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Original Article

The Expression Pattern and Clinicopathological Importance of Hsa_circ_000425 in Colorectal Cancer

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Although colorectal cancer (CRC) is one of the most common cancers, the exact molecular mechanism of this cancer is not yet known. Circular RNAs (circRNAs), a class of non-coding RNAs, are newly identified and their role in the pathogenesis of various cancers has been shown. In this research, we studied the expression pattern and clinical importance of hsa_circ_000425 in CRC patients. After evaluation of hsa_circ_000425 expression rate in 4 CRC cell lines and 100 paired CRC tissues, the potential correlation between hsa_circ_000425 expression rate and clinicopathological parameters of CRC patients was analyzed. Additionally, receiver operating characteristic (ROC) curve was drawn to study the diagnostic value of hsa_circ_000425. A significant downregulation of hsa_circ_000425 was observed in both CRC tissues and cell lines. In addition, this downregulation was significantly associated with differentiation and lymphatic metastasis. The area under the ROC curve of hsa_circ_000425 was 0.839 ($P < 0.001$). hsa_circ_000425 may have a role in the pathogenesis of CRC and might act as a potential biomarker for the diagnosis and treatment of CRC; although further molecular studies must be performed in this regard.

Key words: Circular RNA, hsa_circ_000425, colorectal neoplasm

Colorectal cancer (CRC) is the third most common cancer and the fourth leading cause of death in the world. Despite the fact that colon cancer treatments, including surgery and chemotherapy, have made significant progress and increased the life expectancy of patients, diagnostic

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methods for this cancer, especially the use of various biomarkers, have not progressed much, and there is much room for improvement. Therefore, the identification of new biomarkers can increase the diagnostic and therapeutic power of this disease (1,2).

One of the popular biomarkers in recent years is circular RNAs (circRNAs). These are a class of endogenous noncoding RNA (ncRNA), and are formed by either back-splicing or covalent binding (3,4). Studies have shown that circRNAs participate in different cell signaling pathways, and may become one of the greatest biomarkers in diagnostic and prognostic of different cancers; however, little is known about their roles in human CRC (3-5).

Human hsa_circ_0001563 with alias hsa_circ_000425 is one of the newly introduced circRNAs with 355 nt in spliced sequence length. Its gene is located at chr5:1709043171–179043526, and its also called *HNRPNPH1*. In this research, we selected this circRNA based on previous bioinformatics analysis suggesting its association with CRC (6).

In this context, hsa_circ_000425 expression levels were investigated in 4 CRC cell lines and 100 paired tissues. Then, we evaluated the association between hsa_circ_000425 expression levels and clinicopathological parameters of studied patients. For evaluation of the diagnostic value of this circRNA, a receiver operating characteristic (ROC) curve was built.

Materials and methods

Cell culture

Four CRC cell lines (SW480, SW620, HCT8, and Lovo) and NCM460 as normal human colon epithelial cell were purchased from the Cell Bank of Pasteur Institute (Tehran, IRAN). The cells were incubated in Roswell Park Memorial Institute-1640 medium (RPMI-1640) (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum. Cells were cultured under standard condition, incubated at 37°C under 5% CO_2.

Clinical information of the patients

In this study, 100 samples of cancerous tissue of colon and adjacent normal tissue from patients with CRC were taken from the surgical center of Vellayat hospital of Qazvin province and Shariati hospital in Tehran during the year 2020. These patients had undergone surgery, and had not received any chemotherapy drugs or radiotherapy before the operation. Also, 33 tissue samples were taken from non-cancerous patients who were candidate for colonoscopy. 68.3% of patients were male, 31.7% of them were female. The mean age was 60 ± 2.1 years among the patients and 48 ± 1.8 years for controls. This study was approved by the Ethics Committee of Qazvin University of Medical Sciences (IR.QUMS.REC.1398.218) and the consent forms had been studied and signed by each patient. Patients' documents have been carefully studied and their pathological information was collected.

