High expression of Wnt7a protein predicts a poor survival in patients with colorectal carcinoma

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Abstract

Background: Colorectal cancer is one of the most common malignant tumors in China, and the number of new cases and the number of cases of deaths has increased annually. However, its pathogenesis is still unclear. Wnt7a is a member of the wingless-type MMTV integration site family, and it plays an important role in tumorigenesis and development by controlling cell proliferation and differentiation as a secreted glycoprotein. Whether Wnt7a has the properties of an oncogene or not is an important focus for future research as this target has diverse roles in different tumors.

Methods: Wnt7a protein expression in normal colorectal mucosa and colorectal tumors was detected via immunohistochemistry and Western blot analysis. Univariate and multivariate analyses were used to explore the associations between Wnt7a staining score and various clinical parameters.

Results: Wnt7a was strongly expressed in colorectal cancer tissues but weakly expressed in adjacent normal mucosa and colorectal adenomas. The level of Wnt7a expression was correlated with lymph node involvement ($P < 0.001$), Duke stage ($P < 0.001$), and cell differentiation ($P < 0.001$). Knockdown of Wnt7a inhibits proliferation of colon cancer cells and inhibits the ability of both colon cancer cell lines to migrate.

Conclusions: Collectively, our results present evidence that Wnt7a is associated with an unfavorable prognosis of colorectal cancer.

Background

Colorectal cancer (CRC) is one of the most common malignant tumors in the world. Its global comprehensive incidence rate ranks third among men in malignant tumors and second among women [1]. Colorectal cancer is the second leading cause of cancer death [2, 3]. In China, the number of new cases of colorectal cancer and the number of cases of death has also increased year by year. From 2008 to 2013, the incidence of colorectal cancer in China has risen from 14.6/100,000 to 17.2/100,000. The mortality rate has risen from 6.18/100,000 to 7.76/100,000 [4]. In China, more than 50% of patients with colorectal cancer miss the ideal time for treatment, and the 5-year survival rate is less than 40% [6]. Therefore, early identification and diagnosis of colorectal cancer as well as early intervention can improve the survival rate of colorectal cancer. Several markers have been known to be either positive or negative indicators of the disease, which indicates these complementary biomarkers may contribute to risk assessment and aid in the personalized treatment of CRC patients [7-9,15]. Histological and serological samples are also be easily collected from patients. Thus, the discovery of more prognostic biomarkers for CRC can help predict patient outcomes and provide a novel therapeutic target.

Wnt7a is closely related to a variety of tumors. (1) Wnt7a exerts a tumor suppressor effect in various cancers. Ochoa-Hernández et al. [10] demonstrated its role in leukemia, showing through the construction of overexpressing virus that Wnt7a is expressed in leukemia cell lines and Wnt7a expression in normal peripheral blood T lymphocytes is significantly higher than in leukemia cells. Calvo et al. [11] and Ohira et

al. [12] found that Wnt7a may play a tumor suppressor role in lung cancer (especially non-small cell lung cancer), finding that Wnt7a is down-regulated in non-small cell lung cancer. Ohira et al. [12] also found that Wnt7a plays an important tumor suppression role in lung cancer, which may be related to the absence of E-cadherin. (2) Wnt7a also plays a carcinogenic role. Yoshioka et al. [13] injected Wnt7a ovarian cancer cells and Wnt7a-expressing ovarian cancer cells into nude mice, and then found that Wnt7a ovarian cancer SKOV3ip1 was knocked out. Tumor lesions and cell invasion in the cell group were relatively small. Carmon and Loose [14] found in endometrial cancer cells by co-immunoprecipitation that Wnt7a activates the canonical Wnt/β-catenin signaling pathway by binding to Fzd5, which ultimately leads to cell proliferation. In colorectal cancer, it has been shown that high expression of Wnt7a protein predicts poor survival in patients with colorectal carcinoma [15]. However, the role that Wnt7a plays in colorectal cancer remains unknown.

In the present study, we detected the expression of Wnt7a protein in CRC patients and analyzed the correlations between the expression and clinicopathological prognostic variables and survival.

