

## Original Article

# Decreased expression of hsa\_circ\_001988 in colorectal cancer and its clinical significances

Xuning Wang<sup>1\*</sup>, Yue Zhang<sup>2\*</sup>, Liang Huang<sup>1\*</sup>, Jiajin Zhang<sup>1\*</sup>, Fei Pan<sup>3\*</sup>, Bing Li<sup>1\*</sup>, Yongfeng Yan<sup>1\*</sup>, Baoqing Jia<sup>1\*</sup>, Hongyi Liu<sup>1\*</sup>, Shiyu Li<sup>2\*</sup>, Wei Zheng<sup>1\*</sup>

<sup>1</sup>Department of Surgical Oncology, Chinese PLA General Hospital, Beijing, China; <sup>2</sup>Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing, China; <sup>3</sup>Department of Gastroenterology and Hepatology, Chinese PLA General Hospital, Beijing, China. \*Equal contributors.

Received October 14, 2015; Accepted November 28, 2015; Epub December 1, 2015; Published December 15, 2015

**Abstract:** Circular RNA (circRNA) is a type of RNAs which, unlike the better known linear RNA, forms a covalently closed continuous loop. They have emerged recently as a new player in governing fundamental biological processes. However it remains elusive about the correlation of hsa\_circ\_001988 abundance with colorectal cancer. To investigate the circular RNA expression in colorectal cancer, the targeted hsa\_circ\_001988 was selected from next generation sequence data base generated in house and then designed divergent primers to amplify hsa\_circ\_001988 and sequenced it for validation. The expression of hsa\_circ\_001988 in 31 matched colorectal cancer tissue and normal colon mucosa was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). We used  $\Delta C_t$  method and investigated the differences between tumor tissues and normal colon mucosa by paired t-test. One-way analysis of variance was conducted to analyze the relationship between hsa\_circ\_001988 expression level and clinic pathological factors of patients. Receiver operating characteristic (ROC) curve was built by SPSS to evaluate the diagnostic values. The expression of hsa\_circ\_001988 was significantly correlated with differentiation ( $P < 0.05$ ) and perineural invasion ( $P < 0.05$ ). The area under ROC curve of hsa\_circ\_001988 was 0.788 ( $P < 0.05$ ). Those results indicate that hsa\_circ\_001988 may become a novel potential biomarker in the diagnosis of colorectal cancer and a potential novel target for the treatment of colorectal cancer.

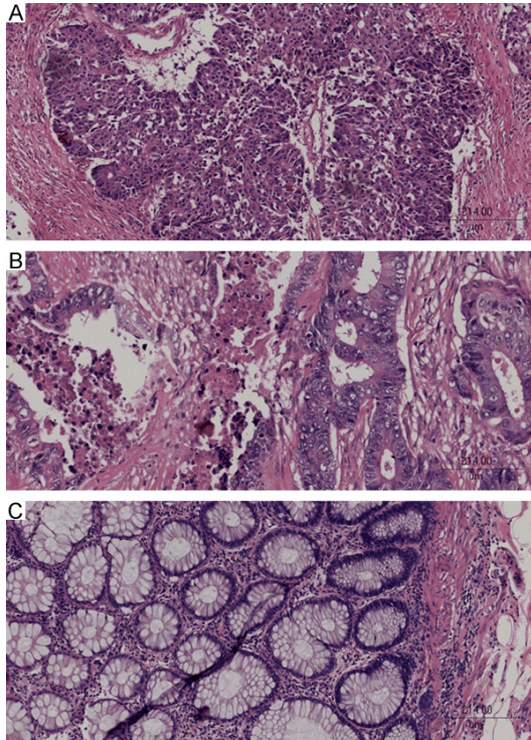
**Keywords:** Circular RNA, hsa\_circ\_001988, colorectal cancer, biomarker, differentiation

## Introduction

Colorectal cancer (CRC) is one of most common cancers worldwide, representing almost 10% of the global cancer incidence, leading a high mortality [1]. Although there was several treatment options to colorectal cancer, CRC patient's survival is still poor because of lacking effective tools for early diagnosis and limited capacity for optimal therapeutic decision making. Thus, it's valuable to find an ideal biomarker and therapy target to improve the diagnosis rare and treatment.

