CircRNA hsa_circ_0004585 as a potential biomarker for colorectal cancer

Objective: Circular RNAs (circRNAs) are involved in regulating of carcinogenesis of various cancer cells. However, the function of circRNAs in colorectal cancer (CRC) has remained largely unknown. This study investigated the characteristic expression of circRNAs in CRC and adjacent normal tissues, analyzed the miRNAs related to candidate circRNAs, and studied the correlation between circRNAs with clinical data of CRC.

Methods: Human CircRNA microarray has been applied to screen the expressions of circRNAs of the CRC tissues and adjacent normal tissues. Quantitative real-time polymerase chain reaction (qRT-PCR) verified the candidate circRNAs in CRC tissue and patients’ peripheral blood. The circRNA array data were analyzed by GeneSpring 13.0 (Agilent) software. The diseases, pathways and functional enrichment analysis of these genes were performed using the KEGG system. In addition, the circRNA-miRNA network was constructed based on the miRanda-3.3 software. Statistical analysis was performed with SPSS23.0, GraphPad Prism, and SigmaPlot software.

Results: In total, 13,198 circRNAs were identified as distinct between CRC and adjacent normal tissues, including 6,697 upregulated and 6,501 downregulated genes. Based on scores, six of them were selected for further verification in CRC tissues and peripheral blood. The hsa_circ_0004585 expression was significantly upregulated both in CRC patients tissue and peripheral blood. Hsa_circ_0004585 was positively correlated with patient’s tumor size, indicating the function of hsa_circ_0004585 in CRC carcinogenesis and metastasis.

Conclusion: Hsa_circ_0004585 could be a potential biomarker for diagnosis of CRC.

Keywords: circRNA, colorectal cancer, prognosis biomarker

Introduction
Colorectal cancer (CRC) is one of the most frequently occurring digestive tract cancers, and the leading cause of morbidity and mortality. It is estimated that over 1.3 million new CRC cases are diagnosed every year.1 The diagnosis and treatment of CRC have continuously improved, but the mortality remains high. Therefore, to screen an efficient diagnostic marker and therapeutic target is an urgent problem.

Circular RNAs (circRNAs) are a new type of non-coding RNA, which form a covalently closed continuous loop. CircRNA does not have the 3‘ and 5‘ ends, but has a large number of miRNAs binding sites.2 The majority of circRNAs are exonic circRNAs, which are derived from exonic regions of known protein-coding genes by back-splicing. CircRNAs are abundant and stable in exosomes and plasma, providing a more convenient method for detection. Some researchers reported that circRNA can be a new diagnostic biomarker and therapy target for cancer, due to its stable and highly specific expression in different diseases and tissues.
such as breast cancer, lung cancer, colorectal cancer, liver cancer, gastric cancer, and non-small cell lung cancer. Based on the reports, circRNA plays an important role in oncogenesis and influences the cancer cells proliferation, migration, and apoptosis.

CircRNAs play their function mainly by sponging miRNA. For example, circNT5E as a “sponge” of miR-422a regulates tumorigenesis in glioblastoma. CircNT5E controlled multiple pathologic processes, including cell proliferation, migration, and invasion by directly bound miR-422a and inhibited miR-422a activity. Circular RNA CiRS-7 provide a promising prognostic biomarker and a potential therapeutic target of CRC. CiRS-7 abrogates the tumor suppressive effect of miR-7 on gastric cancer. However, the potential functions and the molecular mechanism of the majority circRNAs in different cancers are still not clear.

In this study, we investigated the characteristic expressions of circRNAs in CRC and adjacent normal tissues, and analyzed the clinical value of candidate circRNA in CRC. We found that hsa_circ_0004585 could be a potential biomarker for CRC diagnosis.