Methods

Patients and tumor specimens

Eighty specimens from the Department of Gastroenterology and General Surgery of Beijing Tiantan Hospital affiliated with Capital Medical University from June 2013 to March 2017 were collected and confirmed by pathology as colorectal adenocarcinoma. There were 49 males and 31 females; aged 24–73 years, mean age (54.88 ± 10.32) years old, 35 cases less than 65 years old, 45 cases older than 65 years; 26 cases of rectal adenocarcinoma, 54 cases of colon adenocarcinoma; 51 cases with diameter ≤ 5 cm, 29 cases with diameter > 5 cm; 55 cases with infiltration into serosal layer, 25 cases without infiltration into the serosal layer; 25 cases with highly differentiated adenocarcinoma, 22 cases with moderately differentiated adenocarcinoma, 33 cases with low differentiated adenocarcinoma; 28 cases without lymph node metastasis, 52 cases with lymph node metastasis; 9 cases with distant metastasis, 71 cases with no distant metastasis; Dukes staged 32 cases in A + B stage and 48 cases in C + D stage. All cases occurred with no other tumors, no tumor bleeding, intestinal perforation, intestinal obstruction, acute or chronic infection. In the control group, 20 normal tissues and 40 benign colon adenomas were selected. The appropriate paraffin tissue specimens were selected and serially sectioned 4 μm thick for immunohistochemical staining, screened by two pathologists, and the final results were confirmed.

Immunohistochemistry staining

Wnt7a immunohistochemical analysis was performed on 80 CRC specimens. Firstly, the paraffin-embedded and formalin-fixed tissues were cut into 4-mm sections and dried at 70°C for 2 hours. After dewaxing and hydration, the antigen was rinsed with phosphoric acid buffer saline (PBS) and soaked in 3% hydrogen peroxide for 10 min. The antigen was extracted from the citrate buffer (pH 6.0). Finally, after incubation with secondary antibodies for 30 min, staining with 3, 3-diaminobenzidine (DAB) for 10 min, slight back-dyeing with 10% Mayer's hematoxylin, dehydration, and pilling.

Immunohistochemical evaluation

The staining was assessed by a semi-quantitative analysis and a protein level score which is equal to the positive cell proportion score added to the cell staining score. A well-stained area was selected and observed in 10 high-power fields continuously, and more than 50 cells per field were observed. The procedure outlined in Wang et al. [16] was then followed to calculate the proportion of stained cells. If the proportion of positive cells < 10%, the score is 0; if the proportion of positive cells is between 10–40% the score is 1; if the proportion of positive cells is between 40–70% the score is 2; if the proportion of positive cells is ≥ 70% the score is 3. The scores designate: no coloration (0 points); yellow staining (1 point); brownish yellow staining (2 points); yellowish brown staining (3 points). Final protein level scores are as follows: 0 is negative, 1–3 is +, and 4–6 is ++. Two pathologists analyzed the stained tissue sections and they were blinded to the patient's clinical parameters.

Cell culture

Colorectal cancer cell lines HT-29 and HCT-116 were cultured in a constant temperature and humidity incubator containing 5% CO_2 at 37°C using complete medium (containing McCoys5A medium, 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin). in. According to the cell growth condition, the medium was cultured once every 2–3 days, and when the cells covered most of the surface of the bottom wall of the bottle, the cells were passaged or collected.

Plasmid construction

Expression of Wnt7a was inhibited by small hairpin RNA (shRNA) technology constructing a lentiviral vector. According to the design principle of shRNA target sequence screening, combined with the gene sequence of human Wnt7a and website analysis, a specific shRNA target sequence was selected and determined. Using the NCBI database to perform Blast analysis on the identified target sequences, it is shown that there is no homology with other known genes, and then the sense and antisense strands of the synthetic oligonucleotide are designed to be annealed, that is, they are separately dissolved in double steaming. In water, the equimolar number was mixed and heated at 95°C for 5 min, and naturally cooled to 37°C to form a double-stranded oligonucleotide. The annealed shRNA sequence was inserted into the digested pSIH1-H1-Puro vector, and the ligated product was transformed into E. coli DH5α competent cells, and monoclonal sequencing was performed.

