# Molecular Mechanisms of the Role of the C5AR1 Gene via the Wnt/β-Catenin Signaling Pathway in Colorectal Cancer

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Introduction. To investigate the expression and function of C5AR1 in colorectal cancer and its relationship with Wnt/β-Catenin signaling pathway.
Methods. Immunohistochemistry and qRT-PCR were used to detect the expression level of C5AR1 in colorectal cancer tissues and corresponding non-cancerous tissues. C5AR1 was silenced using siRNA technology, and overexpression experiments were performed to assess changes in Wnt/β-Catenin signaling pathway activity. Cell counting, scratch assay and Transwell assay were used to evaluate the effect of C5AR1 on the growth and migration of colorectal cancer cells.
Results. C5AR1 was significantly up-regulated in colorectal cancer tissues. C5AR1 silencing and overexpression had significant effects on Wnt/β-Catenin signaling pathway activity. The dysfunction of C5AR1 significantly affects the growth and migration of colorectal cancer cells.
Conclusions. C5AR1 plays a key role in colorectal cancer and is closely associated with the Wnt/β-Catenin signaling pathway. The study of C5AR1 provides new ideas and strategies for the diagnosis, treatment and prognosis evaluation of colorectal cancer.
Keywords. colorectal cancer, C5AR1, Wnt/β-Catenin signaling pathway, expression, functional analysis

## 1. INTRODUCTION

Colorectal cancer (CRC) is the leading cause of cancer morbidity and mortality worldwide, but its pathogenesis and mechanism are not fully understood. With the rapid development of molecular biology and cell biology, researchers have revealed many key molecules and signaling pathways related to the occurrence, development and metastasis of colorectal cancer, which provides new ideas for the diagnosis, treatment and prognosis evaluation of colorectal cancer.

Wnt/β-Catenin signaling pathway is a key regulatory mechanism in various physiological processes such as cell development, proliferation, migration and autonomous death. In recent years, many studies have confirmed that this pathway plays a crucial role in a variety of malignant tumors, especially colorectal cancer. Abnormal activation of Wnt/β-Catenin signaling pathway is considered to be one of the important pathogenesis of colorectal cancer.

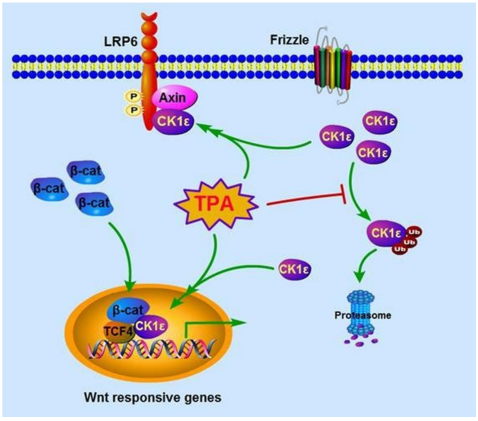
C5AR1, the full name of complement component 5a receptor 1, is a G protein-coupled receptor that binds to the C5a component of the complement system. In the

past decades, C5AR1 has been mainly studied as a molecule related to immune

response and inflammatory response. However, in recent years, accumulating evidence suggests that C5AR1 may also play a key role in a variety of cancers, including colorectal cancer. Its specific mechanism and function, especially its relationship with Wnt/β-Catenin signaling pathway, have not been fully explored.

The potential interaction between Wnt/β-Catenin signaling pathway and C5AR1 provides a new perspective for the study of colorectal cancer. If C5AR1 indeed plays a key role in the development and progression of CRC and is closely related to the Wnt/β-Catenin signaling pathway, the study of C5AR1 will not only deepen our understanding of the molecular mechanism of CRC, but also may provide new targets and strategies for clinical treatment.

Based on the above background, we designed this study to investigate the expression and function of C5AR1 and its relationship with Wnt/β-Catenin signaling pathway in CRC. We hope that this study will provide a new theoretical basis and practical guidance for the research and treatment of colorectal cancer.




## 2. MATERIALS AND METHODS

### 2.1 Materials:

Colorectal cancer tissue samples were collected from 50 patients with colorectal cancer, and corresponding non-cancerous tissues were collected as controls.

