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Original Article

Upregulated serum miR-675 predicts poor prognosis for colorectal cancer

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Abstract: Circulating microRNAs (miRNAs) have been shown to be promising biomarkers for various types of cancers including colorectal cancer (CRC). The aim of this study was to investigate the potential diagnostic and prognostic value of serum miR-675 in CRC. The expression levels of serum miR-675 in CRC patients and healthy controls were compared by real-time PCR. Then the clinical significance of serum miR-675 in CRC was further evaluated. The serum miR-675 levels in CRC patients were significantly higher compared to healthy volunteers. Serum miR-675 was able to accurately distinguish CRC patients from healthy controls as well as CRC patients with and without recurrence. In addition, increased serum miR-675 level was positively correlated with lymph node metastasis and clinical stage. Moreover, CRC patients in the high serum miR-675 group suffered worse 5 year overall survival and recurrence free survival than those in the low serum miR-675 group. Furthermore, serum miR-675 was an independent prognostic factor for CRC. In conclusion, serum miR-675 might serve as a promising biomarker for early detection and prognosis prediction of CRC.

Keywords: Biomarker, colorectal cancer, early detection, miR-675, prognosis

Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death worldwide [1]. The primary reason for the high mortality of CRC is due to the liver and lung metastasis. The application of regular screening and the progress in the therapy have significantly improved the clinical outcome of CRC [2]. However, the prognosis of CRC remains poor, especially in patients with distant metastasis [3]. Thus, it is imperative to identify novel molecular markers for early detection and prognosis prediction for CRC.

microRNAs (miRNAs) are endogenous, evolutionary conserved, small non-coding RNA molecules with 18-25 nucleotides in length, which regulate gene expression at the post-transcriptional level [4, 5]. A large body of evidence supporting the crucial role of miRNAs in regulating a majority of cellular processes has accumulated [6, 7]. Deregulation of miRNAs is found in many different types of human cancers, indicating these small molecules are important modulators of cancer initiation and development [8, 9]. For instance, the expression level of miR-375 was significantly reduced in CRC tissues. In addition, overexpression of miR-375 inhibited the migration and metastasis capacity of CRC cells both *in vitro* and *in vivo* by targeting Frizzled 8 [10]. miR-20a-5p was upregulated in human CRC tissues and high miR-20a-5p levels indicated poor overall survival in patients with CRC. miR-20a-5p promoted the malignant behaviors of CRC cells by downregulating smad4, suggesting that miR-20a-5p functioned as an oncomiR in CRC [11].

miR-675, which is excised from exon one of H19, has been shown to be involved in CRC tumorigenesis. miR-675 was overexpressed in both colon cancer cell line and CRC tissues. Moreover, the known tumor suppressor retinoblastoma was a direct target of miR-675, indicating miR-675 might play an oncogenic role in CRC [12, 13]. Liquid biopsy has become a promising technology, which can provide real-

