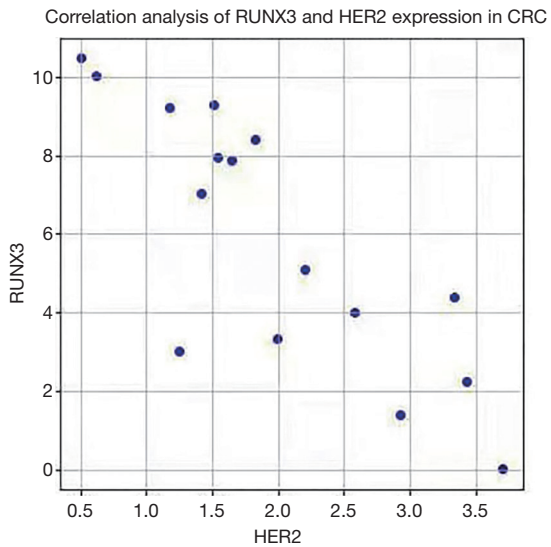

Figure 6 Correlation between RUNX3 and HER2 expression in CRC tissue. CRC, colorectal cancer.

(VEGF) (14,15). Highly methylated or point mutated of *RUNX3* could reduce RUNX3 expression in CRC (16,17).

HER2, a well-studied proto-oncogene, is the coding product of ERBB2 on chromosome 17 with a molecular weight of ≈185 kD. HER2 protein is a transmembrane glycoprotein with tyrosine kinase activity. HER2 expression is very low in normal human tissues, and high expression usually occurs only during the fetal period. The monomer of HER2 is inactive and the heterodimer has strong growth promoting ability. The upregulation of VEGF by HER2 can promote the formation of new blood vessels and increase the invasiveness of tumors, and the anti-invasion barrier of the body is destroyed (18). The ligand binds to form the dimer, and then phosphorylated phosphokinases are activated. Intracellular adaptor molecules recognize the phosphorylated sites, and intracellular signaling pathways such as Ras/MAPK, PI3K/Akt, or DAG/IP3 are coupled, resulting in cell growth and proliferation. At the same time, a series of negative feedback mechanisms, such as endocytosis, circulation and degradation, dephosphorylation, and serine/threonine phosphorylation in the intimal domain, attenuate the growth signal and destroy the regulatory mechanism, such as HER2 mutation or amplification, leading to continuous growth and carcinogenesis. Tumor proliferation, differentiation and metastasis can also be regulated by STAT, PLCg and other signaling pathways (19).

Previous studies have shown that the expression of RUNX3 in normal tissues is higher than in CRC (2-6). The study of HER2 is relatively mature in breast cancer, and prognosis is related to its expression level. The targeted drug Herceptin has become the standard treatment for patients with HER2-positive breast cancer (20), and its expression in gastric cancer is associated with tumor invasiveness and poor prognosis (21). There are many studies on the expression of RUNX3 and HER2 in CRC, but there are some differences in the reports (7-12). There are few studies of simultaneous investigation

