

Activation of WNT7b Autocrine Eases Metastasis of Colorectal Cancer via Epithelial to Mesenchymal Transition and Predicts Poor Prognosis

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Abstract

Background: Aberrant activation of the Wnt/ β -catenin signaling pathway is one of the most frequent abnormalities in human cancer, including colorectal cancer (CRC). Previous studies revealed pivotal functions of WNT family members in colorectal cancer, as well as their prognostic values. Nevertheless, the prognostic role and mechanisms underlying WNT7b in colorectal cancer development remains unclear.

Methods: In this study, WNT7b expression was measured by immunohistochemical staining of 100 cases of surgically resected human colorectal cancerous tissues as well as matched adjacent normal tissues constructed as tissue microarrays. In vitro studies, we attempted to substantiate the WNT7b expressional pattern previously found in immunohistochemistry staining. We used the colorectal cancer cell-line HCT116 and normal colorectal cell-line CCD-18co for immunofluorescence staining and nuclear/cytoplasmic separated western blotting. We measured epithelial–mesenchymal transition (EMT) markers and migration capacity of HCT116 in the context of WNT7b knocked-down using short interfering RNA. Finally, clinical and prognostic values of WNT7b activation levels were examined.

Results: WNT7b was expressed in the nucleus in adjacent normal tissues. In CRC tissues, nuclear expression of WNT7b was similar; however, membrane expression was strikingly enhanced. Consistently, in vitro analysis confirmed the same expression pattern of WNT7b. Compared with CCD-18co cells, HCT116 cells displayed higher levels of WNT7b membrane enrichment, as well as higher migration capacity with a sensitized EMT process. Either partial knockdown of WNT7b or blockade of the Wnt/ β -catenin signaling pathway reversed EMT process and inhibited the migration of HCT116 cells. Finally, elevated secretion levels of WNT7b were significantly associated with lymphatic and remote metastasis and predicted worse prognosis in the CRC cohort.

Conclusion: In summary, we demonstrated that the membrane enrichment of WNT7b autocrine probably contributes to CRC metastasis by activating EMT process through the Wnt/ β -catenin signaling pathway. High levels of membrane WNT7b autocrine secretion predicts poor outcome in patients with CRC. This molecule is a promising candidate for clinical CRC treatments.

Background

Colorectal cancer (CRC) is one of most prevalent malignant neoplasms of the digestive system [1]. Therapeutic approaches to CRC include surgical removal of polyps and early diagnosis, and these have been reported to improve prognosis and prolong survival [2]. Nevertheless, the treatment and prevention of CRC remains a global challenge because of the high risk of invasion and metastasis during CRC progression [3]. Recurrences of cancer in the liver, lung or peritoneal are major outcomes of CRC treatment failure. Therefore, the identification of critical molecules regulating CRC metastasis is urgently needed to improve treatment.

Epithelial–mesenchymal transition (EMT) is a developmental process by which epithelial cells are converted to mesenchymal cells during embryogenesis, tissue remodeling, wound healing, and tumor metastasis [4, 5]. During EMT, epithelial cells acquire mesenchymal cell properties and show reduced intercellular adhesion and increased invasion [5]. In cancer cells, EMT is abnormally regulated by extracellular stimuli derived from the tumor microenvironment, including growth factors and inflammatory cytokines, along with intra-tumoral physical stresses such as hypoxia [6]. EMT programming allows tumor cells to adapt to the constant changes of the tumor microenvironment, in so doing to successfully metastasize.

The wingless/integrase-1 (Wnt) pathway is a major homeostatic signaling cascade in development and stem cell homeostasis [7, 8]. In the canonical or β -catenin–dependent signaling branch, secreted Wnt ligands engage a transmembrane receptor system consisting of the Frizzled family core and LRP5/6 co-receptors to inhibit a multiprotein β -catenin destruction complex [9]. Consequently, cytosolic β -catenin is relieved from constitutive proteasomal degradation and induces the transcription of target genes through association with TCF/LEF family transcription factors. Specificity of the Wnt signaling output is achieved primarily via the differential expression of a wide range of Wnt ligands and receptors that exert overlapping but nonredundant functions [10].

The expression of WNT7B and its co-receptors are largely restricted to specific tissues, especially the developing brain, where they contribute to blood–brain barrier formation and maintenance through activation of Wnt/ β -catenin signaling [11, 12]. Increased expression of WNT7B and subsequent Wnt pathway activation have been observed in several cancers, including prostate cancer [13], pancreatic cancer [14], and breast cancer [15]. Nevertheless, as is the case with most Wnt ligands, it remains largely unresolved how WNT7b expression is regulated as well as its underlying mechanisms in CRC.

Therefore, in the present study, we measured WNT7b expression in CRC using immunohistochemistry staining (IHC) and described the expression patterns of WNT7b, and further verified these using in vitro studies.

Methods

Ethics Statement

This study was reviewed and approved by Medical Ethics Committee of the Suqian First People's Hospital. The approval of our ethics committees is available in Supplemental Data 1.