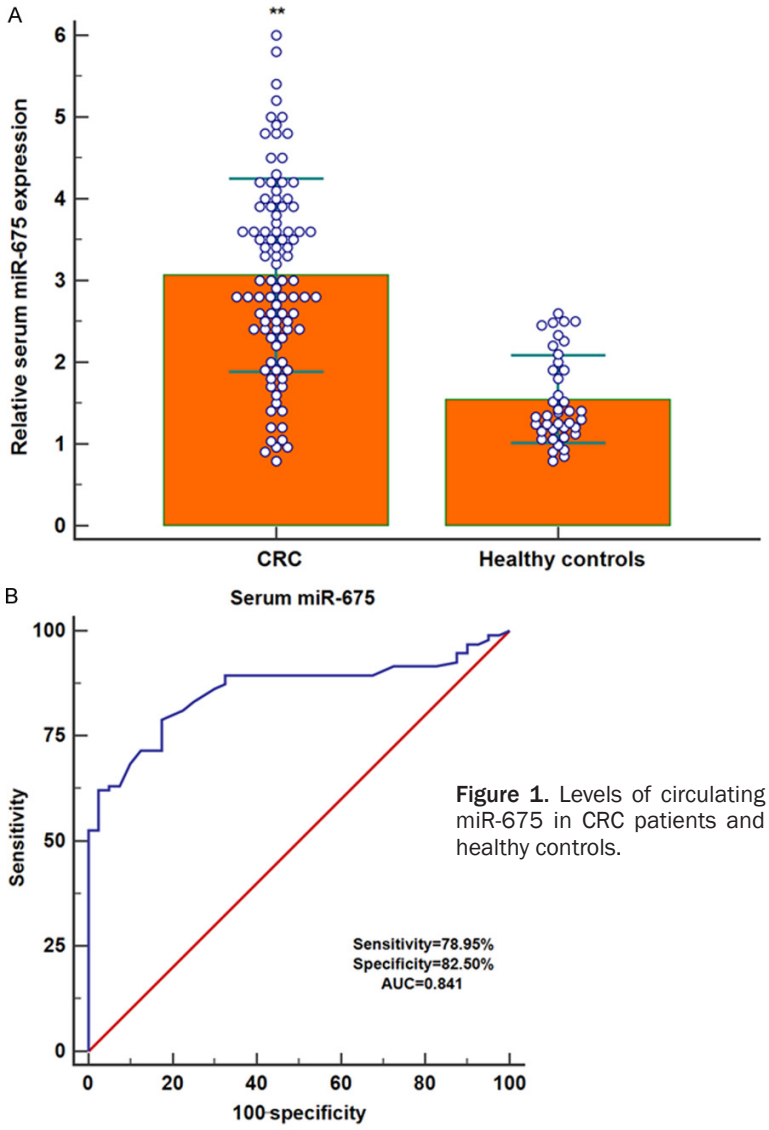

Figure 1. Levels of circulating miR-675 in CRC patients and healthy controls.

time, non-invasive, ongoing picture of a patient's disease status [14]. However, the expression of serum miR-675 in CRC and its potential clinical significance was unclear. The aim of current study was to investigate the diagnostic and prognostic value of serum miR-675 in CRC.

Materials and methods

Patients and serum specimens

This research was conducted in compliance with the Helsinki Declaration and was approved by the Ethics Committee of Cancer Hospital of China Medical University, Liaoning Cancer Hospital & Institute. Written informed consent was obtained from all patients or their relatives. All CRC patients were pathologically confirmed. CRC patients who received anticancer intervention including immunotherapy, chemotherapy or radiotherapy before surgical treatment were excluded. The healthy control subjects were matched to the CRC patients according to age and gender. Baseline data were retrieved from patient medical records. The tumor-node-metastasis (TNM) stage was assessed based on the criteria of the American Joint Committee on Cancer. Overall survival (OS) time was calculated from the date of surgery to the date of death, or the last known follow-up. Recurrence-free survival (RFS) was defined as the time from the initial surgery until the first evidence of local, regional, or distant tumor progression.

Whole blood sample of each participant was collected and centrifuged at 3000 g for 10 min at 4°C. Serum supernatants were transferred in an RNase free Eppendorf tube and stored at -80°C until further use.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from serum samples using the mirVana PARIS Kit (Ambion, Carlsbad, CA, USA). Purity and concentration of RNA were determined from OD260/280 readings by a dual beam UV spectrophotometer (Eppendorf AG, Hamburg, Germany). The first strand cDNA was synthesized from total miRNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). miR-675 expression was quantified by performing qRT-PCR using TaqMan MicroRNA Assay kits (Applied Biosystems) on an ABI 7500 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: predenaturing at 95°C for 10 min, followed by 40 cycles of denaturing at 95°C for 30 sec, annealing at