Plasmid transient transfection

The cells were cultured in DMEM medium containing 10% newborn calf serum and double antibody, and HT-29 cells and HCT-116 were seeded in 12-well plates in DMEM medium containing no double antibody and 10% fetal bovine serum. A cell cell density of 80% during dyeing, and transfection after 24 hours of culture was achieved. A total of 2 μg of the plasmid with 50 μL serum-free and antibiotic-free DMEM medium were mixed, and then added to 1 μL Vigorous with 50 μL serum-free antibiotic-free DMEM medium and left to stand for 15 min at room temperature. The cells were harvested in a 12-well plate at

37°C 5% CO_2 for 48h. Wnt7a shRNA lentiviral particles were obtained, and intestinal cancer cells HT29 cells (i.e., cells infected with Wnt7a shRNA, HT29-shWnt7a-b, HT29-shWnt7a-c) were obtained, and intestinal cancer HT29 cells and HCT-116 were infected with eGFP shRNA lentivirus as control. Blank reagents were used.

Western blot

Total protein was extracted and lysed with RIPA buffer containing protease inhibitor cocktail (Roche). The proteins were separated by SDS-PAGE electrophoresis and transferred to membrane. Membranes were probed with specific primary antibodies against β-actin (Santa Cruz), Wnt7a (abcam), and detected by horseradish peroxidase-conjugated secondary antibodies.

qRT-PCR

Colon cancer cells were harvested 48 h after transfection, and Wnt7a total RNA was extracted according to the Trizol instructions. qRT-PCR analysis was performed using GoScriptTM Reverse Transcription System and GoTaq® qPCR Master Mix to detect Wnt7a expression levels, and the results were normalized by the expression level of phosphoglycerate dehydrogenase (GAPDH). The Wnt7a primer sequence was: upstream primer: 5'-CCTGGGCCACCTCTTTCTCAG-3', downstream primer: 5'-TCCAGCTTCATGTTCTCCTCCAG-3'. The product size was 573 bp. Using GAPDH as an internal reference, the upstream primer: 5'-GCATCCTGGGCTACACTGAGC-3', the downstream primer 5'-GGTACATGACAAGGTGCGGC-3', and the length of the PCR product fragment was 368 bp. The results of qRT-PCR were analyzed and their values relative to the number of critical cycles were calculated and then converted to fold changes relative to GAPDH, normalized using the $2^{-\Delta\Delta Ct}$ method.

Plate clone formation assay

The cells in the logarithmic growth phase were digested with 0.25% trypsin and blown into individual cells, and the cells were suspended in DMEM medium containing 10% fetal bovine serum for use. The cell suspension was diluted as a gradient. Each group of cells was inoculated with 10 mL of 37°C pre-warmed culture medium at a gradient density of 50, 100, and 200 cells per dish, and gently rotated to disperse the cells evenly. It was then incubated in a cell culture incubator at 37°C 5% CO_2 and saturated humidity for 2 to 3 weeks. When macroscopic clones appear in the culture dish, the culture was terminated. The supernatant was discarded and carefully immersed twice in PBS. Then, 5 mL 4% paraformaldehyde was added to fix the cells, after which the appropriate amount of Giemsa staining solution was applied for 10 to 30 minutes, and slowly washed away with running water and air dried. The plate was inverted and a grid of transparencies was overlaid. The clones were counted directly. Finally, the clone formation rate was calculated. Clonal formation rate = (number of clones / number of cells inoculated) × 100%.

Sphere formation assay

The colorectal cancer cells (HT-29 HCT-116) were digested with typsine and filtered with a 40-um strainer. The cells were grown in serum-free DMEM/F-12 (Gibco) supplemented with 10 ng/mL human recombinant bFGF (basic fibroblast growth factor; R&D), 10 ng/mL epidermal growth factor (EGF; Gibco) and B27 (Gibco). The cells were cultured in 2 mL medium in each well of a 6-well ultralow attachment plate and medium was added every 3 days. The size of tumorsphere were evaluated after 7 days of culture. Each experiment was performed in triplicate and measured by two investigators.

Statistical analysis

SPSS 19.0 was used for statistical analysis. Measurement data that conform to the normal distribution are expressed as mean ± standard deviation (x ± s), independent sample t-test or one-way ANOVA were used for comparison between groups, and non-normal distribution is expressed by median and interquartile range; In terms of percentage, the comparison between the positive rates was performed by χ^2 test; the correlation analysis of the two variables was performed by χ^2 test or Spearman correlation analysis, the test level was 0.05, and the two-sided test was used. $P < 0.05$ was statistically significant.

Results

The expression of Wnt7a was assessed in normal colonic mucosa, colorectal adenoma and colorectal cancer tissues (Fig. 1). The positive expression of Wnt7a in normal colonic mucosa and colorectal cancer tissues increased, the difference was statistically significant ($P < 0.01$), there was no significant difference in Wnt7a expression between colorectal adenoma and normal colonic mucosa ($P > 0.05$) (Table 1).