Patients and human tissue samples

Human CRC tissue microarrays (TMAs) were constructed from 100 cases of surgically resected colorectal tumor tissues (2016–2018), along with matched cases of adjacent normal tissues from the Pathology Department, Suqian First Hospital, Jiangsu Province, China. All tissue specimens were reviewed using hematoxylin and eosin staining; representative areas free from necrosis and hemorrhage were selected in

the paraffin blocks. We took 1-mm diameter cylinders from intratumoral or peritumoral tissues (1–2 cm from the tumor edge) and transferred to the TMA by the Outdo Biotech Company, Shanghai, China. The relevant clinical data was collected using retrospective medical chart reviews. Survival data were collected every three months, with the final update on 10/31/2019. All protocols were reviewed and approved by the academic ethics committee.

The demographic data and post-surgical follow-up of the 100 CRC cases are shown in Table 1. The majority of patients were diagnosed (post-surgically) with stages II and III according to the American Joint Committee on Cancer (AJCC) staging 8th edition (72/100), 19 cases were diagnosed as AJCC stage I, and nine cases were AJCC stage IV as have been found with liver metastasis prior to the surgical resection.

Immunohistochemistry and scoring

Archived paraffin-embedded tumor tissues and adjacent normal tissues were constructed for tissue microarrays and IHC. IHC was performed using the polymer HRP detection system (Zhongshan Goldenbridge Biotechnology, Beijing, China) according to the manufacturer's instructions. The primary antibody was anti-WNT7b (Catalog#: AF3460, R&D system) at 1:1000 dilution. The second antibody, was anti-goat (Catalog#: 6403-05, BioVision) at 1:2000 dilution. All TMA slides were scanned using the Leica Aperio AT2 digital slide scanner.

The scoring of WNT7b immunostaining was based on both the intensity and percentage of positively membranal staining cells. The final expression score of a single sample was equal to the intensity grade (0, 1, 2, and 3) multiplied by the percentage level (0–100%). Each CRC sample was compared with its paired adjacent normal tissue. TMA slides was evaluated by two independent pathologists who were blinded to patient information. If there were discrepancies, results were jointly assessed by both investigators and the final score was formed by consensus.

Cell cultures

HCT116 and CCD-18co cells were purchased from Tongpai Biotechnology Company, Shanghai, China. Cells were maintained in 5% CO₂ at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies/Gibco, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies/Gibco, NY, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin (Life Technologies/Gibco, MD, USA). Cells (1 X 10⁶) were seeded into dishes (10-cm diameter) for 72 h at each passage.

Transient transfection

For transient *WNT7b* gene knockdown, cell-lines were transfected with 10–20 nM of duplexed siRNAs using Lipofectamine 2000 (Invitrogen/Life Technologies, NY, USA). The RNA interference sequence is listed in the Supplementary Data 2.

Western blot

Cells were harvested at 90% confluence. For regular immunoblots, cell lysates were obtained using RIPA Lysis Buffer (Millipore, MA, USA). Separate nuclear and cytoplasmic protein extraction was performed NE-PER Nuclear and Cytoplasmic Extraction Reagents (Millipore, MA, USA). Cell lysates were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). The membranes were probed with primary antibodies overnight at 4 °C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immune complexes were detected using enhanced chemiluminescence (Cell Signaling Technology, MA, USA). GAPDH was used to correct for differences in loading of the proteins from the control and experimental groups. Detailed antibody information is displayed in Supplementary Data 3.

Immunofluorescence assay

Cells grown on cover slips in 24-well plates were fixed in 4% paraformaldehyde for 20 min, then the cells were permeabilized with 0.5% Triton X-100 (Solarbio, Beijing, China) for 20 min at room temperature. The HCT116 cells and CCD-18co cells were incubated at 4 °C overnight with the primary antibody (Catalog #: AF3460, 1:1000 dilution, R&D system) after blocking with 3% nonfat dry milk in PBS for 1 h at room temperature. Then the cells were incubated for 1 h at 37 °C with Fluorescein (FITC)-conjugated AffiniPure Fab Fragment (1:200 dilution, Jackson ImmunoResearch Laboratories, PA, USA) as the secondary antibody. The nuclei were stained by DAPI (Sangon Biotech, Shanghai, China) for 5 min after washing with PBS. Images were captured using a confocal microscope (Olympus, Tokyo, Japan).

Transwell assay

Transwell assays were performed using growth factor-reduced, Matrigel-coated filters (8 mm pore size, BD, Franklin Lakes, NJ, US) in 24-well plates. Cells were trypsinized and seeded onto the upper chambers of the Transwells (3×10^4 cells/well) in supplement-free DMEM medium. The lower chambers of the Transwells were filled with DMEM medium containing 100 ng/mL of EGF. The chambers were incubated at 37 °C with 5% CO₂ for 24 h. At the end of incubation, cells on the upper surface of the filter were removed using a cotton swab. Cells migrating through the filter to the lower surface were fixed with 4% paraformaldehyde for 10 min and stained with 0.1% crystal violet for 5 min. Migrated cells were viewed and photographed using a phase-contrast microscope (Olympus).

Statistical analyses

The χ^2 test was used to analyze the significance of migrated cells in the Transwell assay using SPSS. Fisher's exact test were used to analyze the significance of lymphatic and remote metastasis between each group based on membrane upregulated expression of WNT7b by SPSS. For survival analysis, we compared Kaplan–Meier survival curves between different thresholds of membranal upregulated expression of WNT7b using the log-rank test on GraphPad Prism 7.