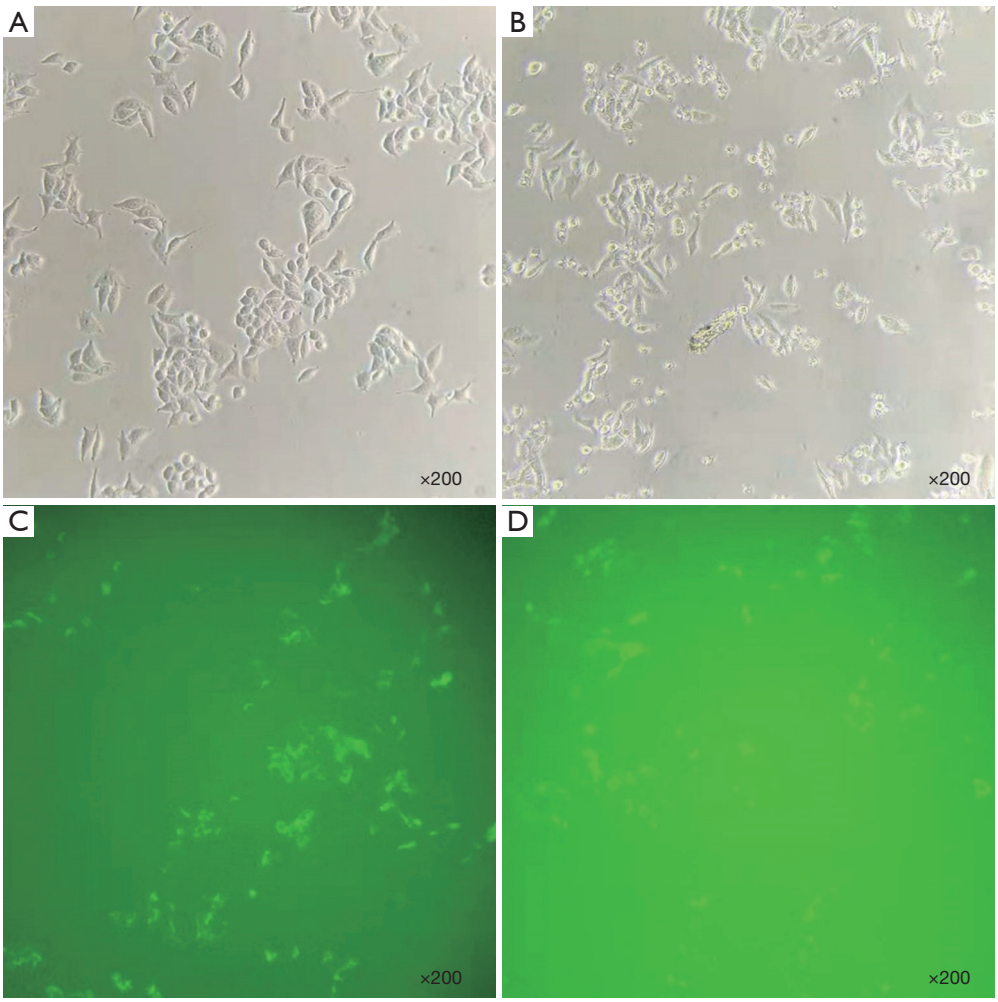

Figure 7 Si-RUNX3 transfected in RKO and HCT-116. (A,B) Bright field, (C,D) dark field. Magnification: ×200.

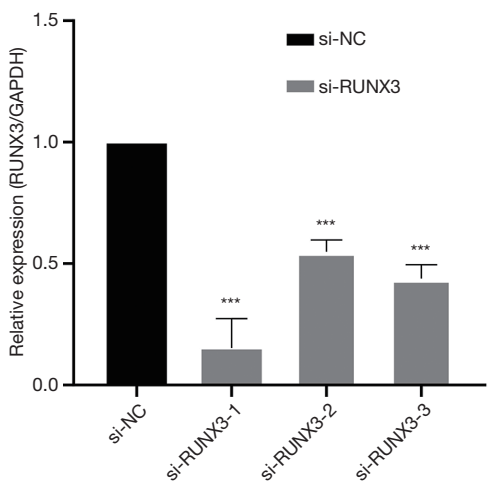

Figure 8 Knockdown efficiency of si-RUNX3 in RKO. ***, represent for P<0.001.

of RUNX3 and HER2 expressions in CRC, and the relationship between RUNX3 and HER2 in CRC has not been reported. In this study, immunohistochemistry was used to detect the expression and locations of RUNX3 and HER2. The relative expression level of mRNA was detected at the molecular level by RT-qPCR, and the relative expression level of the proteins was detected by western blot. This study showed that the mRNA and protein of RUNX3 in CRC were lower than in the adjacent normal tissues, while the mRNA and protein of HER2 in CRC were higher than in the adjacent normal tissues.