Correlation between immunohistochemical Wnt7a expression and clinicopathological features is shown in Table 2. The positive expression of Wnt7a protein was not related to the gender, age, lesion location, path length, depth of invasion, and distant metastasis ($P > 0.05$), but was related to the Duke stage, cell differentiation, and lymph node metastasis. Clinical pathological parameters were associated ($P < 0.01$). The lower the degree of differentiation, the higher the stage and the colorectal cancer with lymph node metastasis, the higher the positive rate of Wnt7a.

Colorectal cancer cell lines (HT-29 HCT-116) were cultured in vitro, and the expression level of Wnt7a colon cancer cell lines was detected by qRT-PCR (Fig. 2). The expression of Wnt7a was down-regulated by gene transfection. The effects of Wnt7a on the proliferation and differentiation of HT-29 and HCT-116 cells were observed.

To investigate the potential role of Wnt7a in colon cancer tumorigenesis, we first silenced Wnt7a in the colon cancer cell lines HT29 and HT116 (Fig. 2). Western blot and real-time PCR confirmed that shWnt7b and shWnt7c effectively silenced Wnt7a expression at the protein and mRNA levels. According to the gray ratio of the Western blot, the expression level of Wnt7a in the two cells was significantly lower than that in the control group ($P < 0.01$).

The number of clones larger than 50 cells was counted using a microscope (Fig. 3). Clonal formation rate was calculated, which is equal to the number of clones divided by the number of cells inoculated, multiplied by 100%. These plate cloning results showed that Wnt7a knockdown in HT29 colorectal cancer cell lines inhibited the proliferation of colorectal cancer cells. These results demonstrated that silencing Wnt7a inhibited the cancer stem cell activities of colon cancer.

After culturing in sphere formation medium, the knockdown of Wnt7a decreased the amount of tumor spheres in HT29 and HT116 cells (Fig. 4).

Discussion

Early detection, early diagnosis, prognosis and discovery of new methods for the treatment of advanced colorectal cancer will greatly reduce the incidence and mortality of colorectal cancer. Therefore, we studied indicators related to the occurrence, development and metastasis of colorectal cancer from the signaling pathway.

The conclusion of this study suggests that the positive expression of Wnt7a in colon adenocarcinoma is significantly higher than that in colorectal adenoma and normal colorectal mucosa. It is suggested that Wnt7a may be related to the occurrence and development of colorectal cancer, inhibit its biological activity, or may play a role in the prevention and treatment of colorectal cancer. In addition, by studying the relationship between Wnt7a and clinical pathological parameters of colorectal cancer, it suggests that Wnt7a can be used as a reference index to evaluate the malignant degree and metastasis of colorectal cancer, and provide a reference for the prognosis of colorectal cancer. The present study demonstrates that Wnt7a protein overexpression may be strongly linked to poor survival outcomes in colon cancer. However, the number of experimental studies is small, the results of research may have some deviation, and follow up studies have not been done. Therefore, the prognosis cannot be accurately evaluated and further optimization is needed. In addition, to investigate the potential role of Wnt7a in colon cancer tumorigenesis, Wnt7a was silenced in the colon cancer cell lines HT29 and HT116. Western blot and real-time PCR confirmed that shWnt7b and shWnt7c effectively silenced Wnt7a expression at protein and mRNA levels. Therefore, we speculate that by promoting the growth and metastasis of cancer cells, Wnt7a plays a key role in the transformation process from adenoma to cancer. Our results are consistent with previous reports, suggesting that the overexpression of RNA in human colorectal tumors by Wnt7a may lead to the occurrence and development of malignant tumors [17].Further, overexpressed Wnt7a may be closely related to the poor prognosis of colon cancer. This study also showed that the knockdown of Wnt7a decreased the tendency toward tumor sphere formation in HT29 and HT116 cells. Unexpectedly, the results of a Plate clone formation assay displayed that silencing Wnt7a in HT29 and HT116 cells increased the clone formation rate. These results demonstrated that silencing Wnt7a inhibited the cancer stem cell activities of colon cancer.

