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2025,Volume 10, Issue 2
Advances in Precision Medicine
Online ISSN: 2424-9106
Print ISSN: 2424-8592

# Study on the Role of Circular RNA hsa_circ_0001591 in Colorectal Cancer

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**Abstract:** *Objective:* To validate and characterize the expression of circular RNA hsa_circ_0001591—identified by high-throughput sequencing on the DNBSEQ platform—as differentially expressed in colorectal cancer (CRC) tissues versus adjacent non-tumor tissues and to explore its association with clinicopathological features and prognostic potential. *Methods:* RT-qPCR was performed on 13 paired CRC and matched adjacent samples to confirm elevated hsa_circ_0001591 levels, functional knockdown attempts were made in CRC cell lines with PCR verification of silencing efficiency, and statistical analyses were conducted to correlate RNA expression with CD31/CD34 status, patient age, and short-term postoperative outcomes. *Results:* Ten of 13 CRC specimens showed significantly higher hsa_circ_0001591 expression in tumor versus adjacent tissue, and elevated levels correlated with CD31/CD34 positivity ($P = 0.041$) and age > 70 years old ($P = 0.033$), whereas circRNA knockdown *in vitro* was inconclusive due to its inherent stability and no significant differences in short-term postoperative status were observed. *Conclusion:* hsa_circ_0001591 is upregulated in CRC and linked to markers of vascular invasion, suggesting a potential inhibitory role in angiogenesis, and its miRNA/mRNA interaction network may drive CRC pathogenesis, making it a promising molecular biomarker and therapeutic target.

**Keywords:** Colorectal cancer; Whole transcriptome sequencing; Gene differential expression; Circular RNA: hsa_circ_0001591

***Online publication:*** June 28, 2025

# 1. Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors of the digestive tract worldwide. In recent years, the incidence and mortality rates of CRC have been showing an increasing trend. According to the 2022 Global Cancer Burden data released by the International Agency for Research on Cancer of the World Health Organization, in the 2022 global new cancer incidence data, colorectal cancer ranked third in the number of new cases, followed by lung cancer and breast cancer. In terms of death cases, CRC ranks second and lung cancer ranks first. In 2022, the total number of newly diagnosed cancer cases in China reached 4.824 million, among which the number of new cases of CRC was 517,000, ranking second among all types of cancer, only after lung cancer. In the same year, there were 2.574 million cancer deaths in China, among which 240,000 were deaths from CRC, ranking fourth [1]. Circular RNA (circRNA) is a newly discovered type of endogenous non-coding RNA. circRNA does not have a 5'cap and a 3' polymerized A tail, presenting a closed loop. This unique closed-loop structure endows circRNA with high stability and makes it less prone to degradation by nucleases [2,3]. In recent years, with the continuous in-depth research on circRNA, its functions in organisms have been constantly discovered and confirmed, including acting as miRNA sponges, having certain translation functions, participating in transcriptional regulation functions, and the interacting with circRNA and proteins, etc. Acting as miRNA sponges, circRNAs can target and adsorb miRNAs to regulate gene expression, and this function has been widely recognized [4–6].

The study first analyzed 20 pairs of specimens using high-throughput sequencing technology to precisely screen out circRNAs that were significantly differentially expressed in colorectal cancer tissues and adjacent tissues. Based on the CeRNA mechanism, the range of circRNAs with differential expression was further narrowed. Eventually, the researchers selected has_circ_0001591 with upregulated expression as the key research object. The real-time fluorescent quantitative PCR (RT-qPCR) technique was once again employed to verify the accuracy of the upregulated hsa_circ_0001591 obtained by sequencing. Finally, cell experiments were carried out on this gene to further explore the role of hsa_circ_0001591 in colorectal cancer cell lines. This study is expected to identify the mechanism of tumor occurrence and development, provide new theoretical support for the early diagnosis and treatment of colorectal cancer, and explore potential new therapeutic targets, contributing to the improvement and breakthrough of the diagnosis and treatment level of colorectal cancer. Through the one-year follow-up of the last 13 patients after the operation, the influence of differentially expressed hsa_circ_0001591 on prognosis was analyzed. This study gained an in-depth understanding of the function and mechanism of action of hsa_circ_0001591 in colorectal cancer, providing new ideas and methods for the diagnosis, treatment and prognosis evaluation of colorectal cancer.