Materials and methods
Tissue and plasma samples
Both plasma and tumor tissue samples were collected from the Surgery Department of the General Hospital of Ningxia Medical University (Yinchuan, China). The study obtained approval from hospital ethics committee, which was conducted in accordance with the Declaration of Helsinki. All the patients signed their consent for donating their samples for the research. A total of 50 CRC tissue samples and adjacent normal tissues and 142 CRC and 142 healthy persons’ peripheral blood samples were collected from June 2017 to August 2018. Colorectal cancer tissue samples were obtained at the site of surgery and stored directly in liquid nitrogen. RNA is usually isolated within a week and then reverse transcribed to cDNA for further research. Blood samples were collected from patients with 2 mL aseptic and RNase-free tubes and treated with Trizol reagent within half an hour to isolate the RNA. Detailed clinical, pathological, and molecular characterization of these patients were collected accordingly.

RNA isolation
Total RNA was isolated from patient tissue and blood samples using the Trizol reagent (Invitrogen) and purified with mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA) according to the protocol. The purity and concentration of RNA were determined by using spectrophotometer NanoDrop2000 (Thermo Fisher Scientific), the OD260/280 ratio around 1.9 to 2.0. RNA integrity was determined by 1% formaldehyde denatured gel electrophoresis.

Microarray analysis
Human CircRNA Array v2 microarray (Beijing Capital Bio Biotechnology Corporation, China) has been used for circRNA microarray profiling. The circRNA array data was analyzed by GeneSpring 13.0 (Agilent) software. Four CRC tissues and adjacent normal tissues were used for microarray analysis. The RNA integrity was determined by Bioanalyzer 2100 (Agilent). RNA digestion, amplification, and labeling were performed according to protocol. The labeled RNAs were hybridized on the microarray (Human circRNA array, version 2.0. Beijing Capital Bio Technology) containing 162,351 human circRNA probes. Differentially expressed circRNAs were detected by the filter criteria fold-change ≥2, P-value<0.05, Fluorescence value≥100.

Reverse transcription-quantitative PCR (RT-qPCR)
The cDNA was synthesized by the Superscript Reverse Transcription System (Invitrogen). RT-qPCR was performed using TB Green qPCR Mastermix (TaKaRa, Japan) on a LightCycler® 480 real-time PCR Platform (Roche). The qRT-PCR reaction was in a total volume of 20 μL systems, including 0.8 μL/10 μM forward/reverse primers, 10 μL TB Green qPCR Mastermix, 2 μL cDNA, and 6.4 μL double-distilled water. The cycling program is 95°C for 30 seconds, followed by 40 cycles of 95°C for 8 secondss and a pre-selected annealing temperature for 30 seconds. GAPDH expression was used as control in qPCR. The primers (Table 1) were synthesized by Sangon biotech (Shanghai) Co. Ltd. The relative expression of genes was calculated using the ΔΔCT method.

Bioinformatics and data analysis
The circRNA array data were analyzed for data summarization, normalization, and quality control by using the GeneSpring software V13.0 (Agilent). To select the differentially expressed genes, we used threshold values of ≥2 and ≤−2 fold change and a Benjamini-Hochberg corrected P-value<0.05. The data was Log2 transformed and median
centered by genes using the Adjust Data function of CLUSTER 3.0 software. Scatterplot and Volcano Plot were analyzed by using ggPlot2 software (R). CircRNA structure was performed by circPrimer1.2. The circRNA-miRNA network was constructed based on the miRanda-3.3 (http://www.microrna.org/microrna/home.do). The miRNA-binding sites of circRNAs were predicted by the miRanda and Circ-Interactome (https://circinteractome.nia.nih.gov/index.html) bioinformatics databases. All differentially expressed circRNAs were annotated its function in terms of the diseases, pathways, and functional. Enrichment analysis of these mRNAs were performed using the KEGG orthology-based annotation system (KOBAS) 3.0. KEGG analysis was performed to determine the involvement of target genes in different biological pathways.