Results

IHC labeling of WNT7b expression pattern in CRC and adjacent normal tissues

To evaluate the expression of WNT7b in primary CRC, we performed immunohistochemical staining of TMA slides of both CRC and adjacent normal tissues (Catalog #: AF3460, R&D system, 1:1000 dilution) (Fig. 1a). In adjacent normal tissues, WNT7b was expressed in the nucleus (99/100, 99%). Weak and sporadic positive membrane staining was also observed in 67 peritumoral cases (67/100, 67%). However, in CRC tissues, nuclear expression levels of WNT7b were similar; interestingly, membrane expression levels were strikingly enhanced. All samples presented positive membrane WNT7b staining (100/100, 100%). Some of the matched CRC and adjacent normal tissues are shown in Fig. 1b. This result suggests that WNT7b autocrine secretion may have been activated during colorectal cell transformation.

CRC cells showed enhanced WNT7b autocrine secretion

To confirm the WNT7b expression pattern found on IHC staining, the colorectal cancer cell-line HCT116 and the normal colorectal cell-line CCD-18co were selected for subsequent studies. First, using immunofluorescence staining, we observed that membrane WNT7b expression was dramatically higher in the CRC cell-line HCT116 than in the normal colorectal cell-line CCD-18co (Fig. 2a). Western blotting analysis confirmed this feature when we split cytoplasmic (containing membrane protein) and nuclear protein lysates (Fig. 2b). In the nuclear lysate, similar levels of WNT7b expression were detected between HCT116 and CCD-18co cells while cytoplasmic WNT7b expression levels were elevated in HCT116 cells. These findings confirmed the WNT7b expression pattern in CRC and also suggested that high level of WNT7b autocrine secretion may stimulate malignant transformation of colorectal cells.

WNT7b promotes cell migration by inducing EMT in HCT116 cells

It is unclear as to whether WNT7b is involved in canonical β -catenin-dependent signaling, or the self-dependent signaling pathway in CRC. This is because WNT7b and its co-receptors depending on the tissue. We initially checked how WNT7b was involved in canonical Wnt/ β -catenin pathway in CRC cells. Because the canonical Wnt/ β -catenin pathway is known to drive EMT, we firstly compared EMT markers between CCD-18co cells and HCT116 cells. As shown in Fig. 3a, HCT116 cells presented higher WNT7b expression levels with activated EMT compared with CCD-18co cells. However, partial knockdown of WNT7b expression using small interfering RNA reversed the EMT process in HCT116 cells (Fig. 3b). Additionally, Wnt/ β -catenin pathway-specific antagonist Dickkopf-1 (DKK1) was used to determine whether this reversion was regulated by the Wnt/ β -catenin pathway. By blocking the Wnt/ β -catenin pathway, HCT116 cells exhibited decreased WNT7b expression and inhibited EMT (Fig. 3c). These findings suggest that WNT7b is associated with EMT in CRC cells via the Wnt/ β -catenin pathway. Finally, knock-down of WNT7b significantly downregulated the migratory capacity of HCT116 cells (Fig. 3d and e). These findings suggest that WNT7b is involved in promoting cells migration via the Wnt/ β -catenin pathway and by enhancing EMT.

Membrane-upregulated WNT7b was significantly associated with lymphatic and remote metastasis in the CRC cohort

Because the function of WNT7b in manipulating CRC cell migration was identified, we further studied the correlation between WNT7b membrane enrichment level and metastatic rates in CRC patients. The CRC cohort was divided into three subgroups based on following strategy: Because each sample was assessed by comparing the WNT7b labeling score in CRC tissue with paired adjacent normal tissue, the grouping strategy was based on varied WNT7b upregulation scales. The classifications were the Slight Upregulation Group ($1 \leq$ elevated times ≤ 3 , S Group, n = 28), the Moderate Upregulation Group ($3 \leq$ elevated times ≤ 10 , M Group, n = 19), and the Widely Upregulation Group ($10 \leq$ elevated times, W Group, n = 53) (Fig. 4a). We performed correlation analyses between WNT7b upregulation and the TNM stages of CRC patients (Table 2). Statistical analysis suggested that high levels of WNT7b upregulation were significantly related to lymphatic (p=0.000) and remote (p=0.047) metastasis (Fig. 4b and c). This finding was consistent with in vitro studies that showed that abnormally upregulated WNT7b autocrine secretion was associated with CRC cell migration, thereby enabling metastasis of CRC.

WNT7b expression was related with poor post-surgical survival rates in CRC patients

To examine the prognostic values of the multiple WNT7b upregulation levels identified above,

we used Kaplan–Meier survival analysis. As shown in Fig. 5, post-surgical survival rates analysis showed significantly differentiated outcomes among subgroups. High levels of WNT7b upregulation predicted worst post-surgical survival rates. This finding supported our hypothesis that activation of WNT7b autocrine was an intrinsic step in the procedure of CRC progression and predicted poor outcome in CRC patients.