# 2. Background and methods

## 2.1. Experimental background

### 2.1.1. Sequencing conclusion

Based on the ceRNA theory, the combination with the most significant shared miRNA was screened out from numerous circRNA-mRNA relationship pairs, and then the Cytoscape software was used to construct the circRNA-miRNA-mRNA regulatory network that could reflect differential expression. After a detailed comparison of the circRNAs in this network with the significantly different circRNAs determined by high-throughput sequencing, hsa_circ_0001591 with an up-regulated expression was successfully identified. The network

construction results are shown in **Figure 1**. This gene is expected to become an important breakthrough point for in-depth exploration of the pathogenesis of colorectal cancer.

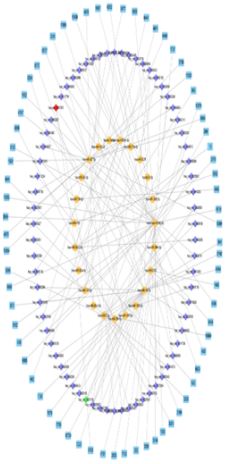

**Figure 1**. ceRNA co-expression network. The red rhombus is hsa_circ_0001591, the orange circle is miRNA, and the blue rectangle is mRNA.

As shown in **Figure 2**, hsa_circ_0001591 is located at 22.1 of the short arm of chromosome 6, with its coordinate position being (chr6:27,100,817-27,101,314), its length is 14057nt, its host gene is HIST1H2AG, and the transcription is: NM_021064, exon 1 forms a loop independently.

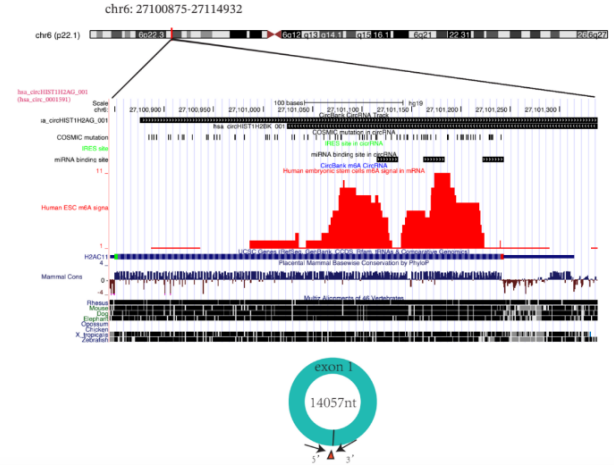


**Figure 2.** shows the characteristics of the upregulated hsa_circ_0001591.

**Table 1.** Relationship between differentially expressed hsa_circ_0001591 and clinicopathological information

| Clinical Feature | Total | High Expression | Low Expression | P Value |
|---|---|---|---|---|
| Male | 11 | 6 | 5 | 0.157 |
| Female | 9 | 8 | 1 | |
| ≥70 years old | 8 | 6 | 2 | 0.537 |
| <70 years old | 12 | 11 | 1 | |

| | | | | |
|---|---|---|---|---|
| Left Colon 13 | 10 | 3 | 0.593 | |
| Right Colon | 7 | 6 | 1 | |
| With Lymph Node Metastasis | 7 | 6 | 1 | 1.000 |
| Without Lymph Node Metastasis | 13 | 10 | 3 | |
| CEA Positive | 7 | 6 | 1 | 1.000 |
| CEA Negative | 13 | 10 | 3 | |
| Stage I or II | 14 | 10 | 4 | 0.517 |
| Stage III or IV | 6 | 5 | 1 | |
| P53 Positive | 11 | 8 | 3 | 0.642 |
| P53 Negative | 9 | 5 | 4 | |
| CD31, CD34 Positive | 10 | 5 | 5 | 0.033 |
| CD31, CD34 Negative | 10 | 10 | 0 | |
| D2-40 Positive | 14 | 10 | 4 | 1.000 |
| D2-40 Negative | 6 | 5 | 1 | |

The statistical table in **Table 1** reveals the relationship between the clinicopathological data of each colorectal cancer patient in the high-throughput sequencing results and the differentially expressed hsa_circ_0001591. Combined with these pathological data, the study further analyzed the possible association between the pathological conditions of patients with colorectal cancer and the differentially expressed hsa_circ_0001591. Finally, the study found that whether CD31 and CD34 were positive was correlated with the differentially expressed hsa_circ_0001591 ($P$ = 0.033), with a $P$ value < 0.05 (statistically significant). It provides a key clue for further revealing the mechanism of hsa_circ_0001591 in the occurrence and development process of colorectal cancer [17]. **Figure 3** shows the pathological diagrams of positive CD31 and CD34.