In this study, survival analysis showed that RUNX3 was associated with 5-year DFS of CRC, and the 5-year DFS of RUNX3-positive CRC patients was higher better than that of -negative CRC patients. The mRNA and protein of RUNX3 were closely related to the diagnosis, treatment

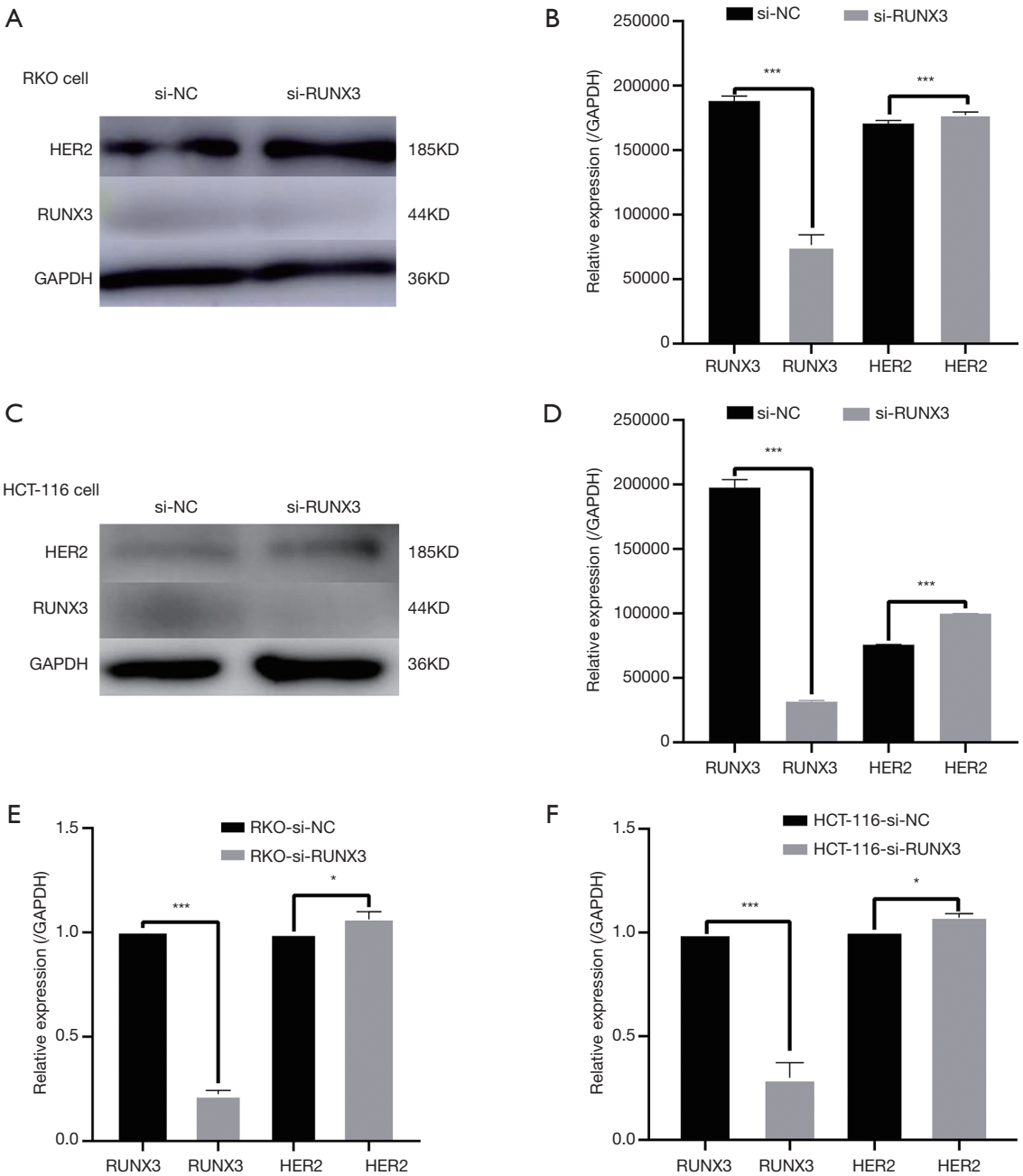

Figure 9 RUNX3, HER2 protein (A-D) and mRNA (E,F) relative expression in RKO and HCT-116. *, P<0.05; ***, P<0.001.

and prognosis of CRC. Detection of RUNX3 mRNA and protein can be used in the diagnosis of CRC and can determine prognosis (22). Recently, the detection of serum HER2 has been proved to be a quantifiable examination method by Siravegna *et al.* (23), indicating that the mRNA relative expression of serum RUNX3 can be an indicator for early screening of CRC.