Circular RNAs are novel RNA molecules which formed mainly by back-splicing with covalently joined 3'- and 5'-ends [2]. Although circular RNA have been reported more than 20 years [3], such molecules mostly were considered as by-products of splicing errors [4, 5]. With the devel-

opment of novel bioinformatics' approaches and deep sequencing, lots of circular RNAs were found in eukaryotic cells. Recent studies have mainly found three kinds of circular RNAs: exonic circRNA, circular intronic RNA and retained-intron circRNA [5-7]. Several possible functions have been claimed: regulator of translation [7], miRNA sponges [8], binding protein [9] and RNA transport [10]. Some of circular RNAs could act as microRNA sponge to compete endogenous RNA, affecting expression of related gene. The exotic circRNA of CDR1 as (cerebellar degeneration-related protein 1 anti-sense) have been shown to bind miRNA-7, inducing impaired midbrain development in zebra fish [8]. Furthermore, circular RNAs are related to many diseases [11]. For an example, the abundance of certain circRNA are associated with gastric cancer [12]. However little is known about relationship between the expres-



**Figure 1.** A. Poor differentiation adenocarcinoma, HE,  $\times 100$ . B. Moderate differentiation adenocarcinoma, HE,  $\times 100$ . C. Well differentiation adenocarcinoma, HE,  $\times 100$ .

sion level of hsa\_circ\_001988 and colorectal carcinoma.

We selected hsa\_circ\_001988 as targeted circular RNA to explore relationship between the expression of this circular RNA and colorectal cancer for following reasons. (1) It's based on our own next generation sequence data. (2) Hsa\_circ\_001988 has been predicted by Memczak et al, but they didn't validate the existence of hsa\_circ\_001988 [8]. (3) FBXW7 (F-box and WD repeat domain-containing 7), also called hCDC4, is located on chromosomal region 4q3 which is a tumor suppressor [13]. Depletion of FBXW7 is often observed in a variety of tumors including colon cancer [14]. As a result, we firstly validated the existence of hsa\_circ\_001988 and analyzed association between this circular RNA expression and clinical significances in colorectal cancer patients.

## Materials and methods

### *Patients and clinical information*

Following informed patient consent and ethical approval, total of 62 specimens (tumor tissues

and adjacent normal mucosa) were obtained from CRC patients during operation and were quickly put into RNA later (Ambion, life technologies, USA). Then we put those specimens into  $4^{\circ}\text{C}$  for 12 h and stored at  $-80^{\circ}\text{C}$  until use. Tumors were classified according to the tumor-node-metastasis (TNM) staging system. Histological grade was evaluated following the National Comprehensive Cancer Network clinical practice guideline of oncology (V.1.2012).

Carbohydrate antigen 19-9 (CA19-9) and serum carcinoembryonic antigen (CEA) and were measured using Cobos 8000 (Roche Diagnostics, Basel, Switzerland). Their cutoff values were 35 U/ml and 5 ng/ml respectively.

### *Total RNA extraction and cDNA synthesis*

TRIzol reagent (Ambion, life technologies, USA) was used to extract RNA from the colorectal tissues and normal mucosa, following the manufacturer's instruction. Then, concentration of RNA was estimated by the NanoDrop ND-8000 (Thermo Fisher Scientific, Wilmington, DE). cDNA was synthesized as following method. First, dilute 1-2  $\mu\text{g}$  RNA into 14  $\mu\text{l}$  RNase-free water in a 0.2 mL PCR tube, then add 1  $\mu\text{l}$  random primers and mix well. Second, put the tubes into a PCR thermocycler and incubate at  $70^{\circ}\text{C}$  for 5 minutes. Third, place the plate on ice immediately and incubate for 2 minutes. Forth, prepare the reaction mix as following: 5  $\mu\text{l}$   $5\times$  Buffer, 1.3  $\mu\text{l}$  dNTP (10 mM), 1  $\mu\text{l}$  M-MLV, 0.6  $\mu\text{l}$  RNase inhibitor, 15  $\mu\text{l}$  RNA and random mix, 2.1  $\mu\text{l}$  RNase-free water. Incubate at  $42^{\circ}\text{C}$  for 1 hour,  $70^{\circ}\text{C}$  for 15 minutes and  $4^{\circ}\text{C}$  forever. No template control was conducted at the same time.

### *Quantitative real-time PCR*

Divergent primers were designed with Vector NTI 10. Their sequences were as follows: 5'-CCAGTAGTATTGTGGACCTGCC-3' (sense) and 5'-CCCTCTGACCCAGTAACTCCACT-3' (antisense) for hsa\_circ\_001988; 5'-TCCTC-ACAGTTGCCATGTAGACCC-3' (sense) and 5'-TG-CGGGCTCAATTTATAGAAACCGGG-3' (antisense) for GAPDH. To validate the existence of hsa\_circ\_001988 in colorectal cancer tissues, the product of quantitative real time PCR was sequenced and the sequencing results were aligned with the theoretical sequence. The expression of hsa\_circ\_001988 was analyzed by using  $\Delta\text{C}_t$  method [15]. All the results are

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**Table 1.** The relationship of hsa\_circ\_001988 expression levels ( $\Delta C_t$ ) in cancer tissue with clinical features in colorectal cancer patients