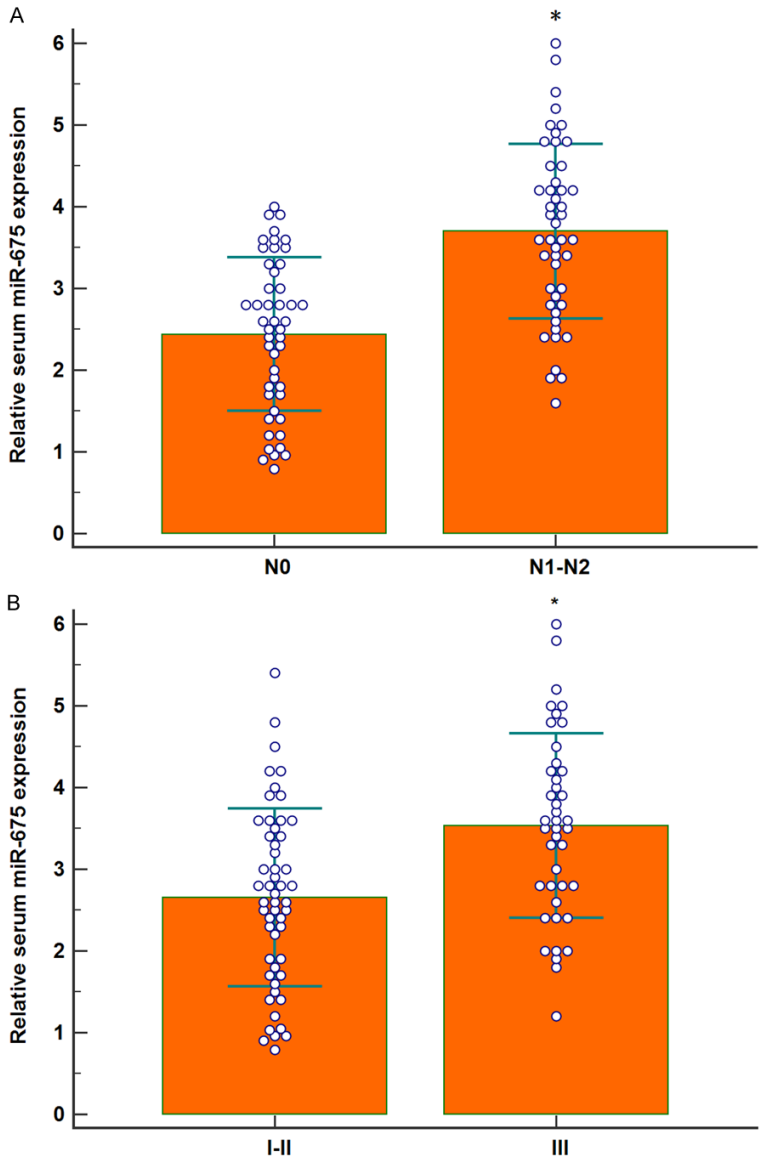

Figure 2. Serum miR-675 was increased in CRC patients with lymph node metastasis or patients in the advanced clinical stage.

60°C for 20 sec, and extension at 72°C for 10 sec. 25 fmol of synthetic *C. elegans* miRNA (cel-miR-39) was used as an internal control. The RT-qPCR data was evaluated using the 2(-delta delta C(T)) method and all assays were carried out in triplicate.

Statistical analysis

Differential serum miR-675 expression between different groups was analyzed using Mann-Whitney U test. Receiver operating characteristic (ROC) curve analysis was used to evaluate whether serum miR-675 expression levels could distinguish CRC patients from healthy control subjects as well as CRC patients with or without CRC recurrence. Area under ROC curve (AUC) was used as an accuracy index for evaluating its diagnostic performance. Chi-squared test was performed to analyze the association between serum miR-675 levels and clinicopathological parameters of CRC. Survival estimates were calculated using the Kaplan-Meier analysis, and groups were compared with the log-rank test. Multivariate analysis was utilized to reveal the independent prognostic factors influencing overall survival in CRC patients. All analysis was performed using MedCalc for Windows, version 15.0 (MedCalc Software, Ostend, Belgium) and GraphPad Prism version 6.0 (GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant when $P<0.05$.

Results

Serum miR-675 was upregulated in patients with CRC

Ninety five CRC patients and forty healthy control subjects were detected by qRT-PCR for serum miR-675 expression. Our results showed that the expression level of serum miR-675 was significantly higher in CRC patients compared to healthy controls (**$P<0.01$) (**Figure 1A**). In addition, ROC analysis revealed that serum miR-675 could effectively discriminate CRC patients from healthy controls, with an AUC value of 0.841 (sensitivity = 78.95%, specificity = 82.50%) (**Figure 1B**).

Then we investigated serum miR-675 levels in CRC patients with different lymph node status as well as various clinical stages. Our results demonstrated that patients with lymph node metastasis had a remarkably increased serum

Table 1. Association between serum miR-675 expression and clinicopathologic characteristics of CRC patients

Parameters	No. of patients	Serum miR-675		*p*
		Low (n = 49)	High (n = 46)	
Gender				0.369
Female	43	20	23	
Male	52	29	23	
Age (years)				0.843
<60	34	18	16	
≥60	61	31	30	
Tumor location				0.251
Colon	50	23	27	
Rectum	45	26	19	
Tumor size				0.432
<5 cm	39	22	17	
≥5 cm	56	27	29	
Node stage				0.001
N0	48	33	15	
N1-N2	47	16	31	
TNM stage				0.013
I	13	10	3	
II	40	24	16	
III	42	15	27	
Differentiation				0.239
Well	11	8	3	
Moderate	58	30	28	
Poor	26	11	15	

miR-675 levels compared to those without lymph node metastasis (*P<0.05) (**Figure 2A**). Similarly, the expression levels of serum miR-675 were higher in the patients with advanced clinical stage (*P<0.05) (**Figure 2B**).