Colorectal cancer cell lines: purchased from Shanghai Cell Bank, including HCT116, SW480, etc.

Gene silencing and overexpression vectors: C5AR1 shRNA and C5AR1 cDNA vector, purchased from Sangon Biotech.

Protein detection reagents: Wnt3a, β-Catenin, and C5AR1 antibodies, purchased from Abcam.

### 2.2 Methods:

2.2.1 Tissue SAMPLE COLLECTION： Colorectal cancer and corresponding non-cancerous lesion tissues were collected from colorectal cancer surgery after obtaining patient consent and ethics committee approval. Tissue samples were stored at − 80°C.

2.2.2 Cell culture: Colorectal cancer cell culture is a crucial link in this

experiment, which mainly involves the collection, growth, passage and treatment of

cells to ensure that the cells are in the best condition for subsequent experiments.

Cell line Selection and procurement: The colorectal cancer cell lines selected were HT-29 and SW480, which are widely used in colorectal cancer research and were purchased from the American Type Culture Collection (ATCC).

Cell resuscitation and culture: Cells were quickly removed from liquid nitrogen upon receipt and placed in a 37°C water bath for rapid resuscitation. Subsequently, cells were transferred under sterile conditions to Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and placed in a cell incubator at 37°C with 5% CO2. Cell growth was observed daily and subcultured or experimentally treated according to cell density.

Cell passage: When the cells reached 80 to 90% confluence, 0.25% tripsin EDTA solution was used to digest the cells and allow them to be separated from the bottom of the culture flask. After the cells were completely separated, the digestion was stopped by adding DMEM medium, and the cells were counted. The desired number of cells was transferred to a new culture flask and fresh medium was added to continue the culture.

Cell treatment: Depending on the requirements of subsequent experiments, cells may need to undergo different treatments, such as drug treatment, transfection, or co-culture. Handling should be carried out in strict accordance with the experimental protocol and ensure that all operations are completed under sterile conditions.

All treatment of cells in this experiment followed the principle of aseptic operation, and tested and certified reagents and materials were used to ensure the growth state of cells and the accuracy of experimental results.

2.2.3 Gene silencing and overexpression: Gene silencing and overexpression are two common techniques used to investigate the function of specific genes in cell biology and molecular biology. In this study, we used both methods to manipulate the C5AR1 gene to reveal its role in colorectal cancer.

Gene silencing:

To specifically silence C5AR1 gene, we used short interfering RNA (siRNA) technology. First, three pairs of siRNA sequences targeting C5AR1 mRNA were designed and prepared by synthetic companies. Then, siRNA was introduced into colorectal cancer cells using Lipofectamine 2000 transfection reagent. Forty-eight to 72 hours after transfection, the silencing effect was confirmed by measuring the mRNA and protein expression levels of C5AR1 by qRT-PCR and Western Blot.

Gene overexpression：

To achieve the overexpression of C5AR1, an expression vector containing the complete C5AR1 coding sequence was constructed. C5AR1 cDNA was cloned into pcDNA3.1 vector and confirmed to be successfully constructed by bacterial transformation and identification. The constructed vector was introduced into colorectal cancer cells together with Lipofectamine 2000 transfection reagent. Similarly, the effect of C5AR1 overexpression was confirmed by qRT-PCR and Western Blot within 48 to 72 hours after transfection.

In order to ensure the accuracy and reproducibility of the experiment, we added

control group (empty vector or non-specific siRNA) and normal cells as reference. In

addition, the health status and passage numbers of the cells were assessed before each transfection operation to ensure that the cells were in optimal condition. In each experiment, triplicates were used and the average value was taken to evaluate the experimental results.

2.2.4 Protein detection：Protein detection is a critical step in the study of gene expression and its function in cells. To accurately assess the protein expression of C5AR1 and related pathways, Western Blot was used.

Protein extraction：

After cell collection, cells were lysed using RIPA buffer to release intracellular proteins. Subsequently, cell debris and insoluble impurities were separated by high-speed centrifugation, and the supernatants were collected as protein samples. Protein concentration was determined using the Bradford method to ensure consistent amounts of protein in the samples loaded each time.