Characteristics	No. Of patients	Mean $\pm$ SD	95% CI	P value
Gender				
Male	19	6.55 $\pm$ 0.94	6.10-7.00	P=0.354
Female	12	6.95 $\pm$ 1.41	6.05-7.84	
Age				
$\leq$ 60	15	6.47 $\pm$ 1.07	5.88-7.06	P=0.280
$>$ 60	16	6.92 $\pm$ 1.19	6.29-7.55	
Diameter				
$>$ 5	8	6.31 $\pm$ 0.80	5.64-6.98	P=0.261
$\leq$ 5	23	6.84 $\pm$ 1.22	6.31-7.37	
Differentiation				
Poor	7	5.59 $\pm$ 0.45	5.17-6.00	P=0.008
Moderate	20	7.04 $\pm$ 1.16	6.49-7.58	
Well	4	6.98 $\pm$ 0.56	6.10-7.87	
Lymphatic metastasis				
Nx&N0	15	6.82 $\pm$ 1.29	6.10-7.53	P=0.601
N1&N2	16	6.60 $\pm$ 1.00	6.06 $\pm$ 7.13	
Invasion				
Tis&T0-T3	24	6.67 $\pm$ 1.12	6.19-7.14	P=0.748
T4	7	6.82 $\pm$ 1.28	5.64-8.01	
TNM stage				
O&I&II	15	6.82 $\pm$ 1.28	6.10-7.53	P=0.601
III&IV	16	6.60 $\pm$ 1.00	6.06-7.13	
PNI				
Negative	28	6.84 $\pm$ 1.11	6.41-7.26	P=0.037
Positive	3	5.42 $\pm$ 0.38	4.46-6.37	
CEA				
Positive	14	6.99 $\pm$ 1.37	6.20-7.78	P=0.202
Negative	17	6.46 $\pm$ 0.87	6.01-6.91	
CA19-9				
Positive	6	6.97 $\pm$ 1.64	5.24-8.70	P=0.527
Negative	25	6.63 $\pm$ 1.01	6.21-7.05	

expressed as the mean  $\pm$  SD of three independent experiments.

### Statistical analysis

Statistical data were analyzed using Statistical Program for Social Sciences (SPSS) 17.0 software (SPSS, Chicago, IL, USA). GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used to plot all graphs. One-way analysis of variance was used to analyze the relationship between hsa\_circ\_001988 expression level and clinic pathological factors in CRC patients. The differences between tumor tissues and normal colon

mucosa were analyzed by paired t-test. Receiver operating characteristic (ROC) curve was constructed to evaluate the diagnostic values.  $P < 0.05$  was considered statistically significant.

### Results

#### General characteristics of the patients

In order to exclude possible influence of cancer types, we just selected patients diagnosed with adenocarcinoma by HE stain. **Figure 1A** shows poorly differentiated adenocarcinoma (HE,  $\times 100$ ), **Figure 1B** shows moderately differentiated adenocarcinoma (HE,  $\times 100$ ), and **Figure 1C** shows well differentiated adenocarcinoma (HE,  $\times 100$ ). The average of 31 patients with colorectal cancer was 59.10 $\pm$ 2.42 years old. The majority of patients with colorectal cancer were man (19, 61.3%). Three patients had perineural invasion (**Table 1**).

#### Existence of hsa\_circ\_001988 in colorectal cancer tissues

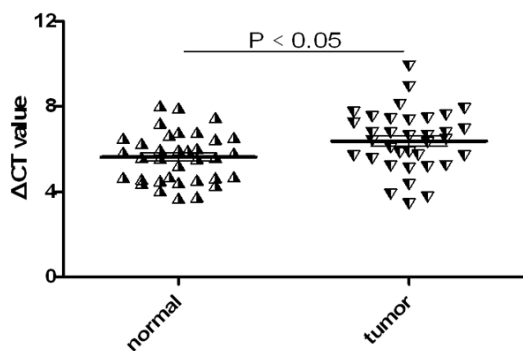
Divergent primers were used to amplify circular RNA. The product of quantitative real

time PCR was sequenced and the sequence of qRT-PCR's product could cross cleavage site. Those results validated the existence of hsa\_circ\_001988 in the tissues.