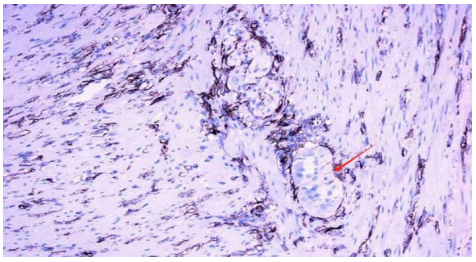

**Figure 3.** Pathological diagrams of positive CD31 and CD34.

### 2.1.2. GO enrichment and KEGG signaling pathway analysis of the HSA_cirC_0001591-related ceRNA regulatory network

Based on the ceRNA regulatory network, the study conducted GO functional classification and KEGG signaling pathway analysis on the mRNA related to hsa_circ_0001591. It can be known from Figure 4 that in the classification of biological processes, this gene is mainly involved in the following pathways: the regulation of blood volume by nicotine response and renal aldosterone, as well as the endogenous coagulation pathway. In terms of cellular components, the related genes are associated with acetylcholine-gated channel complexes, type IV collagen trimers and hemidesmosomes. In terms of molecular functions, the most relevant ones are acetylcholine binding and acetylcholine receptor activity. In addition, through the analysis of the KEGG signaling pathway, the study also found that this gene is related to two pathways: the neuroactive ligand-receptor interaction pathway and the

complement and coagulation cascade reactions.

