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ORIGINAL ARTICLE

Three CircRNAs Function as Potential Biomarkers for Colorectal Cancer

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SUMMARY

Background: **Colorectal cancer (CRC) is one of the most frequently diagnosed cancers worldwide. Based on clinical data, CRC could be cured by surgery with favorable outcomes if diagnosed at an early stage. The present study aimed to determine whether circ-FMN2, circ-LMNB1, and circ-ZNF609 may serve as potential biomarkers for CRC.**
Methods: **Expression levels of circ-FMN2, circ-LMNB1, and circ-ZNF609 were detected in serum samples from 88 CRC patients and 68 healthy volunteers by real-time quantitative PCR (RT-qPCR). The correlation between circRNA expressions and clinicopathological parameters was analyzed subsequently. The ROC curve analysis and survival curves were calculated and compared in order to explore the diagnostic and prognostic values of circRNAs in CRC.**
Results: **The results verified that circ-FMN2, circ-LMNB1, and circ-ZNF609 were significantly elevated in serum samples of CRC patients compared with healthy controls ($p < 0.01$). Increased circ-FMN2, circ-LMNB1, and circ-ZNF609 expressions were markedly positively correlated with histological grade ($p < 0.0001$, $p = 0.0014$, $p = 0.0303$), lymph nodes metastasis ($p < 0.0001$, $p < 0.0001$, $p = 0.0093$), and TNM stage ($p = 0.0055$, $p = 0.0110$, $p < 0.000$). Meanwhile, the ROC curve analysis verified the diagnostic accuracy of circ-FMN2, circ-LMNB1, and circ-ZNF609 with AUC of 0.9153 (95% CI = 0.8707 ~ 0.9599), 0.9627 (95% CI = 0.9351 ~ 0.9903), and 0.8711 (95% CI = 0.8151 ~ 0.9270), respectively. Furthermore, the CRC patients with high circ-FMN2, circ-LMNB1, and circ-ZNF609 had significantly worse outcomes than those with low expression ($p = 0.0267$, $p = 0.0145$, $p = 0.0194$).**
Conclusions: **The present study elucidated that circ-FMN2, circ-LMNB1, and circ-ZNF609 may function as potential diagnostic and prognostic indicators for CRC detection.**
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KEY WORDS

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INTRODUCTION

Colorectal cancer, one of the most commonly diagnosed cancers around the world, estimated over 1.4 million new cases and 690 thousand deaths in 2012 [1,2]. Up to now, the conventional treatments for CRC include surgical resection, radiotherapy, chemotherapy, and other

adjuvant therapies [3]. As for China, CRC ranks as the fifth leading cause of cancer-related deaths, after lung cancer, gastric cancer, liver cancer, and esophageal carcinoma [4]. According to a previous study, the 5-year survival rate of CRC could be up to 90% if diagnosed at an early stage [5]. Unfortunately, CRC is commonly diagnosed at an advanced clinical stage resulting in high mortality due to the rapid metastasis [6,7]. Hence, it is important and urgent to discover and identify novel biomarkers for the diagnosis and prognosis of CRC in order to contribute to clinical detection and treatment. Circular RNA (circRNA), a newly discovered type of single-stranded and non-coding RNAs, recently shown potential as gene regulators [8]. Emerging evidence implied that circRNAs are abundant in various cancers contributing to regulation of tumorigenesis and tumor development. For instance, hsa_circ_100395 was found down-regulated in lung cancer tissues and may inhibit lung cancer progression through the miR-1228/TCF21 pathway [9]. Li et al. [10] suggested that circRNA-101368 expression was significantly elevated in HCC tissues and may modulate the migration of HCC through HMGB1/RAGE signaling. In particular, some circRNAs were reported to be dysregulated in CRC, such as hsa_circ_0000523 [11], hsa_circRNA_102958 [12], and others. Besides, owing to the stability of circRNAs, they may function as potential biomarkers for a number of tumors. Hang et al. [13] pointed out that circFARSA is a novel potential biomarker for non-small cell lung cancer. Another study reported that hsa_circ_0000467 may act as a novel non-invasive biomarker for gastric cancer diagnosis and prognosis with high accuracy [14]. In the CRC realm, circ-FMN2, circ-LMNB1, and circ-ZNF 609 were discovered to be remarkably up-regulated in CRC tissues and cell lines [15-17]. In the present study, we aimed to determine the clinical values of three circRNAs (circ-FMN2, circ-LMNB1, and circ-ZNF609) in CRC diagnosis and prognosis.

MATERIALS AND METHODS

Serum samples

Serum samples were obtained from 88 CRC patients and 68 healthy volunteers between March 2011 and July 2013 at Hiser Medical Center of Qingdao. All the CRC patients had been diagnosed as CRC according to the Union for International Cancer Control (UICC) and the American Joint Committee on Cancer (AJCC) without receiving any adjuvant therapies before the surgical resection. Patients with severe cardiovascular, high blood lipid, and gastrointestinal tract complications were excluded.