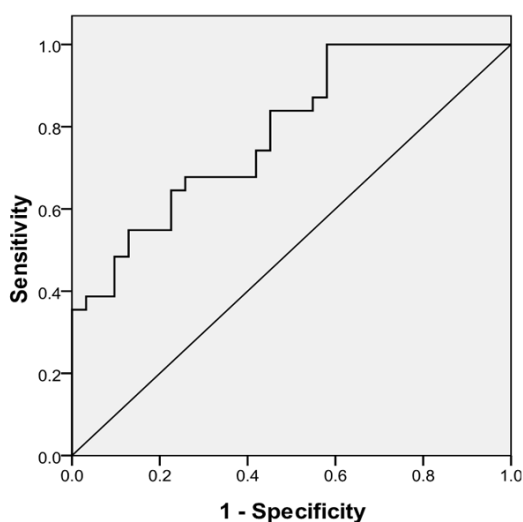
#### Hsa\_circ\_001988 expression and clinicopathologic factors in colorectal cancer patients

Compared with normal samples, the expression of hsa\_circ\_001988 was significantly down regulated (**Figure 2**). To further explore the relationship between hsa\_circ\_001988 and clinical features, we analyzed following clinic pathological factors with colorectal cancer

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**Figure 2.** The expression levels of hsa\_circ\_001988 in colorectal cancer patients. The expression levels of hsa\_circ\_001988 in 31 matched carcinoma and normal samples. Higher  $\Delta C_t$  value indicates lower expression.



**Figure 3.** The ROC curve was constructed by SPSS. Area under curve was 0.788. The sensitivity and specificity was 0.68 and 0.73, respectively.  $P < 0.05$ .

(**Table 1**). The expression level of hsa\_circ\_001988 was related to differentiation ( $P < 0.05$ ) and perineural invasion ( $P < 0.05$ ). Many other clinic pathological features were analyzed, but those features (gender, age, diameter, lymphatic metastasis, TNM stage et al) were not associated with hsa\_circ\_001988 according to statistical analysis.

### ROC curve of hsa\_circ\_001988

To estimate whether hsa\_circ\_001988 could be used as an indicator for diagnosis of colorectal cancer, a ROC curve was built. A total of 31 patients' normal colon mucosa was used as a

control to produce this ROC curve (**Figure 3**). The sensitivity and specificity was 0.68 and 0.73, respectively. The cutoff value was 6.04. The area under the curve was 0.788 (95% CI=0.68-0.90,  $P < 0.001$ ). The Youden index was 0.419. Therefore, hsa\_circ\_001988 could be used as indicator of colorectal cancer.

### Discussion

Circular RNAs are newly found endogenous noncoding RNAs, which had been considered as the results of transcriptional noise [4]. With the development of bioinformatics' methods and sequencing technology, lots of circular RNAs were predicted. Many researchers show great interest in those noncoding RNAs to explore their function. For an example, CDR1 as/ciRS-7 and Sry, which have been studied a lot, act as microRNA sponges [8, 16], while other studies have speculated that circRNAs may have other functions, which may be related to protein and/or RNA transport [10].

Many further studies about relationship between circular RNA and disease were conducted. Such as, Lukiw et al reported CDR1 as associated with Alzheimer's disease [17]. Cyclization of an INK4/ARF-Associated non-coding RNA correlates with atherosclerosis risk [18]. And the study conducted by Ghosal et al. found 105 diseases were connected with circular RNAs from 174 diseases including several carcinomas and neurodegenerative diseases [11]. However it was little known about the relationship between hsa\_circ\_001988 and colorectal cancer. To our knowledge, this is the first report of the existence of hsa\_circ\_001988. Our findings showed that the expression of hsa\_circ\_001988 was decreased in tumor tissues compared with normal mucosa and the average hsa\_circ\_001988 expression of PNI-positive tumors is lower than PNI-negative tumors (**Table 1**). Furthermore, the expression level of hsa\_circ\_001988 was significantly associated with perineural invasion ( $P < 0.05$ ) and differentiation ( $P < 0.05$ ).

It has been identified perineural invasion is a predictor of outcome in colorectal cancer and perineural invasion was negatively associated with survival time and local recurrence in colorectal cancer patients [19, 20]. Besides, the degree of tumor cell differentiation is associated with prognosis. Thus, hsa\_circ\_001988

may play a profound role in differentiation and perineural invasion. It is a potential target to regulate cytological behaviors. Furthermore, hsa\_circ\_001988 may be used as new target to therapy colorectal cancer and predict colorectal cancer cell differentiation and perineural invasion.

However, our study just validated the existence of hsa\_circ\_001988 and analyzed the relationship of between this circular RNA and clinical features in 31 patients. More samples are needed in the future work. And it's necessary to explore the further function of hsa\_circ\_001988.

In conclusion, hsa\_circ\_001988 could be detected in human colorectal cancer tissues and normal mucosa. The expression of hsa\_circ\_001988 was down regulated in colorectal cancers. Furthermore, its expression was associated with differentiation and perineural invasion. Those results suggest that hsa\_circ\_001988 may be a new target for treatment of colorectal cancer and a potential biomarker of colorectal cancer.

#### Acknowledgements

This work was supported by 863 Program of China (No. 2014AA020802).

#### Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Wei Zheng, Department of Surgical Oncology, Chinese PLA General Hospital, Fuxing Road, Beijing 100853, China. Tel: (0086) 10-66937523; E-mail: zhengwei301@sina.cn

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