Discussion

CRC is driven by certain oncogenes and genetic changes. Recent studies reported large-scale screening focused on WNTs and the Wnt/ β -catenin cascade in CRC. Ruan et al. reported that the WNTs family mRNA expression was related to the diagnosis and prognosis of CRC. They analyzed RNA sequencing data from The Cancer Genome Atlas (TCGA) and showed that WNT2 and WNT7b had high diagnostic values in CRC. Their comprehensive prognosis analysis suggested that expression of WNT10B might serve as an independent prognostic biomarker of CRC [16]. Kleeman et al. analyzed 1262 colorectal cancer cases and found that epigenetic suppression of appropriate WNT negative feedback loops were selectively advantageous in ligand-dependent (LD) tumors (identifiable mutations in APC or CTNNB1). They suggest that distinguishing between LD and ligand-independent (LI) tumor types is important; patients with LD tumors retained sensitivity to WNT ligand inhibition and may be stratified at diagnosis to clinical trials of porcupine inhibitors [17]. As more attention turned to WNTs, there were studies of WNT isoform mechanisms in CRC. Aizawa et al. isolated cancer-associated fibroblasts (CAFs) and normal fibroblasts (NFs) from surgical resected CRC tissues and found that WNT2 protein released from CAFs enhanced CRC cell invasion and migration, playing a key role in cancer progression emerging as a potential therapeutic target for CRC [18]. Galbraith et al. and Peng et al. both argued that WNT6a was associated with liver metastasis of CRC and predicted poor outcomes in CRC patients [19, 20]. In the

present study, we were the first to profile WNT7b expression patterns and levels in CRC. We demonstrated that the activation of membrane WNT7b secretion leads to metastasis and predict poor outcome. In mechanistic studies, we found that high levels of WNT7b secretion were associated with activation of EMT through the canonical Wnt/ β -catenin signaling pathway.

The β -catenin dependent signaling pathway is triggered by the binding of Wnt ligands to the LRP-5/6 receptors (low-density lipoprotein receptor) and Frizzled receptors. This in turn activates Disheveled (DVL), causing recruitment of the complex (Axin, GSK3 β , CK1, APC) to the receptor [21–23]. The Wnt-Frizzled-Axin-LRP-5/6 complex sequesters cytosolic GSK3 β , rendering it incapable of phosphorylating β -catenin. The accumulation of unphosphorylated β -catenin in the cytosol migrates to the nucleus, interacting with T cell-specific factor (TCF)/lymphoid enhancer-binding factor (LEF) and co-activators to turn on the Wnt target genes such as c-Myc, cyclin D1 and Cdkn1a, thereby enabling tumor cells to gain growth and metastatic dynamics.

Given the key role of the Wnt/ β -catenin signaling pathway in cancer development and invasion, Wnt/ β -catenin signaling pathway inhibitors were identified. Selective porcupine (PORCN) inhibitors, LGK974 and ETC-159 target the secretion of Wnt ligands; these are being studied in ongoing phase 1/2 trials in metastatic colorectal and head and neck cancers [24]. Specific Wnt ligands and receptors are found to be overexpressed in many tumors; monoclonal antibodies developed against Wnt-1 and Wnt-2 demonstrate Wnt inhibition leading to tumor suppression in melanoma, sarcoma, colorectal cancers, non-small cell lung carcinoma, and mesothelioma [25, 26]. However, there are no drugs approved to target Wnt/ β -catenin, although it has been a compelling target for inhibition in past 30 years. The present study provides an alternative choice for precision therapy of CRC or for combined therapeutic approaches. More evidence is needed.

A major unresolved question in this study is how the expression of WNT7b ligand is controlled, and the upstream signaling regulation remains unclear. Indeed, data regarding upstream regulation of the Wnt signaling pathway is limited. A recent study from Moparthy et al. found that FOXB2, an uncharacterized protein, is a potent regulator of Wnt ligand expression and TCF signaling that drives the neuroendocrine differentiation of prostate cancer cells [13]. Another study also reported that MM-1 competitively binds the *wnt4* gene promoter region, thereby downregulating promoter activity of *wnt4* gene expression [27]. However, WNT7b and its co-receptors are characterized by tissue specific restriction. In particular, WNT7b elicits limited pathway activation on its own, as evidenced by its inability to induce LRP6 phosphorylation and β -catenin stabilization to any substantial degree [28]. By contrast, WNT7b strongly cooperates with other ligands, primarily WNT1, in driving TCF/LEF-dependent gene transcription. The mechanism of the WNT7b-dependent pathway activation is unclear; however, it requires additional coreceptors, namely RECK and GPR124 [29, 30]. Our understanding of WNT7b signaling in CRC is evolving as all the samples from the CRC cohort presented strong WNT7b enrichment in the CRC tissues. Moreover, high levels of WNT7b activation contributed to the risk of metastasis when comparing several WNT7b upregulation scales. Future studies will focus on elucidating mechanisms that regulate higher levels of autocrine WNT7b signaling in CRC.

Conclusion

The membrane enrichment of WNT7b autocrine probably contributes to CRC metastasis by activating EMT through the Wnt/ β -catenin signaling pathway. High levels of membrane WNT7b autocrine secretion predicts poor outcome in patients with CRC. WNT7b could be a promising candidate molecule in clinical CRC treatments.