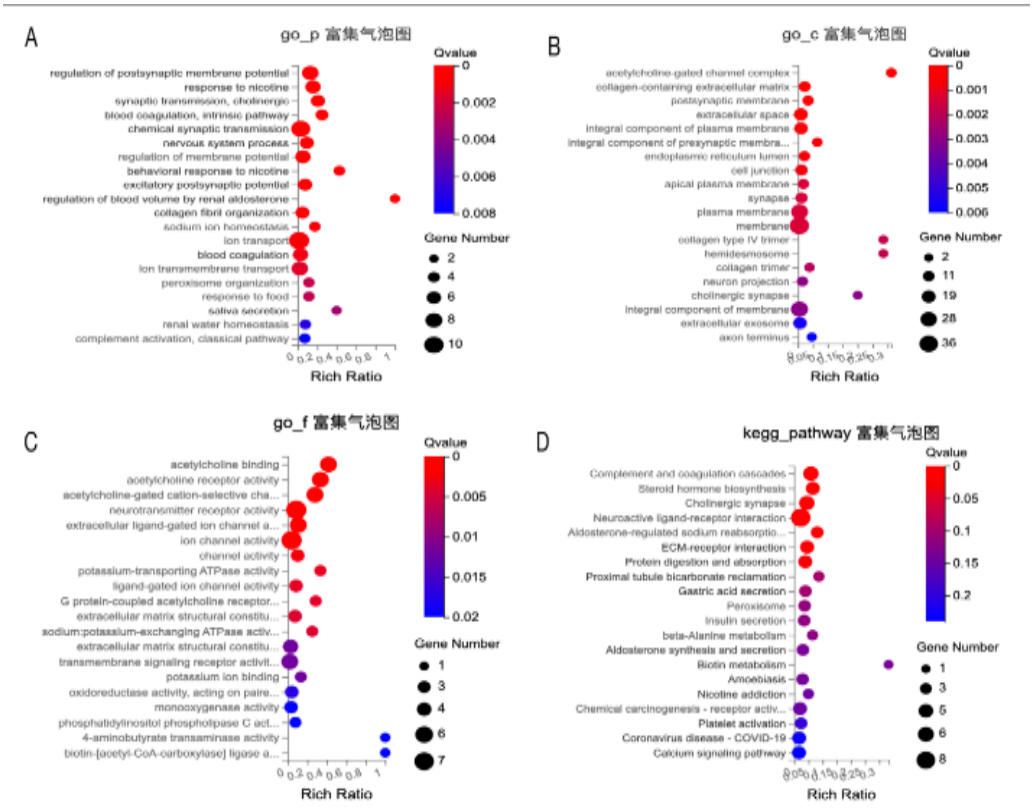


**Figure 4.** ceRNA regulatory network regulated by hsa_circ_0001591.A is gene ontology - the analysis of biological processes; B is gene ontology - analysis of cellular components; C stands for gene ontology - the analysis of molecular functions; D represents the analysis of the KEGG signaling pathway.

## 2.2. Research object

The specimens selected for this experiment were all colorectal cancer patients who underwent surgical treatment in the General Surgery Department of Ordos Central Hospital, and this study was approved by the Theoretical Ethics Committee. Before the operation, inform the patient and their family of the relevant matters, such as sample collection and experiments, and have the family members sign the informed consent form for medical sample collection. In this study, 13 pathological samples were randomly selected from the patients who underwent colorectal cancer surgery in the General Surgery Department of the hospital from January to September 2023 for real-time fluorescence quantitative PCR verification. All the patients included in the study did not receive any treatment for colorectal cancer before the operation, such as chemotherapy, radiotherapy and targeted therapy, etc. All tissue samples diagnosed as colorectal cancer through pathological examination are collected in strict accordance with the norms. First of all, the sample should be washed multiple times with frozen physiological saline to remove impurities such as blood and feces that adhere to the surface. After the cleaning is completed, collect the sample into a 2 mL freeze-drying tube. After confirming the collection of specimens, the study will also conduct detailed information registration for the patient to ensure the accuracy of the sample information. After the registration is completed, immediately place the refrigerated test tubes in liquid nitrogen and put them in the freezer at -80 ℃ within 30 minutes for subsequent experiments.

## 2.3. Research methods

### 2.3.1. Clinical data collection

In this study, based on the diagnostic criteria of colorectal cancer, tumor specimens of eligible patients with colorectal cancer were selected for research. During diagnosis, a comprehensive judgment is mainly made through multiple examination methods, including collecting clinical data, chemical laboratory data, relevant examination reports, pathological reports, as well as general information such as gender, age, lesion location, chronic disease status, and smoking and drinking habits.

### 2.3.2. Statistical methods

For the key circRNAs obtained through screening, this study focuses on exploring their differential expression in cancer tissues and adjacent tissues, and further analyzes them in combination with clinicopathological characteristics. Given that the sample size was 13 cases, during the statistical analysis process, considering the situation where $n < 44$ and $t < 1$, based on statistical principles, the Fisher's exact test method in SPSS 26.0 software was selected for comparative analysis. It was determined that when $P < 0.05$, the differential expression was statistically significant. This result can provide strong data support and theoretical basis for subsequent research, and is conducive to further revealing the potential mechanism of key circRNAs in the occurrence and development process of colorectal cancer.

# 3. Experimental results

## 3.1. External RT-qPCR verification

In this experiment, 13 tumor specimens of colorectal cancer patients who underwent colorectal cancer surgery in our hospital from January to September 2023 and met the conditions were selected for external verification by RT-qPCR. Using GAPDH as the internal control, the primer sequence of the gene is shown in **Table 2**. The relative expression level of circRNA was calculated using the formula of 2-ΔΔCt. The results showed that hsa_circ_0001591 was significantly highly expressed in the colorectal cancer tissues of 10 specimens, while the expression differences in the remaining 3 cases were moderate or low, which was consistent with our previous test results.

**Table 2.** Primer sequence of RT-qPCR.

| Gene Name | Primer Sequence | Length | Tm | GC% | ProductLength |
|---|---|---|---|---|---|
| GAPDH | CCCACTTCTCTCTAAGGAGAAT | 22 | 56.35 | 45.45 | 77 |
| | TACACGAAAGCAATGCTATCAC | 22 | 57.14 | 40.91 | |
| circ1591 | CAGTATGTCTGGACGTGGCA | 20 | 59.75 | 55 | 126 |
| | GCATAGTTGCCTTTGCGGAG | 20 | 59.9 | 55 | |

The clinicopathological information of the 13 specimen patients was further analyzed. The results are shown in **Table 3**. The expression difference of this gene was statistically significant compared with the age of the patients ($P = 0.033$) and the positive or negative status of CD31 and CD34 ($P = 0.041$) ($P < 0.05$). There were no significant statistical differences in terms of gender, lesion location, presence or absence of lymph node metastasis, positive or

negative status of CEA, tumor stage, presence or absence of metastasis, and positive or negative postoperative complications.

