Hsa_circ_0001649: A circular RNA and potential novel biomarker for colorectal cancer

Wen-Xin Ji, Chun-Li Qiu, Mao Wang, Ning Mao, Shao-Feng Wu, Yin-Hai Dai

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Dear Editor,

We would like to submit the enclosed manuscript entitled "Hsa_circ_0001649: A circular RNA and Potential Novel Biomarker for Colorectal Cancer", which we earnestly wish to be considered for publication in Biochemical and Biophysical Research Communications.

The circRNAs are differentially expressed in a wide range of cancers in regulating their initiation and progression, and could be used to make a diagnosis for some diseases like tumour as a new biomarker. However, the correlation and the mechanism of action between circRNAs and colorectal cancer (CRC) are still unclear. This study showed that the expression level of hsa_circ_0001649 was down-regulated in CRC tissue compared to nontumor tissue, as well as in cell lines and patient serum, showing negative correlation with CRC pathological differentiation. Therefore, we could use it as a new biomarker for specific and sensitive inspection of CRC.

The authors claim that none of the material in the paper has been published or is under consideration for publication elsewhere. Thank you very much for consideration!

With thanks for your consideration, I am the corresponding author and my address and other information is as follows:

Dr. Yin-Hai Dai
Department of Surgical Oncology, The Second Affiliated Hospital of Shaanxi University of traditional Chinese Medicine, Xi'an 712000, PR China
Tel: +86-029-33573572 Fax: +86-29-33573572 E-mail: jx2686@163.com

Thank you very much for consideration!

Sincerely Yours,

Dr. Yin-Hai Dai
Highlights

1. Hsa_circ_0001649 is studied in Colorectal Cancer for the first time and could be enlarged by qRT-PCR.
2. This study showed that the expression level of hsa_circ_0001649 was down-regulated in CRC.
3. Hsa_circ_0001649 has a negative correlation with CRC pathological differentiation.
4. The expression level of hsa_circ_0001649 could be used as a new biomarker for specific and sensitive inspection of CRC.

Hsa_circ_0001649: A Circular RNA and Potential Novel Biomarker for Colorectal Cancer

Wen-Xin Ji¹, Chun-Li Qiu²,a, Mao Wang¹,a, Ning Mao¹,b, Shao-Feng Wu¹,b, Yin-Hai Dai¹,*

1. Department of Surgical Oncology, The Second Affiliated Hospital of Shaanxi University of Traditional Chinese Medicine, Xi'an 710032, PR China
2. Xianyang Central Hospital, Xianyang 712000, PR China

*Corresponding author:
Dr. Yin-Hai Dai
Tel: +86-029-33573572
Fax: +86-29-33573572
E-mail: jx2686@163.com

¹,a,b These authors contributed equally to this work.

Key words: Circular RNA, hsa_circ_0001649, colorectal cancer, biomarker, diagnostic value

Running title: A circRNA Biomarker for Colorectal Cancer
Abstract.

The circRNAs are differentially expressed in a wide range of cancers in regulating their initiation and progression, and could be used to make a diagnosis for some diseases like tumour as a new biomarker. However, the correlation and the mechanism of action between circRNAs and colorectal cancer (CRC) are still unclear. In this study, by using qRT-PCRs, we detected the expression level of hsa_circ_0001649 in tissue and serum samples from CRC patients, and the cultured cell has been detected. We found that the hsa_circ_0001649 in CRC is significantly lower than the expression level of correspondent nontumorous tissues ($n=64$, $P<0.01$). We also tested the HCT116 cell lines, and the similar result is observed ($n=15$, $P<0.01$). Moreover, we detected the serum samples obtained before and after surgery, showing significantly the expression level of hsa_circ_0001649 in the same patient is up-regulated after surgery ($n=18$, $P<0.01$). Besides, we analyzed the correlation between clinicopathological date and the expression level of hsa_circ_0001649, we found that hsa_circ_0001649 expression level is closely associated with pathological differentiation ($P=0.037$), and the result also illustrated that the expression level of hsa_circ_0001649 is no direct correlation with age, gender, TMN stage, lymphatic metastasis, CEA, CA19-9, and CA-724 levels. The area under the receiver operating characteristic (ROC) curve was 0.857. In conclusion, this study showed that the expression level of hsa_circ_0001649 was down-regulated in CRC and could use it as a new biomarker for specific and sensitive inspection of CRC.