The present study was approved by the Ethics Committee of XXXX and informed consent was obtained from each participant before collection. Briefly, serum samples were preserved in EDTA-K2 tubes and centrifuged at 1,600 g for 10 minutes. After removing the supernatants, the serum samples were centrifuged at 16,000 g for another 10 minutes then preserved at -80°C until use. Clinicopathological parameters were analyzed and presented in Table 1 - 3.

RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was isolated and extracted using TRI Reagent BD (Sigma Aldrich, St. Louis, MO, USA) under the instructions. Then, cDNA was reverse transcribed from plasma RNA using RevertAid First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania). Afterward, the RT-qPCR analysis was carried out using SYBR Green qPCR Supermix UDG (Invitrogen, Carlsbad, CA, USA) in Real-Time PCR Detection System (Bio-Rad, USA). Glyceraldehyde 3-phosphate dehydrogenase (GADPH) functioned as a housekeeping gene. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method relative to the expression of GAPDH. Primer sequences were as follows: circ-FMN2 forward,
5'-AGACTTGAAAGCTGTTGTGAA-3'
and reverse,
5'-AAGACTTGAAAGCTGTTGTGA-3'.
circ-LMNB1 forward,
5'-GCCAAAATTGAATGCTGTCC-3'
and reverse,
5'-TGAGATAGCCCAGCAATCCT-3'.
circ-ZNF609 forward,
5'-CAGCGCTCAATCCTTTGGGA-3'
and reverse,
5'-GACCTGCCACATTGGTCAGTA'3';
GAPDH forward,
5'-GCACCGTCAAGGCTGAGAAC-3'
and reverse,
5'-GGATCTCGCTCCTGGAAGATG-3.

Statistical analysis

All the data in the present study were presented as the mean ± standard error of the mean (SEM) from at least three independent experiments. SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism were applied to analyze data. The significance of differences between groups was determined by Student's *t*-test and χ^2 test. Receiver operating characteristic (ROC) curves were used to analyze the diagnostic values of certain circRNAs in CRC. The survival analysis was analyzed by the Kaplan-Meier method and compared by log-rank test.

RESULTS

Evaluation of three circRNAs via RT-qPCR analysis

Expression levels of circ-FMN2, circ-LMNB1, and circ-ZNF609 in serum samples and healthy controls were evaluated by RT-qPCR analysis. As shown in Figure 1A - 1C, the data indicated that the expression levels of circ-FMN2, circ-LMNB1, and circ-ZNF609 were significantly higher than those in paired healthy controls (*** $p < 0.001$).

Table 1. Correlation between circ-FMN2 expression and clinicopathological parameters of CRC.

Parameters		Cases	Ratio of circ-FMN2		p-value
			High (n = 44)	Low (n = 44)	
Gender	male	53	28	25	0.8975
	female	35	16	19	
Age (years)	> 60	50	26	24	0.6669
	≤ 60	38	18	20	
Tumor size (cm)	> 5	27	16	11	0.2478
	≤ 5	61	28	33	
Local invasion	T1 - T2	49	25	24	0.8301
	T3 - T4	39	19	20	
Histological grade	moderate	36	9	27	< 0.0001
	poor	52	35	17	
Lymph node metastasis	negative	52	13	39	< 0.0001
	positive	36	31	5	
TNM stage	I + II	41	14	27	0.0055
	III + IV	47	30	17	

Table 2. Correlation between circ-LMNB1 expression and clinicopathological parameters of CRC.

Parameters		Cases	Ratio of circ-LMNB1		p-value
			High (n = 46)	Low (n = 42)	
Gender	male	50	24	26	0.3573
	female	38	22	16	
Age (years)	> 60	47	24	23	0.8079
	≤ 60	41	22	19	
Tumor size (cm)	> 5	30	17	13	0.5528
	≤ 5	58	29	29	
Local invasion	T1 - T2	45	24	21	0.8385
	T3 - T4	43	22	21	
Histological grade	moderate	33	10	23	0.0014
	poor	55	36	19	
Lymph node metastasis	negative	51	15	36	< 0.0001
	positive	37	31	6	
TNM stage	I + II	42	16	26	0.0110
	III + IV	46	30	16	

Clinicopathological features of study subjects

In order to further demonstrate the relationship between three circRNA expressions and the clinicopathological parameters, we divided CRC patients into two groups (high and low) based on the average value of circ-FMN2 (1.053 ± 0.0658), circ-LMNB1 (1.083 ± 0.0517), and circ-ZNF609 (0.9978 ± 0.0554). As shown in Table 1, high circ-FMN2 expression was markedly positively correlated with histological grade ($p < 0.0001$), lymph node metastasis ($p < 0.0001$), and TNM stage ($p = 0.0055$). As shown in Table 2, high circ-LMNB1 expression was markedly positively correlated with histological grade ($p = 0.0014$), lymph node metastasis ($p < 0.0001$), and TNM stage ($p = 0.0110$). As shown in Table 3, high circ-ZNF609 expression was markedly positively correlated with histological grade ($p = 0.0303$), lymph node metastasis ($p = 0.0093$), and TNM stage ($p < 0.0001$). However, there were no significant differ-

Table 3. Correlation between circ-ZNF609 expression and clinicopathological parameters of CRC.