Abbreviations

CRC: colorectal cancer; EMT: epithelial–mesenchymal transition; IHC: immunohistochemistry staining; TMAs: tissue microarrays; AJCC: American Joint Committee on Cancer; DMEM: Dulbecco's Modified Eagle's Medium; DKK1: Dickkopf-1; TCGA: The Cancer Genome Atlas; LD: ligand-dependent; LI: ligand-independent; CAFs: cancer-associated fibroblasts; NFs: normal fibroblasts; DVL: Disheveled; TCF: T cell-specific factor; LEF: lymphoid enhancer-binding factor

Declarations

Ethics approval and consent to participate

This study was reviewed and approved by Medical Ethics Committee of the Suqian First People's Hospital. The approval of our ethics committees is available in Supplemental Data 1.

Author's contributions

SJ, QL, and YW conceived and designed the study. SJ, QL, QW, XS, MW, JL, and JJ performed the cells and pathological experiments. DX and PZ performed the statistical analysis. SJ, YZ, HZ and SP drafted the manuscript. YC and YW reviewed and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1		
Patient characteristics (n = 100)		
Characteristic	Sub-characteristic	Value
Age		66 (range 43–89)
Gender	Male	59
	Female	41
Depth of invasion (T)	T2	35
	T3	63
	T4	2
Lymph node metastasis (N)	N0	39
	N1	55
	N2	6
Distant metastasis (M)	M0	91
	M1	9
AJCC stage	I	19
	II	38
	III	34
	IV	9
Total		100

Table 2

	S Group	M Group	W group	
T2	11	1	23	
T3	16	17	30	P(T) = 0.133
T4	1	1	0	
N0	20	8	11	
N1	7	11	37	P(N) = 0.000
N2	1	0	5	
M0	28	18	45	P(M) = 0.047
M1	0	1	8	
P value was calculated by Fisher's Exact Test				

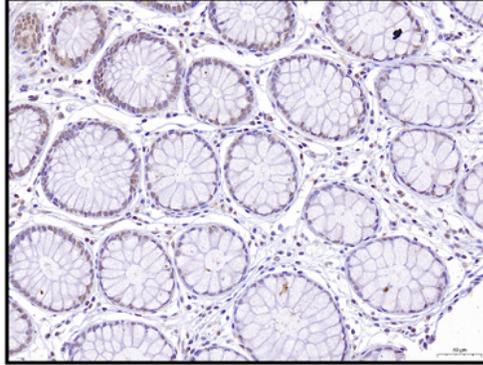
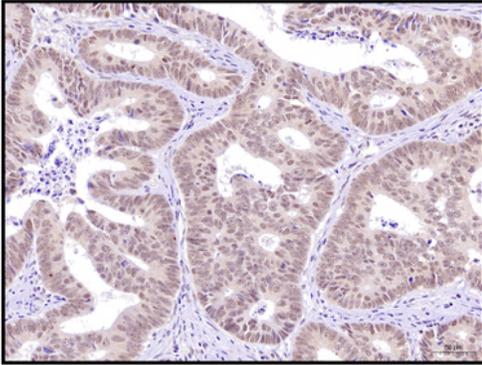
Figures

Figure 1

A

Colorectal carcinoma

Adjacent normal



B

HE staining

IHC staining of WNT7b

Colorectal carcinoma

Adjacent normal

Colorectal carcinoma

Adjacent normal

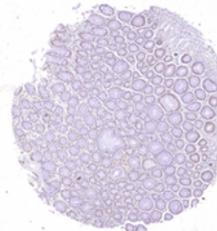
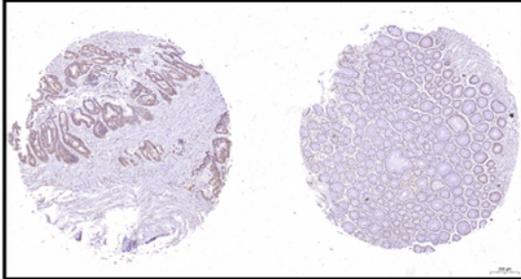
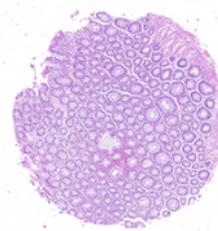
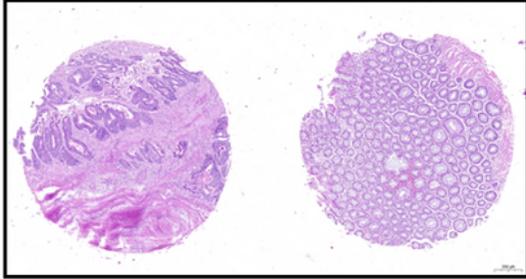
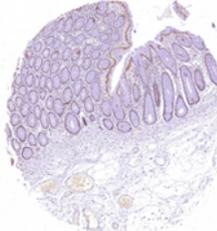
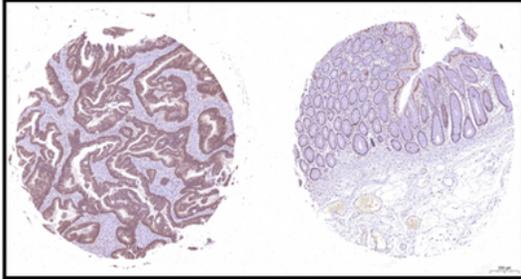
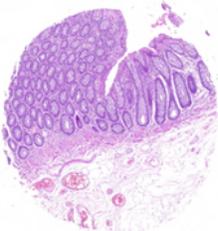
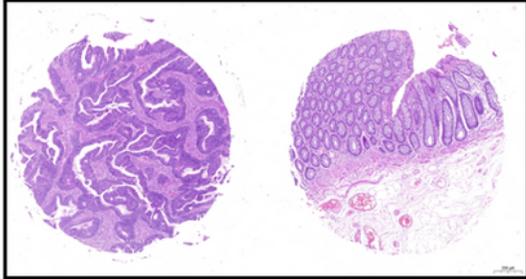
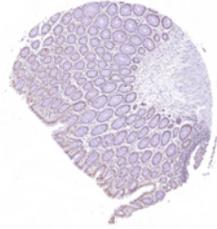
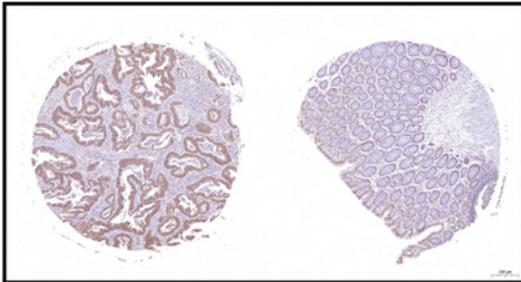
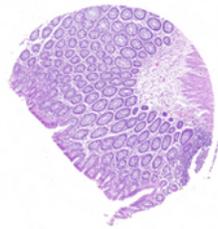
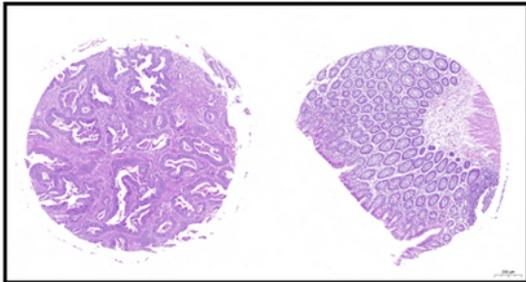