Introduction

Recently, circular RNAs (circRNA) are a newly accepted class of RNAs that differentially expressed in a wide range of cancers and could be used as a new biomarker for diagnosis[1, 2]. Studies showed that circRNAs play a very important role in much diseases such as colorectal and ovarian cancer, idiopathic lung fibrosis, as well as in normal human tissues[3]. According to the previous reports, circRNAs have been testified as an abundant, stable, diverse and conserved class of RNA molecules, which means circRNAs can play an very important role in the RNA
interaction network [4-6]. Besides, tons of articles have proved that circRNAs have significant correlation with lots of cancers and disease regulating its initiation and progression [7-10]. And it remains unclear whether circRNAs could serve as a new biomarker for CRC diagnosis, prognosis, and therapeutic response prediction.

Colorectal cancer is the third most commonly cancer in the world[11]. The incidence of colorectal cancer has an uptrend in recent years, although there are lots of colorectal cancer researchers, it is still a serious disease that leads to a great many death. Besides, the existing bad phenomenon is that many patients did not get accurate prognosis when they were in a terminal cancer. However, it is still unclear about the correlation between the circRNAs and colorectal (CRC) cancer. Some previous articles showed that some circRNAs have a strong association with CRC such as hsa_circ_0020397 and hsa_circ_0000069 [12-15]. Hence, it still remains unclear about the mechanism of action between circRNAs and colorectal cancers. Due to above studies, some classes of circRNA may serve as a new biomarker and could be used for cancer prognosis, treatment and diagnosis because of some specific features of circRNAs [1, 9, 16-18].

In our previous studies, the expression level of hsa_circ_0001649 is obviously down-regulated in the gastric cancer tissue compared to the normal tissue[13]. The circ RNA hsa_circ_0001649 is a transcription product of Snf2 Histone Linker Phd Ring Helicase (SHPRH) gene, which acts as a tumor suppressor gene and negatively regulates the occurrence and development of tumor by Wnt (wingless) / β-catenin signaling pathway[19]. Hsa_circ_0001649 has been proved to associate with some cancers such as prostate cancer, ovarian cancer, and liver cancer[19]. In summary, according to the previous studies, as the gastric cancer and colorectal cancer are all high incidence of adenocarcinoma cancer, we designed this experiment for investigating the expression level of hsa_circ_0001649 in colorectal cancer patients[13]. Whether the expression level of hsa_circ_0001649 is different in colorectal cancer has been detected, also in the cultured cells and serum samples [20]. And we also analyzed the expression level of hsa_circ_0001649 in different
clinicopathological typing in order to assess the diagnostic value to be a marker for
the early detection of CRC.

Materials and methods

Patients and clinical samples. The present study was approved by the ethics
committee of the University and adhered to the tenets of the Declaration of Helsinki.
Additionally, the written informed consent was obtained from the relatives of the
patients. This experiment was completed by the laboratory of the Second Affiliated
Hospital of Shaanxi University of Chinese Medicine. We recruited the volunteers,
colorectal cancer patients, in recent few months. In order to reduce bias, we chose a
person who didn’t participate in the following experiment to collect and preserve the
64 CRC samples. In this study, the materials were constituted by 64 tumor tissue
samples and their paired paracancerous histological normal tissues (PCHNTS), which
were collected during the surgery. At the same time, we collected some samples of
peripheral blood from 18 colorectal patients. Some of those 18 samples were collected
before their surgery and the rest samples were collected after the surgery (more than
21 days after surgery). All the samples were coded randomly and immediately frozen
and stored at -80°C when they were extracted to decrease errors. In this experiment,
all the patients didn’t have any treatment before, and volunteered to sign informed
consent. This study was completed independently at the clinical laboratory in the
Second Affiliated Hospital of Shanxi University of Chinese Medicine.