Intriguingly, our results demonstrated that Wnt7a promoted cancer stem cell activity but inhibited proliferation of colon cancer. In breast cancer, molecules also have dual roles, demonstrated by their

contribution to cancer progression coupled with alternative suppression of tumor growth. An example is PI3K-C2α, for which reduction delays tumor onset but promotes fast-growing tumors [18]. In confirmation, accumulating evidence demonstrated that TGFβ has a dual role in cancer progress: it can act as a tumor oncogene or function as a tumor suppressor gene [19]. These results may lead to new methods for the diagnosis and treatment of colorectal cancer. The results of this study only reveal associations, and further study is necessary to investigate the potential mechanism of Wnt7a in CRC development.

Conclusions

Our results present evidence that Wnt7a is associated with an unfavorable prognosis of colorectal cancer. By studying the signaling pathway and the occurrence, development and metastasis of colorectal cancer, early detection, diagnosis, prognosis and treatment of advanced colorectal cancer are of great benefit, as they are expected to substantially reduce the incidence and mortality of colorectal cancer.

Abbreviations

CRC: colorectal cancer; DAB: Diaminobenzidine; EGF: Epidermal growth factor; GAPDH: Phosphoglycerate dehydrogenase; PBS: Phosphate buffered saline; shRNA: Small hairpin RNA

Declarations

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Beijing Tiantan Hospital, Capital Medical University, Beijing, China.Written informed consent was obtained from all the patients at the time of admission.

Consent for publication

All authors have agreed to publish this manuscript. There are no details on individuals reported within the manuscript, consent for publication of images may not be required.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

PLC designed this study. CCL and HLL carried out experiments in vitro and immunohistochemical, XWD and CCL were responsible for basic experiment. PLC and XWD helped with data analysis. JHS and MX were responsible for the collection of specimens. WXD and HLL contributed to technical support. PLC supervised the project. CCL and DXW wrote the manuscript. All authors read and approved the final manuscript.

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Tables

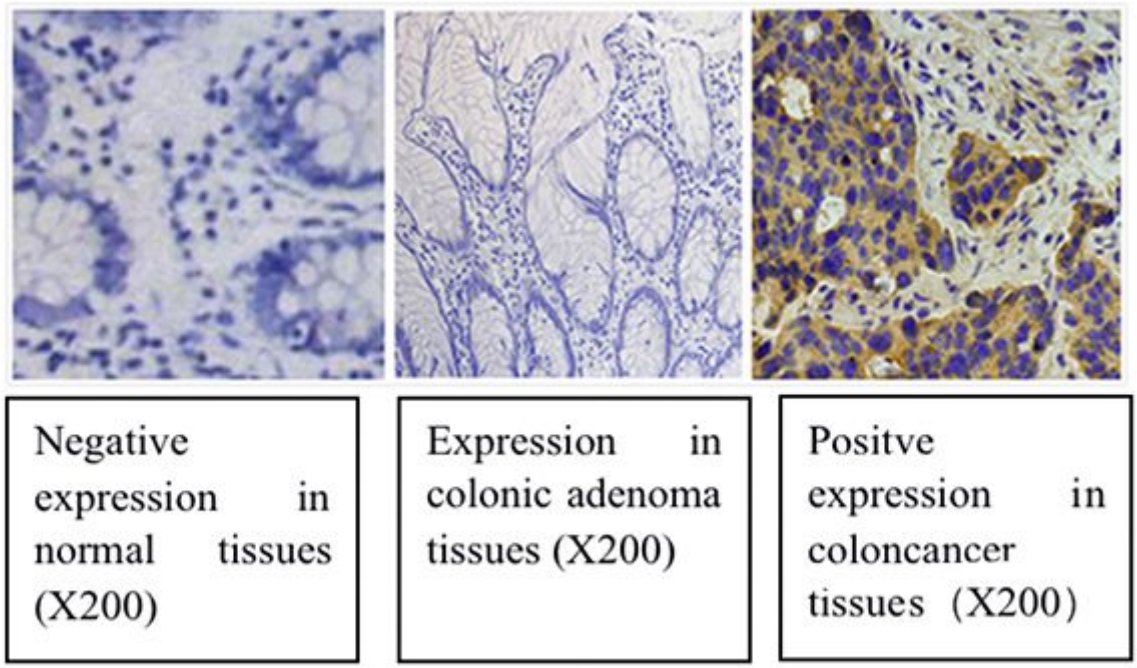
Table 1 Expression of Wnt7a in three different colon tissues.