### Results

#### Profiling of circRNAs from the CRC patients

To find the specific circRNA in colorectal cancer, four CRC tissues samples (CA1–CA4) and adjacent normal samples (AP1–AP4) were selected for investigating the expression of circRNAs in CRC by microarray profiles (Figure 1). Different expression circRNAs between the two groups is displayed in the cluster analysis (Figure 1A). According to the different fluorescence signal values, scatter plots revealed that the differentially expressed circRNAs could separate CRC samples from adjacent normal tissues (Figure 1B). The Volcano plot described the variation in circRNA expression between CRC and adjacent normal tissues (Figure 1C). The figure revealed that the differentially expressed circRNAs could separate CRC samples from adjacent normal samples commendably. As is illustrated in Figure 1, 13,198 differentially expressed circRNAs were identified. Based on the screening criteria as a fold-change ≥2 or ≤-2 and a P-value<0.05, 6,697 were up-regulated and 6,501 down-regulated.

To further verify the candidate circRNA, we use fold-change ≥5 or ≤-5, P-value<0.01 and the Processed Signal ≥100 as classification criteria. The twenty candidate

| circRNA        | Primers type | 5’-3’          |
|----------------|--------------|----------------|
| hsa_circ_0072568 | Forward      | GAACGTGACGAAACAATTTTGGCTG |
|                | Reverse      | GTGCCATTGTCACATCAAAAC |
| hsa_circ_0072566 | Forward      | AGATCACCACATGGCAACCA |
|                | Reverse      | TGGGCAAGACCTGTATGGG |
| hsa_circ_0072567 | Forward      | GCCACCATACAAGACACGGA |
|                | Reverse      | TGGGCAAGACCTGGGCAAT |
| hsa_circ_0074033 | Forward      | CAGCACAACAGACCTCAGGA |
|                | Reverse      | CAAGGAGCCTGAAACAGCT |
| hsa_circ_0074039 | Forward      | CAGCACAACAGACCTCAGGA |
|                | Reverse      | AGTGGTAGGGCTAGTGCA |
| hsa_circ_0004585 | Forward      | CAAGACTGCAAAGGACACACT |
|                | Reverse      | AGAGTGAGCCAGCTGATGG |

**Abbreviation:** RT-PCR, real-time polymerase chain reaction.

**Table 1 Primers for RT-PCR**
circRNAs selected included 11 up-regulation and nine-down-regulation. (Figure 2).

Enrichment analysis
In this study, the related mRNAs (circRNA parental gene) of the differentially expressed circRNAs were annotated by KOBAS 3.0. They were enriched in some disease terms that included 433 in KEGG diseases, 795 in OMIM, and 398 in FunDO. The top 10 disease terms are shown in Figures 3A–C. The KEGG diseases contained digestive system, gastric cancer, as well as laryngeal cancer. The FunDO contained breast cancer. The OMIM contained colorectal cancer, leukemia, and prostate cancer. Based on the enrichment analysis, the differentially expressed circRNAs are involved in different tumorigenesis and development, and have in-depth research values.

Validation differential expression circRNAs
To verify the selected 20 candidate circRNAs, 30 CRC samples and adjacent normal tissues were applied in an independent cohort. Quantitative real-time reverse transcription PCR (qRT-PCR) revealed that hsa_circ_0004585, hsa_circ_0074033, and hsa_circ_0074039 were high expression in the CRC tissue, whereas hsa_circ_0072566, hsa_circ_72567, and hsa_circ_72568 were downregulated (Figure 4). Further validation of the expression of these six candidate circRNAs, peripheral blood samples from 142 patients with CRC, and 142 healthy person were collected.
differently. Based on the results, the expression of hsa_circ_0004585 in CRC peripheral samples were studied, the expression of hsa_circ_0004585 was up-regulated in 121 cases and down-regulated in 21 cases (Figure 5A).