Figure 1

Figure 1

Figure 2

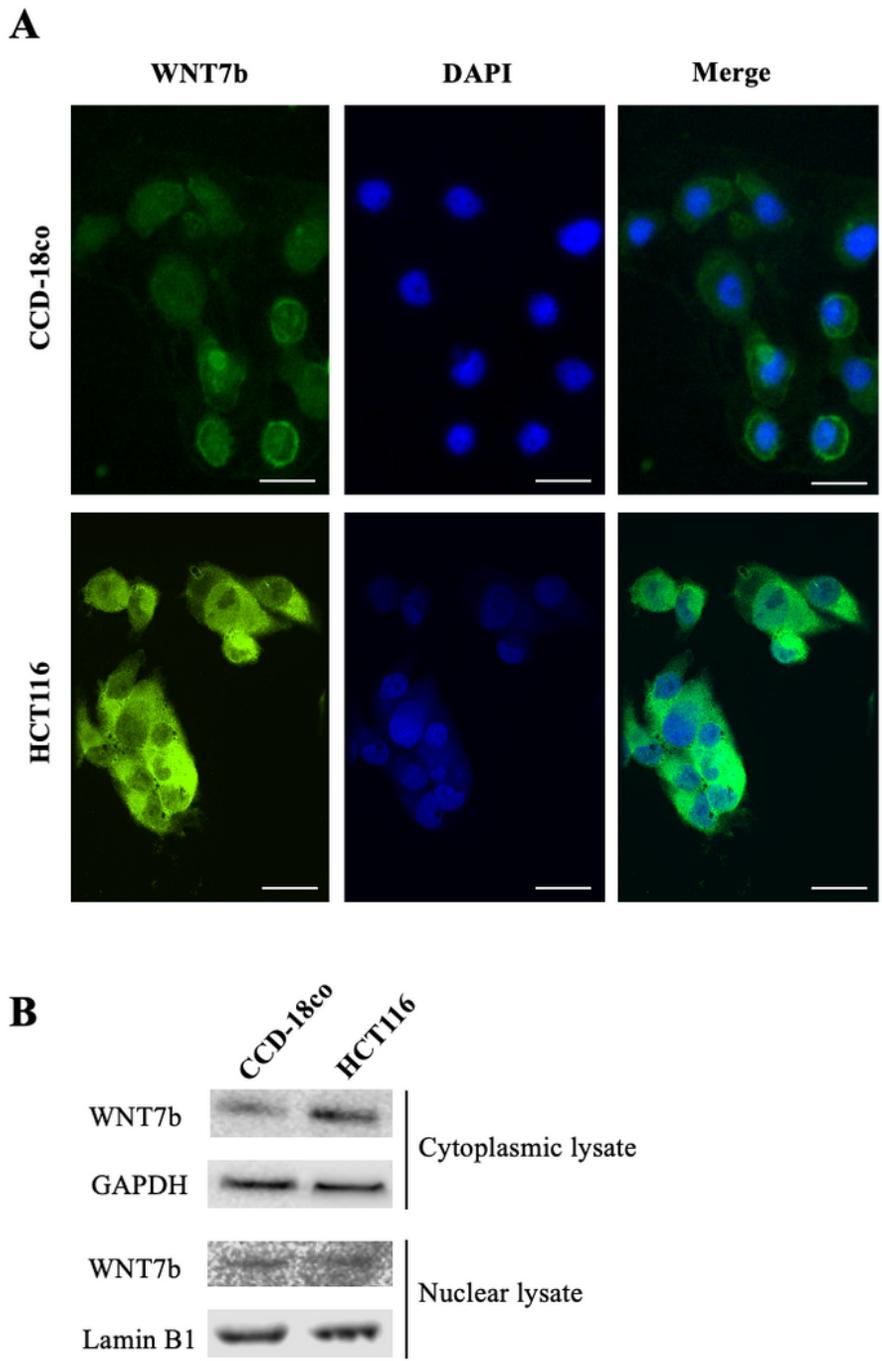


Figure 2

Figure 2