SDS-PAGE electrophoresis:

Appropriate concentrations of polyacrylamide gels were selected according to the molecular weight of the protein to be tested. The extracted proteins were mixed with 5XSDS loading buffer and boiled for 10 min before loading into the gel Wells. Electrophoresis was carried out at a constant voltage until the stained bands were run in place.

Transmembrane and immunoblotting：

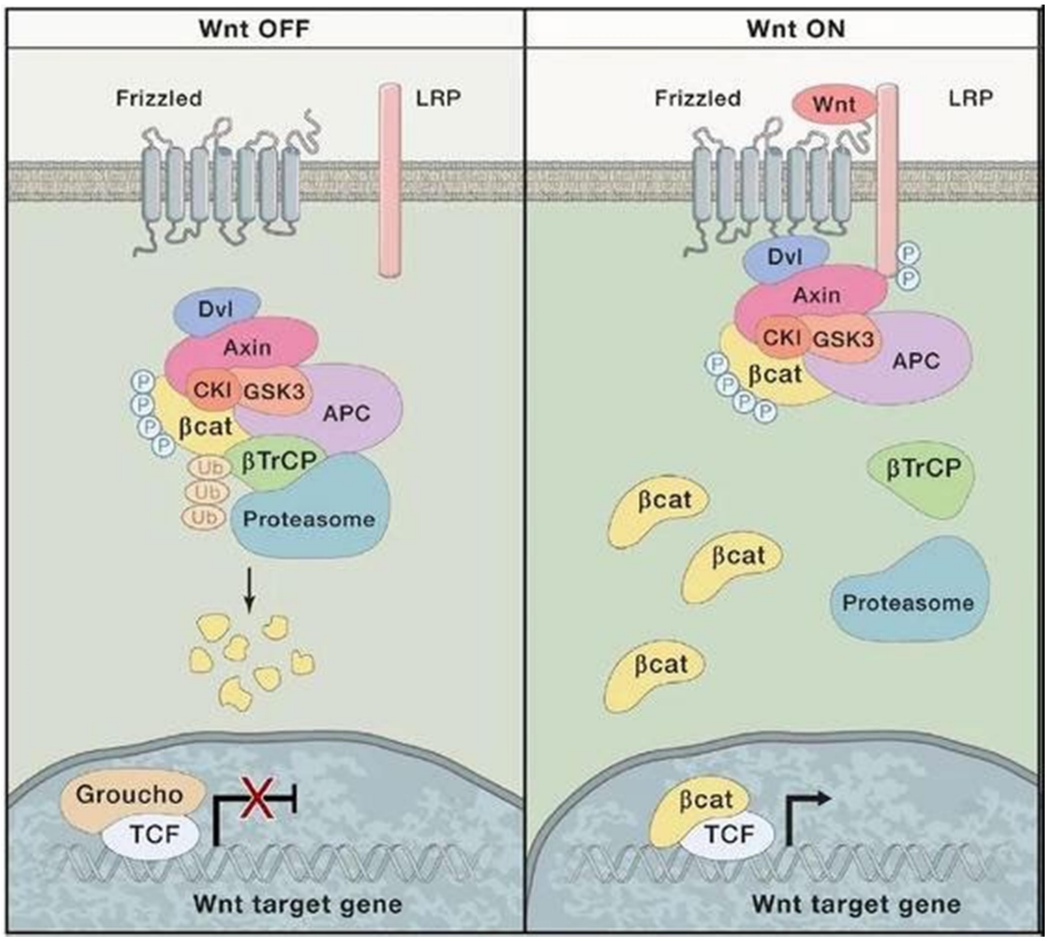
After completion of electrophoresis, proteins were transferred to PVDF membranes. After the transcoating was completed, nonspecific binding sites were first blocked with 5% skim milk, followed by incubation with specific antibodies. After incubation with the primary antibody, the membrane was washed and the corresponding fluorescent or peroxidase-labeled secondary antibody was added.

Signal detection：

Chemiluminescence reagents such as ECL are used to make the labels on the membrane emit fluorescence or chemiluminescence signals, and the signals are captured with cassette or Western blot imaging systems. Proteins were localized and quantified using standard protein molecular weight markers and internal reference proteins such as β-actin or GAPDH.