We further added 20 cases (total 50 cases) of CRC and adjacent normal samples to verify hsa_circ_0004585 expression. The results showed that 46 cases in all were up-regulated and four cases were down-regulated, the positive rate was 92% (Figures 5B and C). The characteristics of the 50 CRC patients are presented in Table 2. The result proved hsa_circ_0004585 expression has good consistency in the CRC tissue and peripheral samples.
ROC curve analysis

To determine the diagnostic values of circRNAs for cancer patients, Receiver Operating Characteristic (ROC) curve analysis was performed in colorectal cancer patients. The results of ROC curve analysis of six circRNAs are shown in Table 3. The results showed that hsa_circ_0004585 has higher diagnostic accuracy in 50 CRC tissues sample with an Area Under the Curve (AUC) of 0.731, \( P=0.000 \). The sensitivity and specificity are 0.851 and 0.511. In 142 peripheral samples, the AUC was 0.707, \( P=0.000 \). The sensitivity and specificity are 0.908 and 0.408 (Figure 6). The results proved that hsa_circ_0004585 have higher AUC and lower \( P \)-values in CRC.

miRNA response element (MRE) bioinformation analysis

CircRNA can act as a “sponge” for miRNAs through their binding sites and modulate the activity of miRNA. In this study, we predicted the hsa_circ_0004585 binding miRNA by the circMIR software (from miRanda and RNAhybrid database). The results showed that hsa_circ_0004585 can bind with many miRNAs, some of the miRNA have multiple binding sites with hsa_circ_0004585, and some of them only have one binding site. According to the number of binding sites, the top 20 miRNA were shown in Table 4. The specific binding site location and information about the top 20 miRNAs with hsa_circ_0004585 is shown in Figure 7.

Discussion

CircRNA, with a stable loop structure, is identified as a new type of non-coding RNA (ncRNA). It can act as competing endogenous RNAs (ceRNA) or as a miRNA “sponge” to regulate the function of mRNA expression, alternative splicing, or protein transcription.\(^{11,12}\) Previous research has proven that circRNA play an
important role in oncogenesis and influences the cancer cells proliferation, migration, and apoptosis.\textsuperscript{13–15}

Because of the stable characteristic of loop structure, many circRNAs being reported have a function in various cancers. For example, circRNA\_100876 is significantly upregulated in non-small cell lung cancer (NSCLC) tissues. The survival time was significantly shorter in NSCLC patients with high circRNA\_100876 expression than those patients with low circRNA\_100876 expression.\textsuperscript{16} Xie et al\textsuperscript{17} found that hsa\_circ\_0074362 had low expression in gastric cancer tissues and gastric cancer cell lines. The expression levels of hsa\_circ\_0074362 were associated with gastric cancer lymphatic metastasis. Zhang et al.\textsuperscript{18} proved that hsa\_circ\_0001649 was down-regulated in hepatocellular carcinoma (HCC) tissues compared with adjacent normal tissues. They also demonstrated that over-expressed hsa\_circ\_0001649 inhibits the proliferation, migration, and invasion, and promotes the apoptosis of HCC cells. Gao et al.\textsuperscript{19} found circ\_0006528 was highly expressed in breast cancer, the expression of circ\_0006528 tightly related to chemotherapeutic resistance in breast cancer, and circ\_0006528 could be a good biomarker for breast cancer prognostic.

Previous research reported that circRNAs have the function of promoting CRC cells growth and metastasis. CircRNA-ACAP2 and Tiam1 were shown to be highly expressed in colon cancer tissues and colon cancer SW480 cells. CircRNA-ACAP2 can act as a “sponge” to miR-21-5p and affect the proliferation, migration, and invasion of SW480 cells by regulating Tiam1 expression.\textsuperscript{20} CircHIPK3 was significantly upregulated in CRC tissues and cell lines, and promoted CRC growth and metastasis. Knockdown of circHIPK3 could inhibit CRC cells proliferation, migration, invasion, and induced apoptosis in vitro and suppressed CRC growth and metastasis in vivo.\textsuperscript{21} Hsa\_circ\_0020397 can enhance CRC cell viability, apoptosis, and invasion by promoting the expression

\begin{figure}[h]
\centering
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\caption{The expression level of hsa\_circ\_0004585. (A) The expression of hsa\_circ\_0004585 in 142 CRC patients’ peripheral samples compared with the normal healthy person group. (B and C) hsa\_circ\_0004585 was significantly upregulated in 92\% (46/50) of 50 colorectal cancer patient tissues and the adjacent normal tissues. \textit{Abbreviation: CT, cycle threshold.}}
\end{figure}
of TERT and PD-L1, which was the target of the miR-138s. CircCCDC66 can act as the "sponge" to promote CRC growth and metastasis.