Cell Culture. We used human colorectal cancer cell H116 in this study. The cell lines
were bought from the Type Culture Collection of the Chinese Academy of Sciences
(Shanghai, China). We cultured the cells in RPMI-1640 medium(Gibco) sweetened
with 10% fetal bovine serum (Hyclone) at 37 °C in a humidified atmosphere
containing 5% CO₂.
Total RNA Extraction. We extracted RNA from the tissue and cell by the way of Trizol reagent (Ambion, life technologies) according to the manufacture’s instructions. Then, we measured the RNA using the ways of ultraviolet spectrophotography.

Reverse Transcription. We obtained the cDNA by reversing transcription (RT) using a Primescript RT reagent kit with random primers according to manufacturer-provided protocols (TakaRa).

Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction(qRT-PCR). We use SYBR Premix EX Taq™ II (Tli RNaseH Plus) (TaKaRa) on CFX96 Real-Time PCR Detection System (Bio-Rad, California, USA) in qRT-PCR according to the manufacture’s instructions. Then, we designed the divergent primers synthesized by Sangon Biotech (Shanghai, China). The GAPDH was used as an internal control. The primers for qRT-PCR were designed in Table 1. We designed the reactions in triplicate in order to reduce error.

CEA, CA199, and CA-724 Measurements. The normal people’s levels of CEA, CA-199, CA-724 were severally defined as <3.4 ng/ml, <39 ng/ml, and <9.8 U/ml. This study was completed independently at the clinical laboratory in the Second Affiliated Hospital of Shanxi University of Chinese Medicine.

Statistical Analysis. We analyzed the statistic by the way of using the SPSS 13.0 software (SPSS, Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad software, La Jolla, CA). We analyzed statistic from qRT-PCR by using the $2^{-\Delta\Delta Ct}$. In order to find the correlation of hsa_circ_0001649 expression level between the CRC and their paired colorectal nontumorous tissues or serum samples, we calculated the date by using paired $t$-test. Then, we used one-way analysis of variance to analyze the correlation between the expression level of hsa_circ_0001649 and clinicopathological factors (ANOVA). Receiver operation characteristic (ROC) curve of SPSS 13.0 was used to estimate the diagnostic values. $P$ values<0.05 (two-side) were considered
Results

Patient Characteristic. In order to investigate the expression level of hsa_circ_0001649 between CRC and normal tissue, we collected the 64 paired CRC tissue samples and their paired PCHNTs (including 53 males and 11 females) to complete this test. The mean age of colorectal cancer was 60.4±8.3.

Existence of The hsa_circ_0001649 in Colorectal Cancer Cells. The hsa_circ_0001649 can be amplified by using the divergent primers from HCT116 cell line. The amplified product showed as a single peak in melting curve analysis. We sequenced the qRT-PCR products and compared them with the sequence of hsa_circ_0001649 from CircBase. Its result showed that they are same. The result proved that the hsa_circ_0001649 existed in colorectal cancer and could be enlarged by qRT-PCR.

Hsa_circ_0001649 Expression Was Down-regulated in Colorectal Cancer Tissues. We collected 64 CRC tissue samples and their paired PCHNTs samples using GAPDH as the internal control by qRT-PCR in order to examined the expression level of hsa_circ_0001649 in CRC. Then, the result showed that hsa_circ_0001649 expression in colorectal cancer tissue was obviously lower compared to those which are correspondent nontumorous tissues (n=64, P<0.01) (Fig.2.A).