Tissue type	N	Wnt7a protein positive expression rate		Positive rate (%)	P
		Positive	negative		
Normal mucosal tissue	20	4	16	20	0.311
Colorectal adenoma	40	13	27	32.5	
Colorectal adenocarcinoma	80	54	26	67.5	
χ^2		21.876			
P		< 0.01*			

Table 2 Correlation between Wnt7a expression and clinicopathological information in primary colorectal cancer.

Characteristic	No. of cases	Wnt7a					
		−	+	++, +++	Positive rate	χ^2	P
Gender						0.890	0.346
Male	49	14	8	27	71.4%		
Female	31	12	6	13	61.3%		
Age (years)						0.033	0.857
< 65	45	15	10	20	66.7%		
≥ 65	35	11	9	15	68.6%		
Location						0.624	0.430
Colon	26	10	5	11	61.5%		
Rectum	54	16	9	29	70.4%		
Tumor size						0.501	0.479
≤ 5 cm	51	18	9	24	64.7%		
> 5 cm	29	8	6	15	72.4%		
Depth						0.932	0.334
Undiluted serosa	25	10	4	9	60%		
Infiltrated serosa	55	16	9	30	70.9%		
Histological grade						10.370	0.001*
Well/moderate	45	25	16	4	44.4%		
Poor	35	7	7	21	80.0%		
Lymph node metastasis						15.631	0.00*
Present							
Absent	52	9	7	36	82.7%		
	28	17	2	9	39.3%		
Vascular invasion						0.003	0.955
Present	9	3	1	5	66.7%		
Absent	71	23	10	38	67.6%		
Dukes staging						5.024	0.025*
A + B	32	15	4	13	53.1%		
C + D	48	11	8	29	77.1%		

Figures



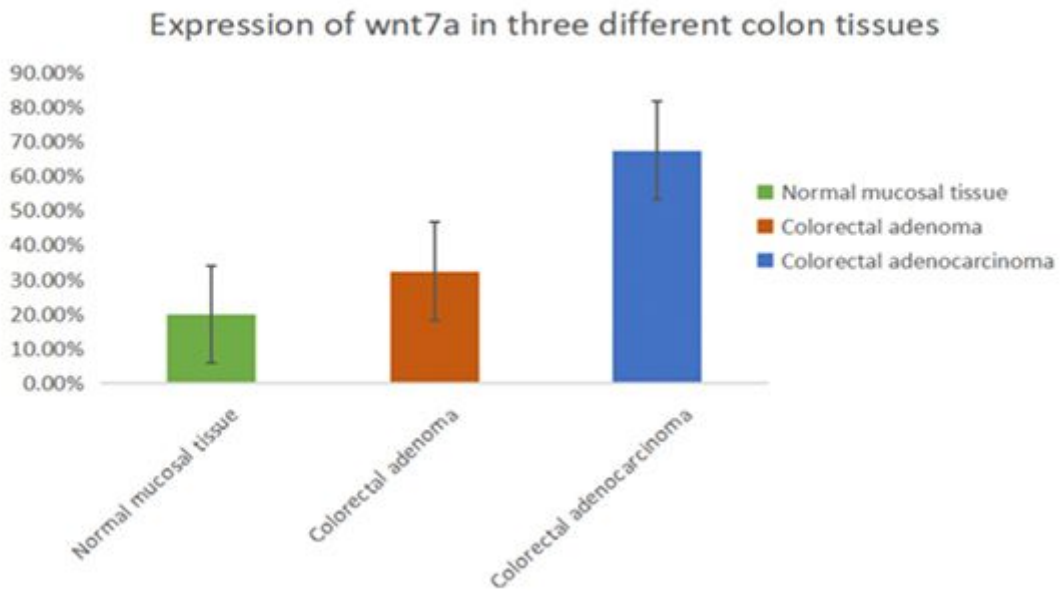


Figure 1

a Immunohistochemical results of Wnt7a in normal tissues, colonic adenoma tissues and colonic adenocarcinoma tissues. b Quantification of expression of Wnt7a in three different colon tissues.