Those studies have revealed new insights into the pathogenicity of CRC and provide circRNA as a novel therapeutic target for the treatment of CRC.

In this study, we first screened the circRNA expression profile in four paired CRC and adjacent normal tissues following a microarray screening, 13,198 up-regulated and down-regulated circRNAs being identified. Then, 20 significantly differential expressed circRNAs, including 11 up-regulation and nine down-regulation, have been selected as candidate molecules. After verifying by RT-PCR in an independent cohort in CRC tissue, adjacent normal tissues, peripheral samples, and healthy person's peripheral samples, hsa_circ_0004585 proved to have a good consistency in the CRC tissue and peripheral samples.

Bioinformatics analysis found that the full-length of hsa_circ_0004585 was 2,019 bp, it was encoded by the KIAA1199 gene. Hsa_circ_0004585 was a novel circRNA derived from exons 2–14 of the KIAA1199 gene, located between 81,166,204 to 81,212,640 of human chromosome 15. Previous research has shown that the KIAA1199 gene was a high expression in CRC tissues, proving that KIAA1199 was an oncogene in CRC. Another researcher reported that the KIAA1199 protein was remarkably increased in CRC tissues and cells, which indicated the KIAA1199 protein involved in the CRC metastasis and reduced the patients' survival.

Although there is research which has proved that circRNA plays an important role in oncogenesis and influences the proliferation, migration, and apoptosis in different cancer, the function of most circRNAs is still unclear. CircRNA can act as a “sponge” for miRNAs through their binding sites and modulate the activity of miRNA. Different circRNAs have different miRNA binding sites, one circRNA could have several miRNA binding sites, one miRNA can bind several circRNA.

In this study, we have predicted the hsa_circ_0004585 binding miRNA through the circMIR software (from miRanda and RNAhybrid database). According to the number of binding sites, the top 20 miRNA have been selected for in-depth analysis.

| Characteristics | No of cases | Mean±SD | P-value |
|-----------------|-------------|---------|---------|
| Age (years)     |             |         |         |
| ≤60             | 19          | 5.26±3.520 | 0.520  |
| >60             | 31          | 6.08±4.782 |         |
| Gender          |             |         |         |
| Male            | 20          | 5.28±4.172 | 0.516  |
| Female          | 30          | 6.14±4.65 |         |
| Tumor size      |             |         |         |
| ≤5cm            | 33          | 6.55±4.725 | 0.044* |
| >5cm            | 17          | 4.06±2.823 |         |
| Clinical stage  |             |         |         |
| I & II          | 20          | 6.25±3.615 | 0.527  |
| III & IV        | 30          | 5.45±4.774 |         |
| CA19-9−        |             |         |         |
| −               | 41          | 5.41±3.996 | 0.208  |
| +               | 9           | 7.41±5.570 |         |
| CEA−           |             |         |         |
| −               | 30          | 5.75±4.453 | 0.970  |
| +               | 20          | 5.80±4.241 |         |

Notes: *P<0.05. Abbreviations: CA19-9, carbohydrate antigen 19-9; CEA, carcino-embryonic antigen.

Table 3 Validation of the selected circRNAs by qPCR and ROC analysis

| CircRNA          | Expression* | AUC       | P-value | Sensitivity | Specificity |
|------------------|-------------|-----------|---------|-------------|-------------|
| hsa_circ_0004585 | 8.47±1.34   | 0.731     | 0.000***| 0.851       | 0.511       |
| hsa_circ_0002568 | 10.37±2.75  | 0.665     | 0.021*  | 0.792       | 0.523       |
| hsa_circ_0002566 | 10.62±2.17  | 0.717     | 0.000***| 0.697       | 0.509       |
| hsa_circ_0002567 | 10.84±1.76  | 0.726     | 0.000***| 0.875       | 0.629       |
| hsa_circ_0074033 | 3.31±2.12   | 0.721     | 0.002** | 0.785       | 0.607       |
| hsa_circ_0074039 | 2.56±1.84   | 0.728     | 0.001***| 0.846       | 0.667       |

Notes: *The expression of each circRNA was calculated from the ΔCt and expressed as mean±standard deviation; *P<0.05; **P<0.01. Abbreviations: AUC, area under the curve; CRC, colorectal cancer; qPCR, quantitative polymerase chain reaction; ROC, receiver operating characteristic.
also used the enrichment to analyze the mRNAs of all differentially expressed circRNAs. We found that there were many diseases related to it.