Association between serum miR-675 levels and clinicopathologic parameters of CRC

The correlation between serum miR-675 levels and clinicopathologic parameters is shown in **Table 1**. Serum miR-675 level was significantly associated with lymph node metastasis (P = 0.001) and TNM stage (P = 0.013). However, it was not correlated with the other clinical variables including age, gender, tumor location, tumor size, M stage and differentiation.

Serum miR-675 level predicted recurrence of CRC

We then analyzed the potential association between serum miR-675 and recurrence of CRC. The data demonstrated that the expression level of serum miR-675 was significantly upregulated in CRC patients with recurrence compared to those without recurrence (*P<0.05) (**Figure 3A**). The ROC analysis showed that serum miR-675 was able to predict the recurrence of CRC with relative high accuracy (AUC = 0.827, sensitivity = 82.93%, specificity = 79.63%) (**Figure 3B**).

Prognostic significance of serum miR-675 in CRC

We also investigated the correlation between serum miR-675 levels and survival duration. Our results showed that CRC patients in the high serum miR-675 group suffered worse overall survival than those in the low serum miR-675 group (P = 0.0052) (**Figure 4A**). Recurrence-free survival of CRC patients with high serum miR-675 expression was significantly lower than that of patients with low serum miR-675 expression (P = 0.0022) (**Figure 4B**).

In addition to TNM stage and lymph node metastasis, high circulating miR-675 (HR = 1.93, 95% CI = 1.09-3.25, P = 0.038) was an independent factor predicting the poor prognosis for overall survival of CRC according to the Cox mutivariate analysis (**Table 2**).

Discussion

In this study, our results showed that serum miR-675 was significantly upregulated in CRC patients especially those with recurrence. In addition, serum miR-675 could effectively distinguish CRC patients from healthy controls as well as CRC patients with and without recurrence. A positive correlation was observed between increased serum miR-675 levels and unfavorable clinicopathological parameters including lymph node metastasis and TNM stage. Moreover, CRC patients with higher serum miR-675 had poorer 5 years overall survival and recurrence free survival. Finally increased serum miR-675 was an independent risk factor for CRC. To the best of our knowledge, this was the first study to demonstrate the diagnostic and prognostic value of serum miR-675. Our study not only further corroborates the tumor promoting role of miR-675 in CRC, but also provides strong evidence to reveal the potential clinical application of serum miR-675 in this malignancy. However, one limi-