Quantitative real time polymerase chain reaction

In order to extract RNA, tissue samples that were frozen at – 80ºC were weighed, and 100 mg pieces were prepared for RNA extraction. Regarding the cultured cells, 1×10^6 of them were used for total RNA extraction by using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The quantity and quality of extracted RNA, was evaluated by Nano Drop 2000c (Thermo, USA). In this case RNA samples with A260/A280 ratios of >2 were selected for quantitative analysis. To improve the purity of hsa_circ_000425, RNase R was used to treatment of total RNA. First strand complementary DNA (cDNA) synthesis was also performed on 500 ng of treated RNA by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Fermentas, Waltham, MA, USA). Hsa_circ_000425 expression was evaluated using quantitative real-time polymerase chain reaction (qRT-PCR) (Rotor-gene, USA). *GAPDH* was used as internal control.

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Primers sequences were: hsa_circ_000425: 5'AGC-AAACCAGTCCAGCTACG3' (forward) (Divergent), and 5'ACATGATTCCGTAAGTAGAGG-CA3' (reverse), *GAPDH*: 5'GAAGGTGAAGGTC-GGAGTC3' (forward), and 5'GAAGATGGTGAT-GGGATTTC3' (reverse) (7).

The reactions were incubated in a 72-well optical strip at 95°C for 15 min (enzyme activation), followed by 95°C for 20 s and 60°C for 60 s (40 cycles). All reactions were run in triplicate. The mean Ct was determined from the triplicate PCRs. Ct values were used to evaluate the expression levels of the hsa_circ_000425. The expression value of mentioned circRNA relative to internal control was determined using the $2^{-\Delta Ct}$ method (8).

Statistical analysis

The results were analyzed by GraphPad software (GraphPad PRISM V 8.4 analytical software). Comparisons of data between pairs of groups were detected by independent Student's *t-test*. Pearson's $\chi 2$ test was used to evaluate clinicopathological variables.

Results

Initially, the expression rate of hsa_circ_000425 was examined in 5 cell line. Our results showed a significant down regulation of hsa_circ_000425 in Sw620 (P <0.01), Sw480 (P <0.01), and Lovo (P <0.001) cell lines, but in Hct8 cell line no significant difference in expression levels of hsa_circ_000425 was observed in comparison with the normal cell line (NCM460) (P = 0.324) (Figure 1).

Comparison of the expression levels between the cancer tissue samples and their adjacent normal tissue sample showed a significant down regulation of hsa_circ_000425 in 68 cancer tissue samples (68%) in comparison with the normal tissues (P < 0.001) (Figure 2).

Correlation between hsa_circ_000425 expression and clinicopathological features

The expression levels of hsa_circ_000425 in the control samples were examined and $2^{-\Delta Ct}$ = 0.95±0.13 was considered as the cut-off value. Then, the CRC tissue samples were divided into low and high hsa_circ_000425 expression groups. In order to investigate whether hsa_circ_000425 can exert a significant effect on the pathogenesis of colon cancer, the relationship between hsa_circ_000425 expression and cilinopathological data was investigated. There were no significant associations with age, gender, invasion (T classification) and -