Based on our results, hsa_circ_0004585 was upregulated both in the CRC tissues and CRC peripheral samples compared with adjacent normal tissues and healthy person’s peripheral blood. ROC curve analysis demonstrated that hsa_circ_0004585 had high diagnostic accuracy in CRC. Our study proved that hsa_circ_0004585 has the potential to be a novel diagnostic marker and therapeutic target for CRC. However, the mechanism of hsa_circ_0004585 in CRC tumorigenesis and metastasis is still unclear, and further research is needed for validation.

Table 4 More binding sites of miRNA with hsa_circ_0004585 top 20

| miRNA ID      | miRanda binding site (position) | Target scan binding site (positions) | Binding numbers |
|---------------|--------------------------------|--------------------------------------|-----------------|
| hsa-miR-299-3p| 1720                           | 1555 1594 1736 241 99 1560 1599 1743 246 104 | 11              |
| hsa-miR-431-5p| 183                            | 170 198 2008 511 977 175 205 2014 516 982 | 11              |
| hsa-miR-4657  | 222 89                         | 1555 1594 1737 240 99 1560 1599 1743 246 104 | 11              |
| hsa-miR-4691-5p| 1276                          | 1293 1453 1527 1873 326 1299 1458 1533 1879 331 | 11              |
| hsa-miR-15b-5p| 884                            | 113 207 23 898 118 213 28 904           | 9               |
| hsa-miR-3912-5p| 869                           | 1470 477 497 882 1475 483 502 888     | 9               |
| hsa-miR-424-5p| 192                            | 113 207 23 898 118 213 28 904           | 9               |
| hsa-miR-497-5p| 884                            | 113 207 23 898 118 213 28 904           | 9               |
| hsa-miR-6736-3p| 548 7                         | 1642 20 332 561 1649 26 337 567       | 10              |
| hsa-miR-6778-3p| 15                            | 253 29 458 542 260 36 463 547         | 9               |
| hsa-miR-6801-5p| 64                            | 126 59 626 79 132 65 632 85           | 9               |
| hsa-miR-6838-5p| 193 884                       | 113 207 23 898 118 213 28 904         | 10              |
| hsa-miR-1262  | 1048 500                       | 1063 1734 520 1069 1739 526           | 8               |
| hsa-miR-23a-5p| 140                            | 153 1922 300 159 1928 305             | 7               |
| hsa-miR-23b-5p| 1908 139                      | 153 1922 300 159 1928 305             | 7               |
| hsa-miR-4701-3p| 508                           | 1063 1734 520 1069 1739 526           | 7               |
| hsa-miR-4709-3p| 29                            | 1854 44 854 1859 51 859              | 7               |
| hsa-miR-4764-5p| 229                           | 100 1992 308 107 1997 314            | 7               |
| hsa-miR-4769-3p| 519                           | 1422 533 699 1427 540 705           | 7               |
| hsa-miR-5695  | 806                            | 1291 1630 819 1297 1636 825           | 7               |

Figure 6 (A) ROC analysis for the expression hsa_circ_0004585 in 50 paired tissue of CRC patients. (B) ROC curves of the CRC person serum for the hsa_circ_0004585 expression.
Abbreviations: AUC, area under curve; ROC, receiver-operating characteristic.
Figure 7 The hsa_circ_0004585 structure and miRNA binding sites, including origin gene KIAA1199, exon2–exon14 composed this circRNA, this gene from Chromosome 15, location between 81,166,204 and 81,212,640, the molecule is 2019 bp and binding miRNA top 20 (Table 3).
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