Figure 3

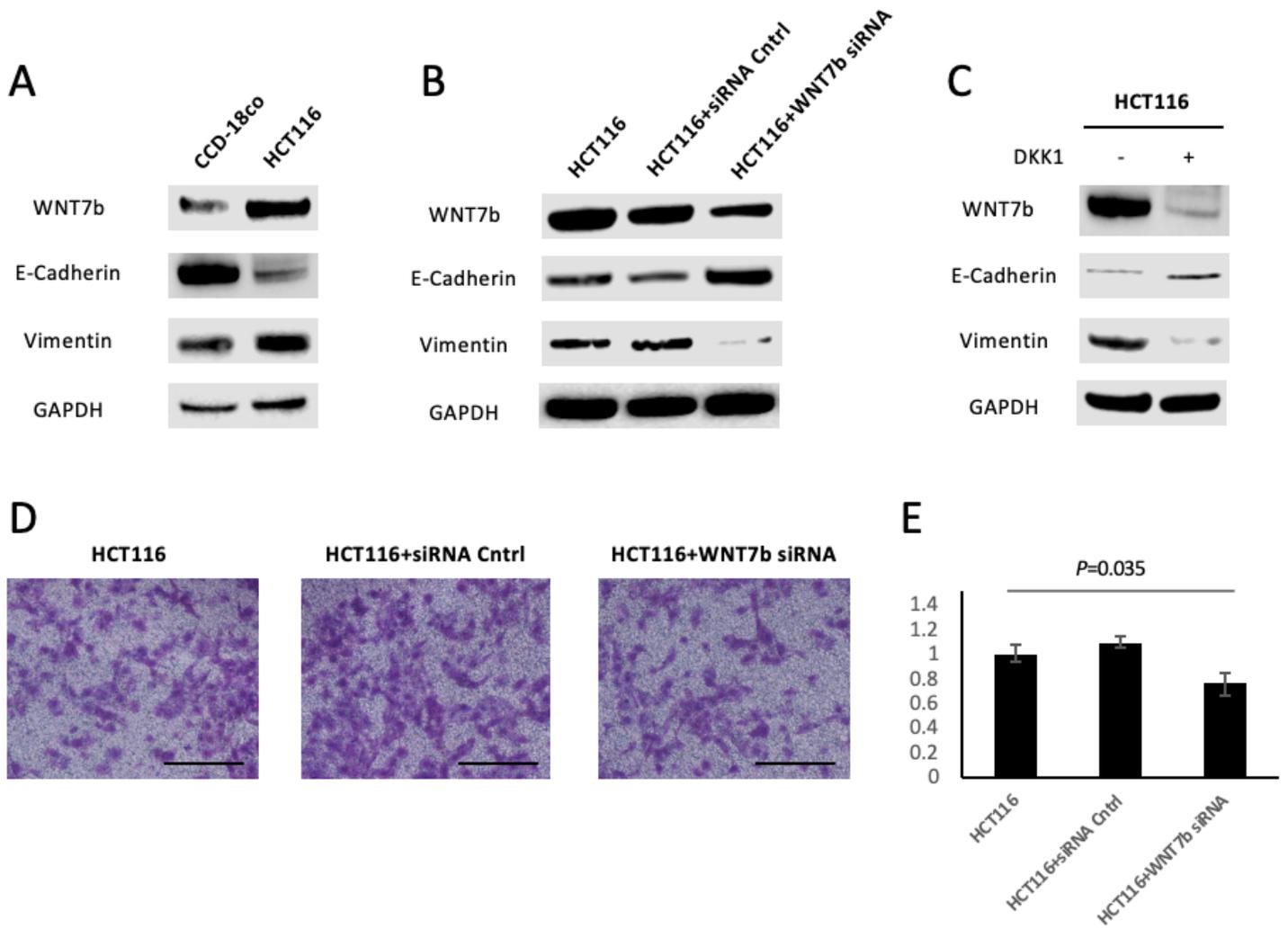
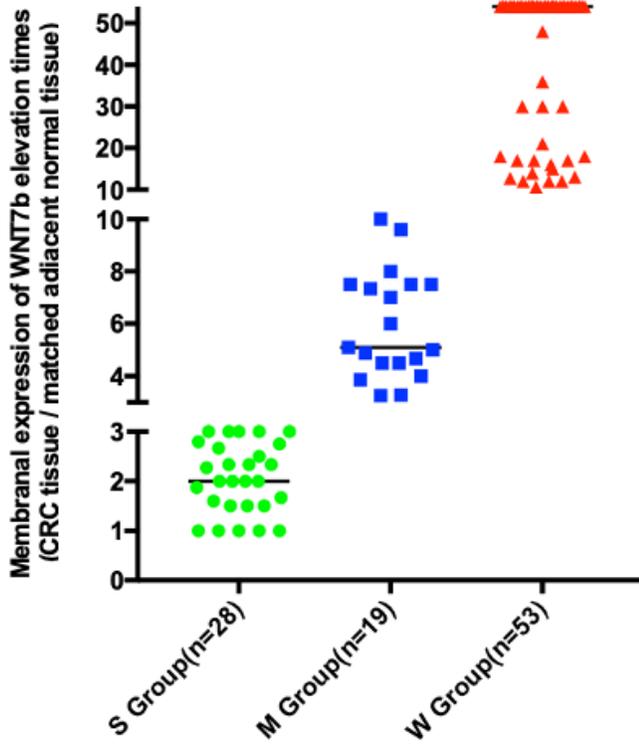