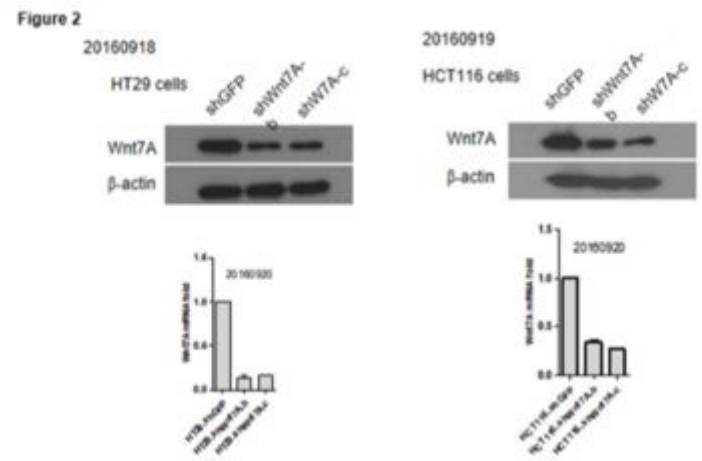


Figure 2

Silencing of Wnt7a in Ht29 and HCT116 cells.

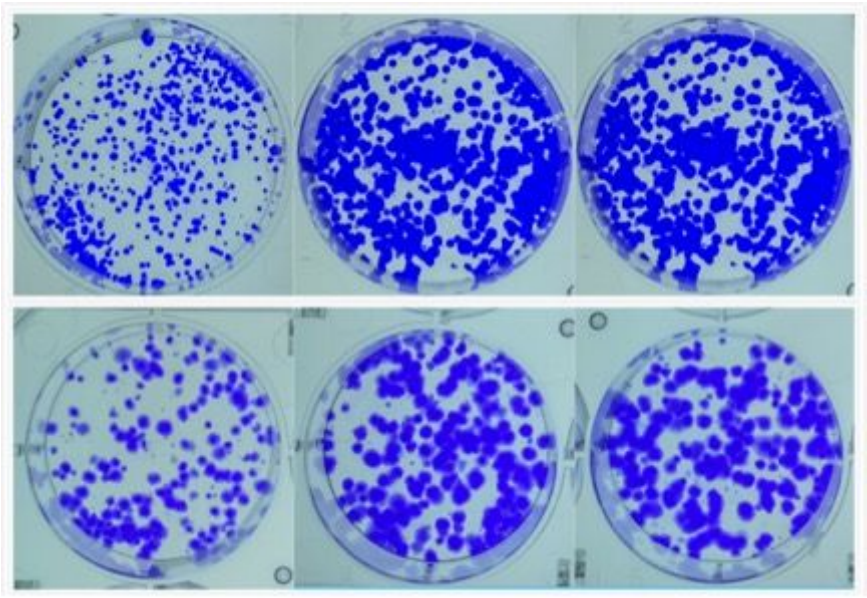


Figure 3

Colony formation assay.

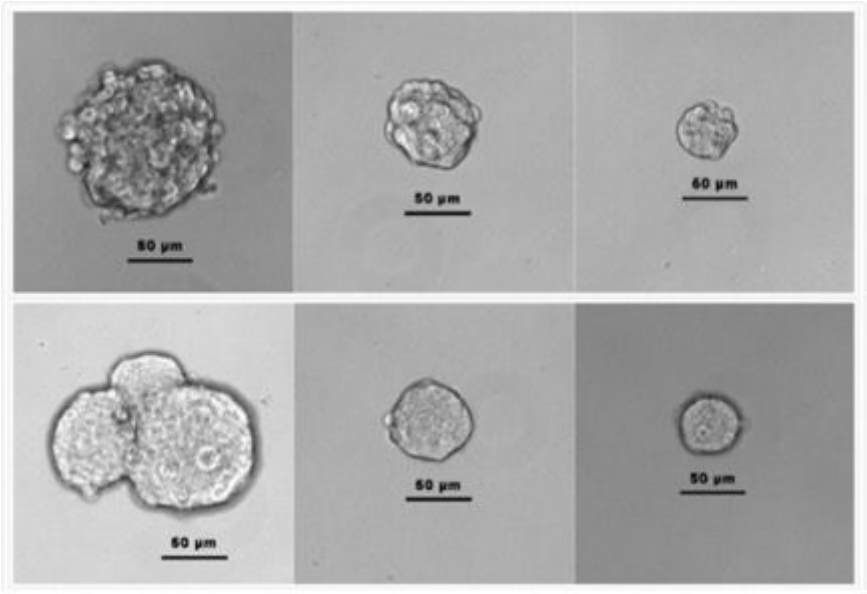


Figure 4

Sphere formation assay.