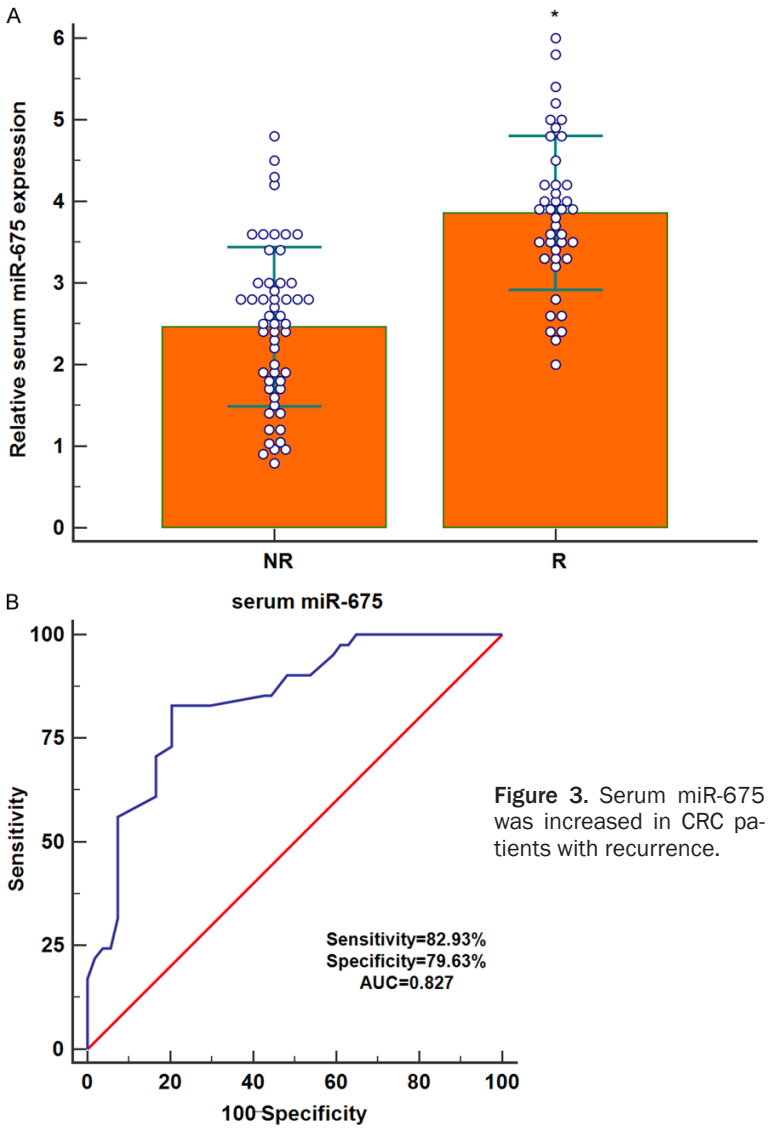

Figure 3. Serum miR-675 was increased in CRC patients with recurrence.

tation of our study was the small sample size. Further studies with large sample size are required to validate our findings.

Similarly to our findings, Costa et al showed that miR-675-5p was overexpressed in metastatic colon cancer cells and promoted the epithelial mesenchymal transition by regulating HIF1α. In addition, higher expression of miR-675-5p was observed in CRC patients with lymph node metastasis compared to those without lymph node metastasis, indicating miR-675-5p could promote the progression of CRC [15]. Recently, H19 enhanced the resistance of colon cancer cells to the treatment with 1,25(OH)2D3 by increasing the expression level of miR-675-5p, suggesting that targeting miR-675-5p might be crucial for the effective treatment [16]. miR-675-5p also played a tumor-promoting role in the other types of cancers. A positive correlation was found between miR-675 and H19 expression in gastric cancer. miR-675 played an important role in mediating the H19-induced gastric cancer progression by targeting RUNX1 [17]. Ectopic expression of miR-675 promoted the proliferation of hepatocellular carcinoma cells, and *vice versa*. Cdc25A was demonstrated to be a downstream target of miR-675 [18].

However, some studies also suggested that miR-675 might function as a tumor suppressor in tumorigenesis. The expression level of miR-675-5p was reduced in non- small cell lung cancer tissues compared to the controls. Down-regulation of miR-675-5p was associated with advanced clinical stage and lymph node metastasis. In addition, miR-675-5p suppression promoted the aggressive behaviors of lung cancer cells both *in vitro* and *in vivo,* and miR-675-5p overexpression led to the opposite results [19]. Both H19 and miR-675-5p were reduced in metastatic prostate cancer cell line, and upregulation of H19 promoted the migration capacity of cancer cells by increasing miR-675-5p expression [20].

In conclusion, our results demonstrate that the expression level of serum miR-675 is especially increased CRC patients with recurrence. Higher serum miR-675 levels are associated with worse clinical outcome. Taken together, these findings indicate that serum miR-675 may be employed as a promising diagnostic and prognosis biomarker of CRC.

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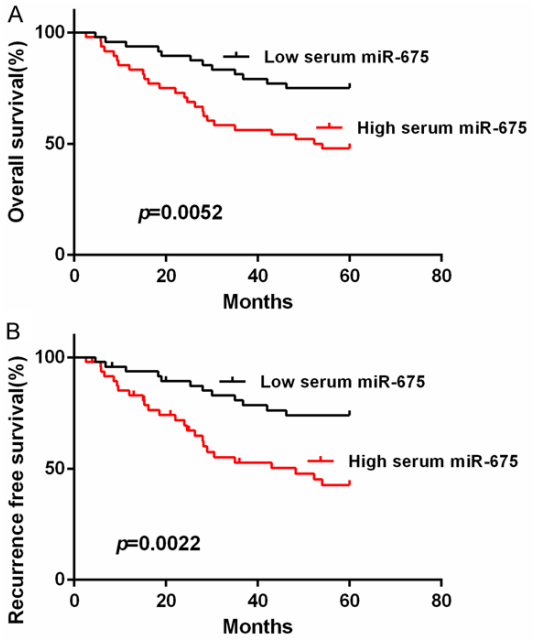

Figure 4. Kaplan-Meier estimates of 5-year overall survival and recurrence free survival in CRC.

Table 2. Multivariate analyses of prognostic factors for overall survival of patients with CRC

Parameters	Overall survival		
	HR	95% CI	*p*
Serum miR-675	1.93	1.09-3.25	0.038
TNM stage	3.16	1.53-5.69	0.006
Node stage	2.38	1.18-3.94	0.014

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Disclosure of conflict of interest

None.

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