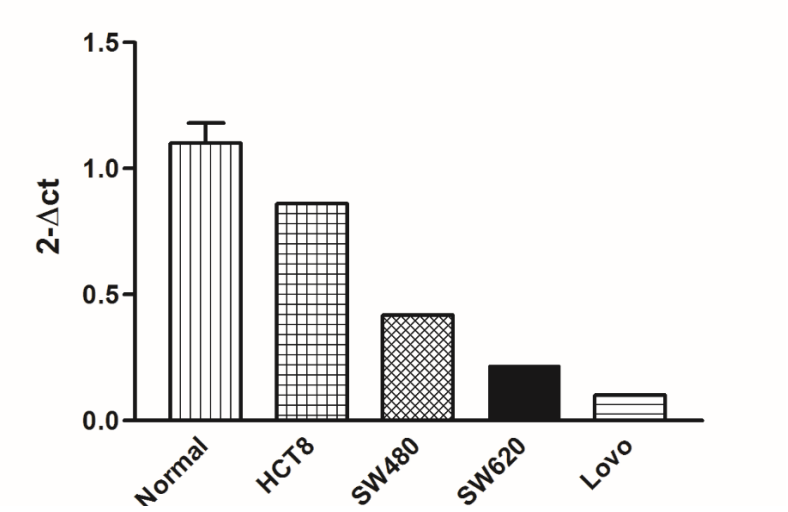

Fig. 1. Expression levels of hsa-circ_000425 in different colorectal cancer cell lines. The expression levels of hsa-circ_000425 in HCT8, SW480, SW620, and Lovo as cancerous cell lines, and normal colon epithelial cell line (NCM460) were evaluated .A significant down regulation of hsa-circ_000425 was observed in Sw620 (P < 0.01), Sw480 (P < 0.01), and Lovo (P < 0.001) cell lines. All values are given as mean ± SD of three independent experiments.

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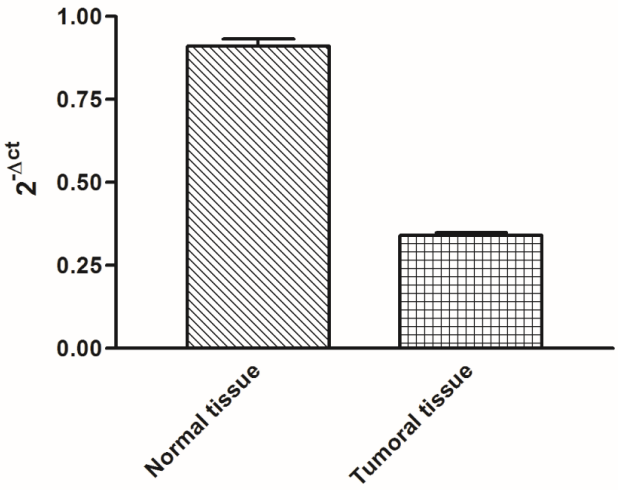

Fig. 2. Expression pattern of hsa_circ_000425 in cancerous and adjacent normal colon tissues. This figure shows the downregulation of hsa-circ_000425 in cancerous tissue (P < 0.001) in comparison with adjacent normal tissues. The values are mean ± SD of three independent experiments.

Table 1. Association of hsa-circ_000425 expression with cilinco-pathological parameters.

Parameters	hsa_circ_000425 expression in tumor		P-value
	Low	High	
Gender			
Male	35	32	
Female	15	18	0.24
Age			
< 55 years	13	11	
≥ 55 years	40	36	0.72
Invasion			
< 5 cm	14	10	
≥ 5 cm	42	34	0.72
Differentiation			
Well+ moderate	17	16	
Poor	48	19	**0.02**
T classification			
1-2	23	35	
3-4	25	17	0.123
N classification			
0	28	24	
1-2	42	12	**<0.001**
M classification			
0	32	30	
1	20	18	**0.23**
TNM stage			
I-II	25	27	
III-IV	20	28	**0.35**

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TNM stage (Table 1). However, a significant poor differentiation was observed in samples with low expression in comparison with those with high expression of hsa_circ_000425 (P = 0.012). Also, significant downregulation of hsa_circ_000425 was observed in samples with lymphatic metastasis (N12) (P < 0.001).

The diagnostic value of hsa_circ_000425 in CRC

ROC curve was used to estimate the diagnostic value of hsa_circ_000425, and distinguish CRC patients from healthy controls. Also, the sensitivity and specificity of hsa_circ_000425 was evaluated. The area under the ROC curve was up to 0.839 (95% confidence interval = 0.789–0.889; P < 0.0001). The specificity and sensitivity were 0.766 and 0.803, respectively. Based on these data hsa_circ_000425 might be a diagnostic biomarker (Figure 3).

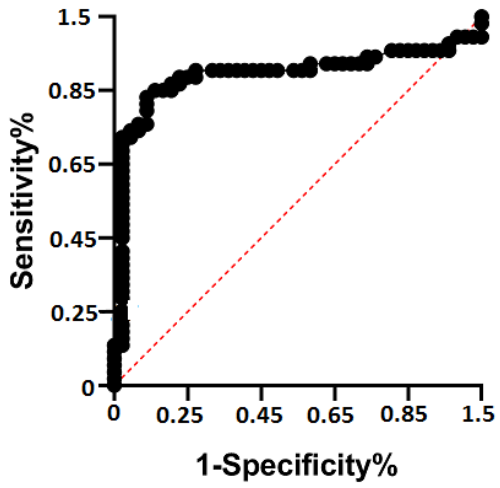

Fig. 3. The ROC curve for differentiation of cancerous tissues from healthy controls. The area under the ROC curve was 0.839 (95% confidence interval = 0.789–0.889; P < 0.001). The specificity and sensitivity were 0.766 and 0.803, respectively.