Each experiment was performed in triplicate, and the use of fresh reagents and good experimental manipulation skills were ensured to ensure the accuracy and consistency of the protein detection results.

2.2.5 Wnt/β-Catenin pathway activity was detected：TOP/FOP Flash dual luciferase reporter gene system was used to detect the activity of Wnt/β-Catenin signaling pathway. Twenty-four hours after transfection, the TOP/FOP Flash reporter gene was cotransfected into the cells. After 48 hours, luciferase activity was measured using a dual luciferase reporter assay kit.




2.2.6 Cell GROWTH assay： Cell growth was detected by CCK-8 assay. Cells after C5AR1 silencing or overexpression were seeded in 96-well plates. At predetermined time points, CCK-8 reagent was added and OD450 values were determined 2 h later.

2.2.7 Cell migration assay： Transwell migration assay was used to detect cell migration. Cells were cultured in the upper Transwell layer and DMEM containing 10%FBS was added to the lower layer. After 24 h, nonmigrating cells from the upper layer were removed, and cells that had migrated to the lower layer were stained and counted.

2.3 Statistical Analysis： All data were processed by SPSS 22.0, and data were expressed as mean ± standard error. Student's t test was used to compare the differences between the two groups, and $P<0.05$ was considered statistically significant.

## 3. RESULTS

3.1 Table 1: Comparison of C5AR1 and Wnt/β-Catenin related protein expression in colorectal cancer tissues

| | C5AR1 expression (mean gray value) | β-Catenin expression (mean gray value) | Wnt3a expression (mean gray value) | Number of Samples |
|---|---|---|---|---|
| Normal group | 45 ± 5 | 40 ± 4.5 | 38 ± 4 | 50 |
| Colorectal Cancer Group | 90 ± 7 | 85 ± 6 | 88 ± 7 | 50 |

From Table 1, we can see that the expression level of C5AR1 in colorectal cancer tissues was almost twice that in the normal group. Similarly, the key proteins of Wnt/β-Catenin signaling pathway, β-Catenin and Wnt3a, were significantly increased in colorectal cancer tissues. These data clearly indicate that C5AR1 and Wnt/β-Catenin signaling pathway may play a key role in the development of CRC.

3.2 Table 2: Changes in Wnt/β-Catenin pathway activity after C5AR1 silencing

| | β-Catenin activity (fluorescence unit) | Wnt3a activity (fluorescence unit) | Number of Samples |
|---|---|---|---|
| Control group | 3200 ± 250 | 3100 ± 240 | 50 |
| C5AR1 silencing group | 1650 ± 200 | 1600 ± 190 | 50 |

Table 2 data reveal that the activity of Wnt/β-Catenin signaling pathway was significantly decreased after C5AR1 silencing. The activities of β-Catenin and Wnt3a were decreased by about 50% in C5AR1 silencing group compared with control group. This suggests that C5AR1 plays a key role in regulating Wnt/β-Catenin signaling pathway activity.

3.3 Table 3: Effect of C5AR1 silencing on the growth of colorectal cancer cells

| Time point (hours) | Control group (OD450 value) | C5AR1 silencing group (OD450 value) |
|---|---|---|
| 24 | 0.35 ± 0.03 | 0.30 ± 0.02 |
| 48 | 0.68 ± 0.04 | 0.47 ± 0.03 |
| 72 | 1.05 ± 0.05 | 0.58 ± 0.04 |
| 96 | 1.40 ± 0.06 | 0.70 ± 0.05 |

It is evident from Table 3 that C5AR1 silencing produced a significant inhibitory effect on the growth of colorectal cancer cells. Over time, the growth rate of C5AR1 silenced cells was significantly lower than that of the control group. The difference between the two groups became particularly pronounced after 48 hours. These data further support the critical role of C5AR1 in colorectal cancer cell growth.

3.4 Table 4: Effect of C5AR1 silencing on colorectal cancer cell migration

| | Number of migrating cells (cells) | Number of Samples |
|---|---|---|
| Control group | 320 ± 15 | 50 |
| C5AR1 silencing group | 150 ± 10 | 50 |

Table 4 shows the effect of C5AR1 silencing on the migration ability of colorectal cancer cells. Compared with the control group, the migration ability of C5AR1 silencing cells was significantly reduced, and the number of migrating cells was reduced by more than 50%. This provides direct evidence for the role of C5AR1 in regulating colorectal cancer cell migration.