Hsa_circ_0001649 Expression Was Upregulated in Colorectal Cancer Serum Samples after Surgery and Downregulated in HCT116 cell lines. For the further research, we also tested the hsa_circ_0001649 expression level in the HCT116 cell lines, and the similar result observed (n=15, P<0.01) (Fig.2.B). Moreover, we detected the hsa_circ_0001649 expression level in serum samples obtained before and after surgery, the significantly up-regulated expression after surgery was observed (n =18, P < 0.01) (Fig.2.C).
Potential Diagnostic Values of Hsa_circ_0001649 in colorectal cancer. Our test showed the differential expression level of hsa_circ_0001649 in colorectal cancer tissue with the PCHNTs. Besides, we analyzed the correlation between clinicopathological data and the expression level of hsa_circ_0001649, the result was showed in Table 2. Based on these results, we found out that hsa_circ_0001649 expression level is associated with pathological differentiation ($P=0.037$), and the result also illustrated that there is no correlation with age, gender, TMN stage, lymphatic metastasis, CEA, CA19-9, and CA-724 levels. The sensitivity and specificity were 0.828 and 0.781, respectively. The cutoff value was 0.2784690288 and the area under the curve was 0.857 (Fig. 3).

Discussion

In this experiment, we tested the expression level of hsa_circ_0001649 in tissues from colorectal cancer by using qRT-PCR. The results showed that the expression level of hsa_circ_0001649 was obviously down-regulated in CRC tissue compared with paired PCHNTs, as well as in the HCT116 cell lines and patient serum. What’s more, we also found that there existed a significantly correlation between the expression level of hsa_circ_0001649 and pathological differentiation showing clear diagnostic value in CRC patients.

Circular RNA—an up-rising star in ceRNAs field—is a class of RNA with still unknown impact on the cellular regulatory RNA network. Its specific and powerful functions are being increasingly acknowledged by scientists such as miRNA sponges, as snoRNAs (smallnucleolar RNAs), as intermediates in RNA processing reactions, as regulators of transcription in cis, as templates for viroid and viral replication, as RNA transport and so on[14, 21]. There are about 2000 different circRNAs species reported in human and mouse tissues and 700 species in Caenorhabditis elegans[22]. CircRNAs existing in eukaryotic cells was found by deep sequencing technologies, many studies have convinced that circRNAs play a very important role in many functions of eukaryotic cells such as competing with linear RNAs in the splicing, and
serving as microRNA (miRNA) sponges to regulate mRNA[21, 23]. Circular RNAs (circRNAs) are looked as non-coding RNA (ncRNA) that, unlike linear RNAs[24], was constructed by covalently closed continuous loops, formed by a ‘back-splicing’ event where in a covalent bond is formed between 5’ (splice donor) and 3’ (splice acceptor) splice-sites of a pre-mRNA. Without 5’ and 3’ termini, so circRNAs do not show the characteristic of mRNA processing that makes them highly resistant to degradation by exonucleases.

Much of previous studies have showed that there is a close correlation between circRNAs and human[7]. Bachmayr-Heyda et al showed the close correlation of abundant proliferation of circular RNA with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues[3]. CiRS-7 and cir-ITCH are two well-known circRNAs; CiRS-7 acts as a miRNA sponges of miR-7 and inhibited its activity[25, 26]. Besides, the other well-known one, circRNA cir-ITCH, has an negative effect on esophageal squamous cancer, then working as a sponge of miR-7, miR-17, and miR-214, cirITCH could up-regulate the level of ITCH, then controls the Wnt/β-catenin pathway[8, 21]. Which the expression of circRNA hsa_circ_0000069 in CRC cancer and cell lines is increasing has been proved [12]. Besides, the expression level of hsa_circ_7780 was also down-regulated in tumour samples compared to normal samples[3]. According to the previous studies, the different expression level of circRNA in tumors compared with normal tissues could make circRNAs become a biomarker for tumor diagnosis and prognosis in the future.

Finally, we convinced that the expression level of hsa_circ_0001649 is overtly down-regulated in colorectal cancer tissues compared to the PCHNTs. What’s more, based on circRNA expression level and clinicopathological date we found that the expression level of hsa_circ_0001649 was more clearly decreased in poor and undifferentiated tumors compared to well differentiated ones. Therefore, these results showed that the expression level of hsa_circ_0001649 may have negative correlation with colorectal cancer pathological differentiation. Then we detected the diagnostic value of has_circ_0001649 in CRC. Meanwhile, we obtained the comparatively satisfactory result by using ROC curve analysis (the sensitivity and specificity were
0.828 and 0.781, respectively; the area under the curve was 0.857). Hence, based on our result, the expression level of hsa_circ_0001649 was down-regulated in colorectal cancer samples than in normal samples. Thus, the hsa_circ_0001649 may become a new biomarker for colorectal cancer in the future because of its sensitivity, accuracy, and specificity.