**Table 3.** The relationship between hsa_circ_0001591 and clinicopathological information in colorectal cancer.

| | | Expression situation（%） | | Total | X2 | p |
|---|---|---|---|---|---|---|
| **Title** | **Name** | **low** | **high** | **7** | **0.258** | |
| | | | | | | **0.612** |
| Gender | female | 2(28.57) | 5(71.43) | 6 | | |
| | male | 1(16.67) | 5(83.33) | 13 | | |
| Total | | 3(23.08) | 10 | 7 | | |
| Age | ≥70 | 0(0.00) | 7 | 7 | | |
| | <70 | 3(50.00) | 3(50.00) | 6 | 4.55 | 0.33* |
| Total | | 3(23.08) | 10 | 13 | | |
| Location | Right half colon | 0(0.00) | 1 | 1 | | |
| | Left half colon | 3(25.00) | 9(75.00) | 12 | 0.325 | 0.569 |
| Total | | 3(23.08) | 10 | 13 | | |
| Whether there is lymphatic metastasis or not | no | 2(20.00) | 8(80.00) | 10 | | |
| | have | 1(33.33) | 2(66.67) | 3 | 0.231 | 0.631 |
| Total | | 3(23.08) | 10 | 13 | | |
| CEA | Yang | 0(0.00) | 3 | 3 | | |
| | Yin | 3(30.00) | 7(70.00) | 10 | 1.17 | 0.279 |
| Total | | 3(23.08) | 10 | 13 | | |
| CD31 | Yang | 2(66.67) | 1(33.33) | 3 | | |
| CD34 | Yin | 1(10.00) | 9(90.00) | 10 | 4.174 | 0.041* |
| Total | | 3(23.08) | 10 | 13 | | |
| Installment | VII | 2(16.67) | 10 | 12 | | |
| | IIV IV | 1 | 0(0.00) | 1 | 3.611 | 0.057 |
| Total | 3(23.08) | 10 | 13 | | | |
| P53 | Yang | 1(20.00) | 4(80.00) | 5 | | |
| | Yin | 1(16.67) | 5(83.33) | 6 | 0.02 | 0.887 |
| Total | | 2(18.18) | 9(81.82) | 11 | | |
| D2-40 | Yang | 1(50.00) | 1(50.00) | 2 | | |
| | Yin | 2(20.00) | 8(80.00) | 10 | 0.8 | 0.371 |
| Total | | 3(25.00) | 9(75.00) | 12 | | |
| Transfer focus | no | 3(25.00) | 9(75.00) | 12 | | |
| | Liver metastasis | 0(0.00) | 1 | 1 | 0.325 | 0.569 |
| Total | | 3(23.08) | 10 | 13 | | |
| Constipation | no | 1(11.11) | 8(88.89) | 9 | | |
| | Mild | 2(50.00) | 2(50.00) | 4 | 2.359 | 0.125 |
| Total | | 3(23.08) | 10 | 13 | | |

*p<0.05**p<0.01

## 3.2. Cell experiment results

(1) RNA was extracted from the SW620 cell line using the Trizol kit. According to the relevant instructions, total RNA was reverse transcribed into cDNA. After purification treatment of cDNA, PCR amplification was carried out. Real-time PCR was performed on the ABI 7500 rapid real-time PCR system (Applied Biosystems), using the SYBRGreen PCR kit. The relative expression level of hsa_circ_0001591 was calculated by the 2-ΔΔCt method and the relative quantitative method.

(2) hsa_circ_0001591 was transfected into the SW620 cell line of CRC by the cationic liposome transfection method, knockdown of the expression level of hsa_circ_0001591 in the SW620 cell line. Furthermore, a series of phenotypic experiments was conducted to verify the effects of hsa_circ_0001591 on the proliferation, migration, and invasion of colorectal cancer cell lines (**Figure 5**).