In this study, the HER2 positive rate in CRC was 54.7%, which was similar to that reported by Wu *et al.* (24), higher than the 32.4% reported by Sun *et al.* (7), and lower than the 75% reported by Wen *et al.* (9), which may be related to whether the samples were fixed in time, whether the samples were repeatedly freeze-thawed (protein degradation results in a negative test), and the judgment of positive results (25).

This study proved that HER2 was highly expressed in CRC, and survival analysis showed that the 5-year DFS of CRC was associated with HER2, and that the 5-year DFS of CRC was lower in HER2-positive patients than in negative patients. Detection of HER2 in CRC patients could be used as a guide for targeted therapy, which could benefit them similarly to breast cancer and gastric cancer.

This study revealed a negative correlation between *RUNX3* and *HER2*, and further verified the correlation by cell experiments. The carboxyl terminal of RUNX3 is rich in proline and serine, which play an important role in transcriptional regulation. HER2 protein is a transmembrane glycoprotein with tyrosine kinase activity, which may affect the phosphorylation of RUNX3 proline and serine. This results in the silencing of RUNX3, resulting in the inability of the bound Smad protein to cross the membrane from within the cytoplasm to specific sites. *HER2* promotes the generation of new blood vessels by upregulating VEGF and increases tumor invasion. The anti-invasion barrier of the body is destroyed, which may affect *RUNX3* ability to regulate the expression of VEGF molecules to inhibit the invasion and metastasis of gastrointestinal tumors. Upregulated *Bcl-1*, *RUNX3* and *HER2* are located on the same chromosome. There may be interactions that lead to RUNX3 methylation or point mutation, but the specific interaction mechanism between RUNX3 and HER2 remains to be further studied.

Above all, RUNX3 and HER2 in the diagnosis of CRC and targeted therapy have important significance. In this study, only the gene and protein levels were detected to analyze their expression, correlation and clinical significance of prognosis in CRC tissue. Moreover, the negative regulatory relationship between RUNX3 and HER2 was confirmed by cell experiments. The interaction mechanism is preliminarily discussed. However, this study has not confirmed the specific interaction pathways between the two genes at the extracellular level, nor has it confirmed the relationship between the two genes and the occurrence and development of CRC at the intracellular level (such as tumor formation experiments in nude mice, etc.). This will be a further research direction.

Acknowledgments

Funding: Clinical Medicine Excellent Talents Training Project of Hebei Provincial Finance Department (361009), Medical Science Key Project of Hebei Provincial Science and Technology Department 2015 (152777237), Medical Science Key Project of Hebei Provincial Health and Family Planning Commission (20210519), Mandatory Project of Zhangjiakou Science and Technology Bureau of Hebei Province (1712005D).

Footnote

Reporting Checklist: The authors have completed the REMARK reporting checklist. Available at https://dx.doi.org/10.21037/jgo-21-403

Data Sharing Statement: Available at https://dx.doi.org/10.21037/jgo-21-403

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://dx.doi.org/10.21037/jgo-21-403). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by Ethics Committee of the First Affiliated Hospital of Hebei North University (No.:K2020291) and informed consent was taken from all the patients.

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(English Language Editor: K. Brown)

Cite this article as: Wu Y, Xue J, Li Y, Wu X, Qu M, Xu D, Shi Y. Expression, clinical significance and correlation of RUNX3 and HER2 in colorectal cancer. J Gastrointest Oncol 2021;12(4):1577-1589. doi: 10.21037/jgo-21-403