Discussion

Despite the fact that 80% of the human genome can be transcribed into RNA, only 3% of them are translated into proteins and the remaining are ncRNAs. NcRNAs are divided into different categories, and some of them have main roles in the pathogenesis of disease. For example the role of microRNAs and long ncRNAs in initiation, progression and metastasis of distinct human (7) malignancy has been approved (9, 10). CircRNAs are a class of single-stranded closed circular RNA molecules that lack 5′-3′ ends and poly (A) tails (11). In 1976, circRNAs were identified in plant specific viruses, and various studies were performed on their structure and function (4). In recent years, results of deep RNA sequencing technique have shown that these RNAs have main roles in biology of eukaryotic cells and are even involved in the pathogenesis of various human diseases, such as nervous system disorders (12), cardiovascular disorders (13), Alzheimer's disease (14), diabetes (15), and cancers (16, 17).

Several studies have demonstrated the bold role of the circRNAs in cancer growth, metastasis, and resistance to therapy (18, 19). Regarding their role in CRC, differential expression of these ncRNAs was shown in CRC tissues (20-22). CircRNAs are involved in different pathways such as regulation, proliferation, invasion, migration, and apoptosis of CRC cells by sponging miRNAs, contributing to peptide translation, regulating cancer-related signaling pathways, and may act either as oncogenes or tumor suppressors. However, miRNA sponging is the main mechanism of circRNAs function reported in CRC cells (23, 24).

In the present study we evaluated the association of hsa_circ_000425 expression with the clinicopathological parameters of CRC patients. To our knowledge, this study is the first study to identify the role of hsa_circ_000425 in CRC. Our results showed that hsa_circ_000425 expression level was significantly down regulated in both CRC tissues and CRC cell lines, and acts probably as a tumor suppressor. Our data indicated that the down regulation of hsa_circ_000425 was significantly associated with poor differentiation and lymph node metastasis. It is clear that tumor cell differentiation is one of the predominant causes for CRC metastasis and recurrence (25, 26). On the other hand, lymph node metastasis can be a predicator

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factor of CRC smay have a role in CRC survival and recurence.

Our data showed a down regulation of hsa_circ_000425 in CRC tissues and cell lines. In line with our results, Liu *et al.* (2018) reported the down regulation of this circRNA in gastric cancer cell line, and they also reported that hsa_circ_000425 binds to miR-17 and miR-106b, (both of which had been demonstrated to be oncogenic) suggesting the tumor suppressive role of hsa_circ_000425 (7). As mentioned above, significant association of hsa_circ_000425 down regulation with lymph node metastasis was observed in this research. Conspicuously, the effect of miR-17 and miR-106b up regulation in lymph node metastasis and poor prognosis was reported (7). Furthermore, we explored the diagnostic value of hsa_circ_000425 by constructing the ROC curve. The area under ROC curve was up to 0.839, and the specificity and sensitivity were 0.766 and 0.803, respectively. Hence, hsa_circ_000425 can be regarded as a potential biomarker in the diagnosis of CRC.

It is worth noting that our data are based on clinical analyzes, and the exact molecular mechanism of action of hsa_circ_000425 in colon cancer pathogenesis should be studied both *in vitro* and *in vivo*.

In conclusion, in this study for the first time we reported that hsa_circ_000425 was significantly downregulated in CRC and associated with poor differentiation and lymphatic metastasis. Besides, our results showed that hsa_circ_000425 may play a crucial role in CRC pathogenesis, and may be a potential biomarker for CRC diagnosis and treatment.

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Conflict of interest

Authors declare that there is no conflict of interest.

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