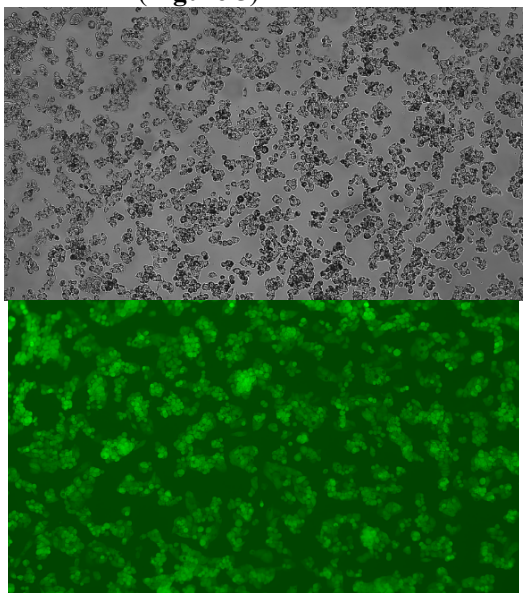

**Figure 5.** The cell culture process and after transfection.

(3) Cells from each group were taken again, and the expression level of hsa_circ_0001591 in the SW620 cell line was detected by RT-qPCR. There were at least three replicates in each group. The CT values of the target gene (hsa_circ_0001591) and the internal reference gene (GAPDH) must be detected simultaneously in each group, and the average value of the three times should be taken.

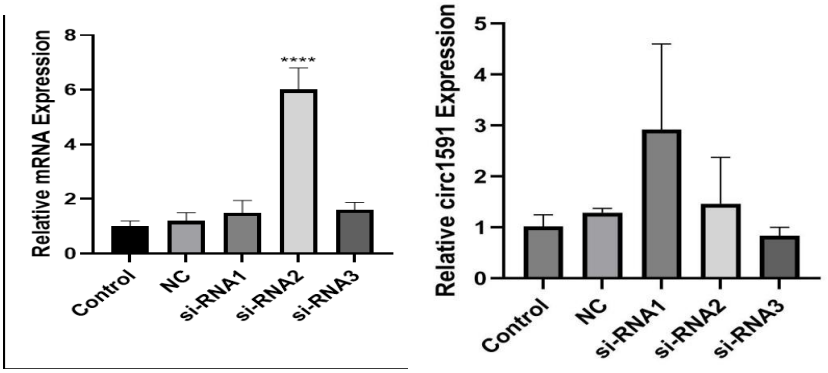

**Figure 6.** Control: Transfection was performed using transfection reagents, but siRNA was not added; NC: Transfection was performed using negative control siRNA; si-RNA1: The first group was transfected with siRNA of the target gene; si-RNA2: The second group was transfected with siRNA of the target gene; si-RNA3: The target gene siRNA was transfected into the third group.

The above figures (**Figure 6**) indicate that after the interference of the target gene hsa_circ_0001591 was introduced into the three groups of cell lines, respectively, and after culture and detection by RT-qPCR, the content of the hsa_circ_0001591 gene was still higher than that of the control group.

## 4. Discussion

Although current surgeries and adjuvant treatments have effectively reduced the overall incidence and mortality of colorectal cancer, effective treatments for advanced colorectal cancer still have little effect and still pose a threat to human health. Further research on the pathogenesis of colorectal cancer is of great significance for the early identification of colorectal cancer and guiding its treatment [7]. As a single-stranded covalent closed RNA molecule, the 3' and 5' ends of circRNA are connected to form a special closed circular structure [8]. Due to this unique closed-loop structure of circRNA, its stability is much stronger than that of linear RNA. To a large extent, it avoids the related linear RNA degradation methods such as decapitation reaction and deadenylation reaction. Therefore, the half-life of circRNA in the cytoplasm can reach more than 48 hours, while the half-life of linear RNA is only 10 hours [9,10]. Furthermore, numerous studies have found that the expression of circRNA in organisms has significant tissue specificity and developmental stage specificity. And in different tissues and cell types, there are significant differences in the expression profiles of circRNA. However, due to the stability, cell type or developmental stage specificity, structural conservation of circRNA, and its ability to play an important regulatory role under different physiological and pathological conditions. Make circRNA potentially become a novel biomarker for biological diagnosis [11]. Therefore, studying the role of circRNAs in tumors is of great significance, especially the differentially expressed circRNAs, which are highly likely to become potential biomarkers for the early diagnosis, prognosis evaluation and targeted therapy of colorectal cancer.