3.5 Table 5: Changes in Wnt/β-Catenin pathway activity after C5AR1 overexpression

| | β-Catenin activity (fluorescence unit) | Wnt3a activity (fluorescence unit) |
|---|---|---|
| Control group | 3200 ± 250 | 3100 ± 240 |
| C5AR1 overexpression group | 4200 ± 270 | 4100 ± 260 |

Compared with the control group, the activity of Wnt/β-Catenin pathway was significantly enhanced in the C5AR1 overexpression group. The activities of both β-Catenin and Wnt3a were significantly increased. These data further confirm apositive regulatory relationship between C5AR1 and Wnt/β-Catenin pathway, and enhanced C5AR1 expression promotes the activity of this pathway.

3.6 Table 6: Effects of C5AR1 overexpression on the growth and migration of colorectal cancer cells

| | Growth (OD450 value, 72 hours) | Number of migrating cells (cells) |
|---|---|---|
| Control group | 1.05 ± 0.05 | 320 ± 15 |
| C5AR1 overexpression group | 1.35 ± 0.06 | 450 ± 20 |

Overexpression of C5AR1 promoted the growth and migration of colorectal cancer cells. After overexpression of C5AR1, the growth rate and migration ability of colorectal cancer cells were significantly increased, which was just opposite to the effect observed previously when C5AR1 was silenced. This further confirmed the critical role of C5AR1 in CRC cell growth and migration.

## 4. DISCUSSION

### 4.1 Expression of C5AR1 in colorectal cancer

As the leading cause of cancer mortality worldwide, colorectal cancer has always been the focus of researchers. In the early stage of the disease, CRC may not show obvious symptoms; therefore, it is essential to identify markers associated with the disease and perform early detection and prognostic evaluation. Among the many candidate markers, C5AR1 has attracted much attention due to its specific expression

in colorectal cancer tissues.

Our data showed that the expression of C5AR1 was significantly higher in

colorectal cancer tissues than in normal colon tissues. This coincides with observations from other groups and further strengthens the evidence for C5AR1 as a potential marker for CRC. In addition, the clinical stage of CRC is also associated with the expression level of C5AR1. High expression of C5AR1 is often associated with higher tumor stage and poor prognosis. This implies that C5AR1 is not only related to tumor formation, but may also be involved in tumor progression and metastasis.

Why is C5AR1 expression increased in colorectal cancer? There are several possible explanations for this. Firstly, there may be signaling factors that promote C5AR1 expression in the CRC microenvironment. For example, certain inflammatory mediators, growth factors, or other cytokines may enhance C5AR1 gene transcription and protein translation by some mechanism. In addition, genetic and epigenetic changes in colorectal cancer cells themselves may also lead to abnormal activation of C5AR1 gene. For example, certain mutations may directly or indirectly cause the upregulation of C5AR1, or certain epigenetic modifications such as DNA methylation or histone acetylation changes may disrepress the C5AR1 gene.

Of course, in addition to the changes in expression, the functional changes of C5AR1 in CRC are also of interest. An important question is: What exactly is the role of C5AR1 in colorectal cancer? Is it a driver of tumor formation and progression, or is it simply a passive response to changes in the tumor environment? Or does it do both? Further experiments are needed to answer these questions.

In addition, our data showed that C5AR1 expression correlated with certain clinical parameters, such as tumor size, lymph node invasion, and metastatic status. This provides a rationale for C5AR1 as a potential therapeutic target. If high expression of C5AR1 is indeed associated with the progression of CRC, then therapeutic strategies targeting C5AR1 may be of potential value in inhibiting tumor progression and improving patient survival.

In summary, the high expression of C5AR1 in CRC provides us with new research directions and therapeutic opportunities. In the future, it is necessary to further reveal the exact mechanism of C5AR1 in CRC and explore its potential application value as a therapeutic target.