Figure 3

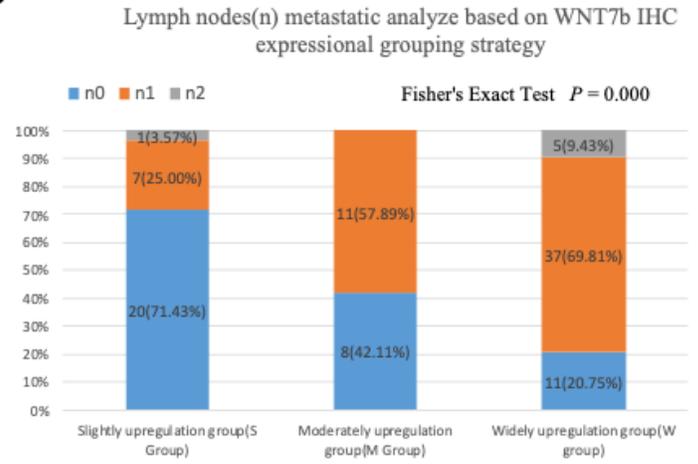
Figure 3

Figure 4

A



B



C

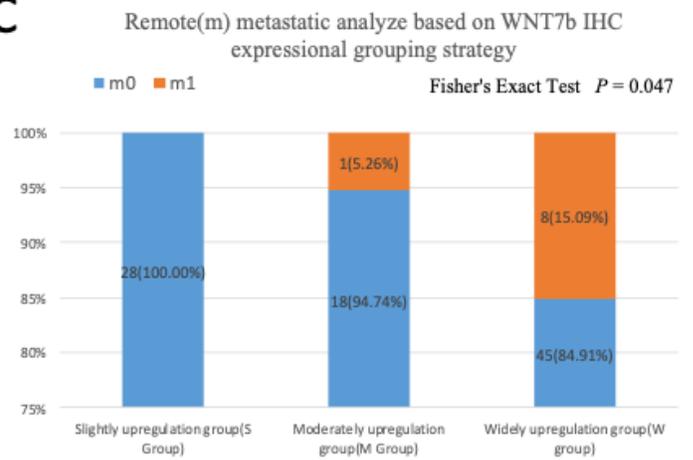


Figure 4

Figure 4

Figure 5

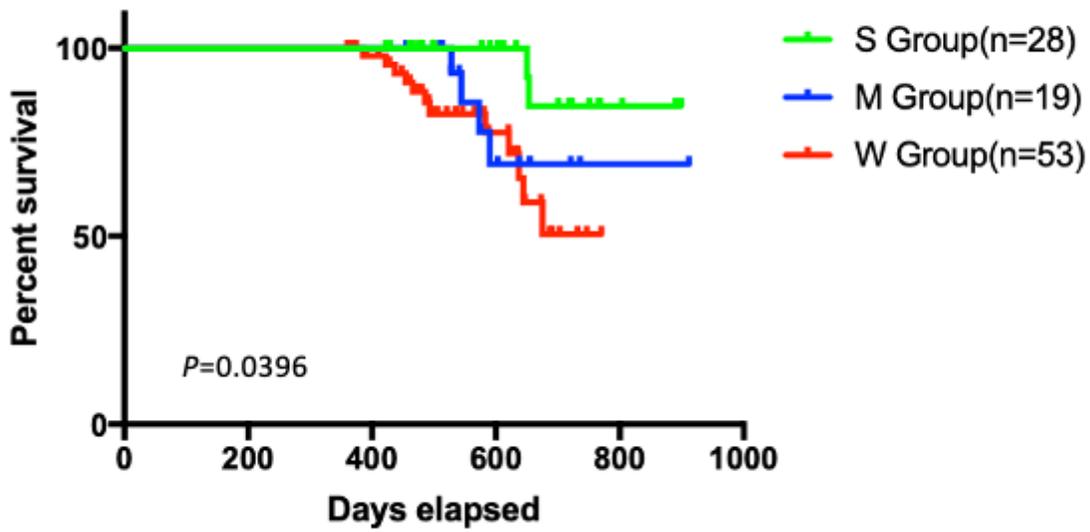


Figure 5

Figure 5

Supplementary Files

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