Through the GO enrichment of the HSA_circ_0001591-related ceRNA regulatory network and the analysis of the KEGG signaling pathway, hsa_circ_0001591, in the role of a molecular sponge, adsorbs miR-497-5p, thereby exerting effects on related genes. In terms of biological processes, it is mainly involved in the regulation of blood volume by renal aldosterone, the body's response to nicotine, and the endogenous coagulation process. In the cellular components, it is involved in the formation of acetylcholine-gated channel complexes, the generation of type IV collagen, and the production of hemidesmosomes. From the perspective of molecular function, this gene is closely related to the binding of acetylcholine and the activity of acetylcholine receptors. According to the analysis of the KEGG signaling pathway, hsa_circ_0001591 mainly involves the complement pathway and the interaction pathway between neuroactive ligands and receptors.

In the process of integrating the pathological data of colorectal cancer patients with hsa_circ_0001591, the study found that the differentially expressed hsa_circ_0001591 had a certain correlation with whether CD31 and CD34 were positive, with a $P$ value $< 0.05$ (statistically significant). This pathological information suggests that in

colorectal cancer tissues, the lower the expression level of hsa_circ_0001591, the more severe the patient's condition. Furthermore, in the external validation of RT-qPCR, the study also found that hsa_circ_0001591 was correlated with whether the age was greater than 70 years old ($P$ = 0.033), with a $P$ value < 0.05 (statistically significant). This provides a new direction for our later continued research on hsa_circ_0001591.

When hsa_circ_0001591 was transferred into the SW620 cell line of CRC, the expression result was not satisfactory. The following factors were comprehensively considered:

(1) Stability of circRNA: circRNA has a stable structure and is not easily degraded. Conventional knockdown methods are difficult to achieve the desired effect. Multiple knockdown methods can be attempted in combination, or more effective strategies for degrading circRNA can be designed.

(2) Intracellular compensation mechanism: Cells may have a compensation mechanism. After circRNA is knocked down, its expression or the expression of related genes is upregulated through other pathways to maintain the functional balance of the cell. It is possible to conduct in-depth research on the related regulatory pathways within cells and explore the methods of inhibitory compensation mechanisms.

In addition, the study conducted a telephone follow-up for the last 13 patients who underwent colorectal cancer surgery in our hospital. Due to the relatively short postoperative time, all of them were about one year. There was no significant change in the survival period of the patients. Only one case developed liver metastasis of colorectal cancer and received regular subsequent chemotherapy. Among the remaining patients, only a few had mild constipation. Considering the patients' advanced age and postoperative intestinal dysfunction. This phenomenon shows no significant difference from the differentially expressed hsa_circ_0001591. However, considering the small number of patients and the short postoperative time, the study are prepared to expand the sample size for further verification in the subsequent experiments and conduct a longer-term follow-up.

## 5. Conclusion

Based on the above experimental results, the study can initially draw the conclusion that the expression of hsa_circ_0001591 in the cancer tissues and adjacent tissues of patients with colorectal cancer is different, that is, it is expressed more in the cancer tissues. Moreover, the differentially expressed hsa_circ_0001591 is somewhat associated with whether the patient has vascular invasion. The GO enrichment of the ceRNA regulatory network and the analysis of the KEGG signaling pathway provide a new research direction for the diagnosis and treatment of colorectal cancer, which will bring new good news to a large number of patients with colorectal cancer.

## Funding

Ordos Science and Technology Plan Project, "Exploration and Functional Validation of Potential circRNA Tumor Markers in Colorectal Cancer" (Project No.: 2022YY015); Joint Scientific Research Fund Science and Technology Project (Project No.: 2023GLLH0256); Ordos Science and Technology Leading Talent Project (Project No.: E CAI Ke Zhi [2022] 711)

## Disclosure statement

The author declares no conflict of interest.

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