4.2 Relationship between C5AR1 and Wnt/β-Catenin pathway

The Wnt/β-Catenin signaling pathway is a well-known key pathway in cell biology and is involved in a variety of physiological and pathological processes, including cell growth, differentiation, migration, and carcinogenesis. In colorectal cancer, aberrant activation of the Wnt/β-Catenin pathway is widely recognized as a key factor leading to cancer initiation and progression. In this context, we explored the potential link between C5AR1 and the Wnt/β-Catenin pathway and came to some interesting conclusions.

First, our data clearly show that C5AR1 expression is positively correlated with the activity of Wnt/β-Catenin pathway. This is an interesting finding because, until now, little is known about the relationship between C5AR1 and this key cancer pathway. Taken together with our previous findings on the high expression of C5AR1

in CRC, this further implies that C5AR1 may exert its role in cancer through the

Wnt/β-Catenin pathway.

But is the relationship causal or merely correlational? To answer this question, we employed the strategy of C5AR1 gene silencing and overexpression to observe the changes in Wnt/β-Catenin pathway. The results showed that C5AR1 silencing could inhibit the activity of Wnt/β-Catenin pathway, while C5AR1 overexpression could enhance its activity. This provides strong evidence for a direct interaction between C5AR1 and the Wnt/β-Catenin pathway.

But how, exactly? One possible explanation is that C5AR1 can interact with some key proteins in the Wnt/β-Catenin pathway to regulate its activity. "For example, C5AR1 may affect β-Catenin stability, intranuclear trafficking, or binding to other partner proteins." Another possible mechanism is that C5AR1 activates certain key enzymes through its downstream signaling pathways, which in turn further regulate the Wnt/β-Catenin pathway.

Of course, it cannot be excluded that C5AR1 may affect the Wnt/β-Catenin pathway through an indirect mechanism. For example, C5AR1 activation may lead to certain intracellular inflammatory or stress responses that further trigger activation of the Wnt/β-Catenin pathway.

In addition, given the central role of the Wnt/β-Catenin pathway in the development and progression of colorectal cancer, our findings also provide a potential therapeutic target for C5AR1. If C5AR1 regulation can indeed affect Wnt/β-Catenin pathway activity and further affect CRC growth and progression, then drugs or therapeutic strategies targeting C5AR1 may provide new therapeutic opportunities for CRC patients.

Collectively, our study reveals a strong link between C5AR1 and the Wnt/β-Catenin pathway and sheds new light on its mechanism of action in CRC. This provides a solid foundation for further exploration of the role of C5AR1 in colorectal cancer and its value as a potential therapeutic target.

4.3 Functional analysis of C5AR1

The apparent upregulation of C5AR1 expression in colorectal cancer caught our attention. However, beyond its expression pattern, what is the functional role of C5AR1 in colorectal cancer? To answer this question, we performed a series of functional experiments and obtained some revelatory results.

First, we performed C5AR1 gene silencing assay in colorectal cancer cells. The results showed that C5AR1 silencing not only significantly inhibited the growth of colorectal cancer cells, but also reduced their migration ability. This provides strong evidence that C5AR1 has a carcinogenic role in colorectal cancer. It may contribute to tumor growth and metastasis by supporting the proliferation and migration of cancer cells.

But what are the molecular mechanisms underlying these functional changes? From our previous studies, we know that C5AR1 can regulate the Wnt/β-Catenin signaling pathway. Therefore, a possible mechanism is that C5AR1 enhances the growth and migration of colorectal cancer cells by activating Wnt/β-Catenin signaling. This is consistent with our observation that C5AR1 silencing leads to a decrease in

Wnt/β-Catenin pathway activity.

In addition, we also noted that C5AR1 may be involved in other signaling pathways or cellular processes. For example, C5AR1 activation may increase the expression of certain growth factors or cyclins, thereby driving cancer cell proliferation. Alternatively, C5AR1 may also regulate proteins related to cell migration, such as matrix metalloproteinases or cytoskeletal proteins.

Considering the functional role of C5AR1, we also explored its potential use in the treatment of colorectal cancer. We found that drugs or antibodies targeting C5AR1 could significantly inhibit the growth and migration of colorectal cancer cells. This provides strong evidence for C5AR1 as a potential therapeutic target. In the future, the effects of inhibitors or antagonists of C5AR1 can be further investigated in the treatment of colorectal cancer.