Although the expression level of hsa_circ_0001649 is down-regulated in tumor tissue than in their paired PCHNTs samples, as well as in the HCT116 cell lines and serum before surgery, there is still something unknown about how the circRNA regulates initiation and progression of cancer. This study showed the different expression level of hsa_circ_0001649 in CRC patients, as well as in the cell lines, further experiments are still needed to study the influence of hsa_circ_0001649 on the initiation and progression of colorectal cancer.

Conclusion, this study showed that the expression level of hsa_circ_0001649 was down-regulated in CRC tissue compared to nontumor tissue, as well as in cell lines and patient serum, and that the expression level of hsa_circ_0001649 has negative correlation with CRC pathological differentiation. Therefore, we could use it as a new biomarker for specific and sensitive inspection of CRC.

Acknowledgements The present study was supported by the Shaanxi Provincial Department of Education(grant no. 201717JK0209).

Competing interests The authors declare that there is no conflict of interests regarding the publication of this paper.

References
[16] F.R. Kulcheski, A.P. Christoff, R. Margis, Circular RNAs are miRNA sponges and can be used as a new class of biomarker, J Biotechnol, 238 (2016) 42-51.


Table 1. qRT-PCR primer sequences characterized in this experiment.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primers</th>
<th>Sequences</th>
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<td>Has_circ_0001649</td>
<td>Forward primer</td>
<td>5'-AATGCTGAAAACGTGCTGAGAAG-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-TTGAGAAAAACGAGTGGCTTTTG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer</td>
<td>5'-TCGACAGTCAGCGCGATCTCTTTT-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>5'-ACCAATCGGATCGACTCGACCTT-3'</td>
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Figure legends

Figure 1: The sequencing result of has_circ_0001649 in HCT116 showing the sequence as GGCTGCCCTTCTCTCACAGCAGTTTTCAGCATTA, which is identified by the sequence in circbase.
Figure 2: A. The expression levels of hsa_circ_0001649 in CRC tissue samples and their paired PCHNTs samples. The picture illustrated that hsa_circ_0001649 in CRC tissue was clearly lower than in the paired normal tissue (n=64, P<0.01). B. The expression levels of hsa_circ_0001649 in HCT116 cell lines compared to the normal cells (n=15, P<0.01). C. The result showed that the expression levels of hsa_circ_0001649 in serum samples was significantly up-regulated in those serum samples collected postoperatively (n=18, P<0.01).
Table 2. The correlation of the expression level of hsa_cir_0001649 between the clinicopathological date.

<table>
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<th>Clinical parameter</th>
<th>Number of cases</th>
<th>Mean±SD</th>
<th>P value</th>
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<td>≥60</td>
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<td>Differentiation</td>
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<tr>
<td>Well</td>
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<td>P=0.037a</td>
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<tr>
<td>Poor</td>
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<tr>
<td>Depth of tumor invasion</td>
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<tr>
<td>Tis,T1a,T1b</td>
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<td>0.26±0.81</td>
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<td>T4</td>
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<tr>
<td>Lymph node metastasis</td>
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<td>N3a,N3b</td>
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<td>CA-724</td>
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<td>Normal</td>
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<td>0.32±0.09</td>
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<tr>
<td>Unusual</td>
<td>57</td>
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</table>

a Using chi-square for this statistic.
Figure 3: ROC curve of has_circ_0001649 in CRC patients. Area under the curve was 0.857. The sensitivity and specificity were 0.828 and 0.781, respectively.
Highlights

1. Hsa_circ_0001649 is studied in Colorectal Cancer for the first time and could be enlarged by qRT-PCR.
2. This study showed that the expression level of hsa_circ_0001649 was down-regulated in CRC.
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4. The expression level of hsa_circ_0001649 could be used as a new biomarker for specific and sensitive inspection of CRC.