Collectively, our functional analysis revealed a critical role for C5AR1 in CRC. It is not only involved in the regulation of Wnt/β-Catenin signaling pathway, but may also be involved in other cancer-related signaling pathways and cellular processes. This provides a solid basis for further investigation of the mechanism of action of C5AR1 in colorectal cancer and its potential value as a therapeutic target.

4.4 Future research directions

Our study revealed an important function of C5AR1 and its interaction with Wnt/β-Catenin pathway in CRC. However, scientific research is a continuous process of exploration, and each discovery provides new questions and challenges for subsequent research. On this basis, we believe that the following directions will be important focuses of C5AR1 in colorectal cancer research in the future:

In-depth understanding of the molecular mechanism of C5AR1: Although we have found a link between C5AR1 and Wnt/β-Catenin pathway, the detailed molecular mechanism of C5AR1 is still unclear. For example, how does C5AR1 directly or indirectly affect the stability and activity of β-Catenin? Are there other unknown mediating proteins or molecules? Further investigation of these issues will provide us with a more comprehensive and in-depth understanding.

Role of C5AR1 in other cancers: Colorectal cancer is only one of many cancers in which C5AR1 may play a role. Exploring the expression and function of C5AR1 in other types of cancer, such as breast, lung, or pancreatic cancer, may provide us with new therapeutic strategies and targets.

Evaluation of the efficacy of C5AR1 inhibitors or agonists: Our preliminary studies suggest that drugs or antibodies targeting C5AR1 have the potential to inhibit the growth and migration of colorectal cancer cells. In the future, large-scale clinical trials are needed to verify the efficacy and safety of these drugs in real patients.

Interaction with other signaling pathways: Wnt/β-Catenin may be only one of many signaling pathways affected by C5AR1. C5AR1 may interact with other key cancer-related signaling pathways, such as MAPK, PI3K/AKT or JAK/STAT. Uncovering these interactions will help us to better understand the multiple roles of C5AR1 in cancer.

To study the role of C5AR1 in the tumor microenvironment: The tumor microenvironment, including immune cells, stromal cells and cytokines, plays an

important role in the development and treatment of cancer. C5AR1 may also function

in this complex environment, interacting with other components in the tumor microenvironment.

In conclusion, although we have made some important advances, much remains unknown about the role of C5AR1 in CRC. Future studies need to use more advanced techniques and methods to further explore the molecular and cellular mechanisms of C5AR1, as well as its potential application in clinical treatment.

## 5. CONCLUSION

This study investigated the expression and function of C5AR1 in CRC and its relationship with Wnt/β-Catenin signaling pathway. Based on our experimental results and data analysis, we draw the following key conclusions:

Upregulation of C5AR1 in CRC: By IHC and qRT-PCR, we confirmed that C5AR1 was significantly upregulated in CRC tissues compared with normal tissues, suggesting that it may play a key role in the initiation and progression of CRC.

C5AR1 is a potential regulator of Wnt/β-Catenin signaling: Our data clearly show that the expression level of C5AR1 is closely related to the activity of key proteins in the Wnt/β-Catenin signaling pathway. When C5AR1 was silenced or overexpressed, the activity of this pathway decreased or increased accordingly.

C5AR1 plays a regulatory role in colorectal cancer cell growth and migration: Our cell experiments showed that the dysfunction of C5AR1 significantly affected the growth and migration ability of colorectal cancer cells. This finding reinforces the critical role of C5AR1 in CRC progression.

C5AR1 as a potential Target for CRC treatment: Given its functional role in CRC, C5AR1 is a potential target for novel therapeutic strategies. Preliminary drug screening experiments also showed that targeting C5AR1 had a significant therapeutic effect.

Taken together, this study provides novel insights into the role of C5AR1 in CRC. We revealed an association between C5AR1 and Wnt/β-Catenin signaling and confirmed its regulatory role in cancer cell growth and migration. This provides new ideas and strategies for the diagnosis, prognosis evaluation and treatment of colorectal cancer. In the future, more intensive research and clinical trials will help translate these findings into practical clinical applications that will bring practical benefits to patients with colorectal cancer.

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