Parameters		Cases	Ratio of circ-ZNF609		p-value
			High (n = 45)	Low (n = 43)	
Gender	male	45	21	24	0.4232
	female	43	23	19	
Age (years)	> 60	42	20	22	0.5282
	≤ 60	46	25	21	
Tumor size (cm)	> 5	47	24	23	0.9884
	≤ 5	41	21	20	
Local invasion	T1 - T2	40	21	19	0.8153
	T3 - T4	48	24	24	
Histological grade	moderate	31	11	20	**0.0303**
	poor	57	34	23	
Lymph node metastasis	negative	49	19	30	**0.0093**
	positive	39	26	13	
TNM stage	I + II	44	7	37	**< 0.0001**
	III + IV	44	38	6	

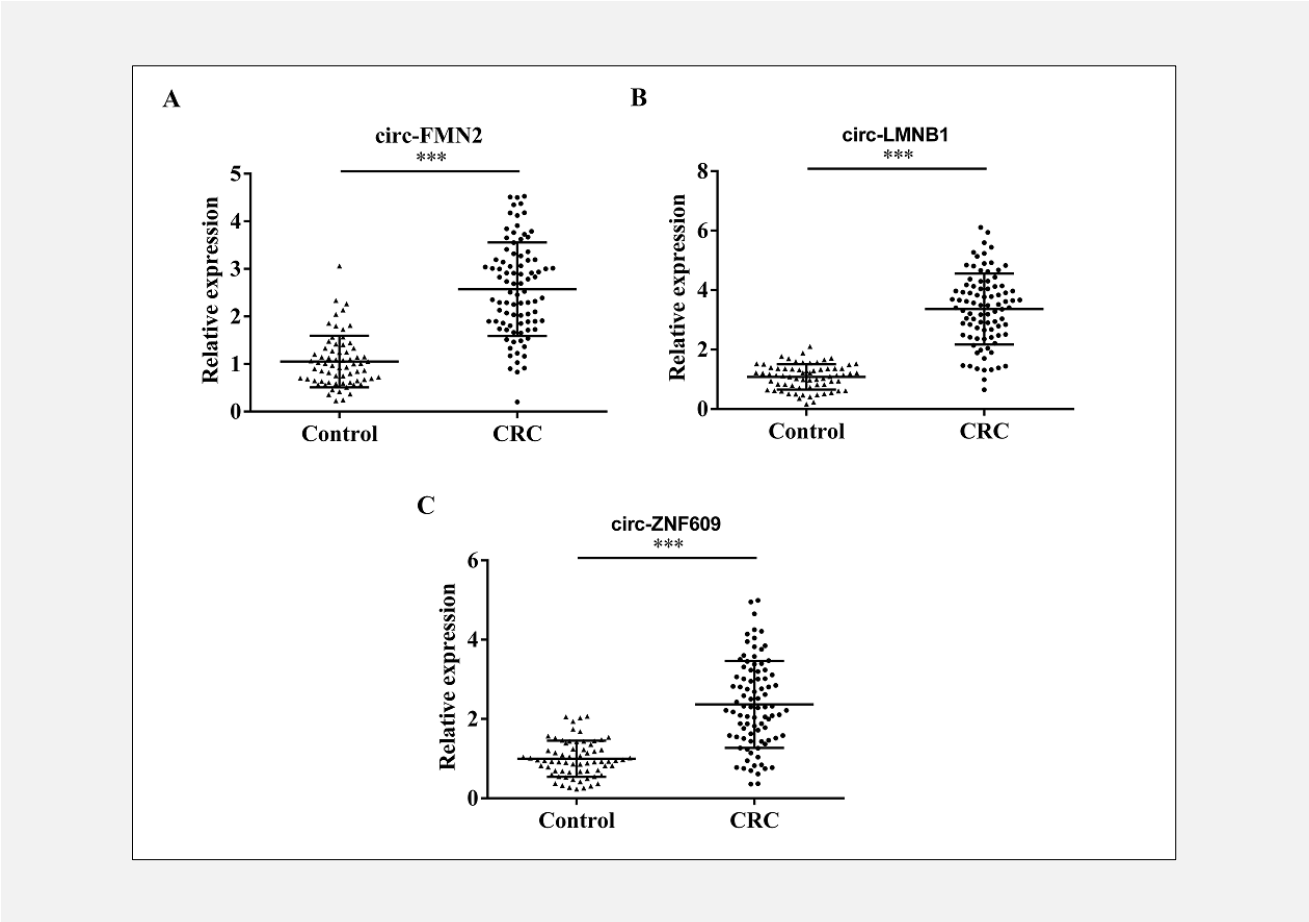

Figure 1. Expression levels of three circRNAs in the serum samples of 68 healthy controls and 88 CRC patients.

A: circ-FMN2; B: circ-LMNB1; C: circ-ZNF609. CRC: colorectal cancer; Control: healthy volunteers; *** p < 0.001.

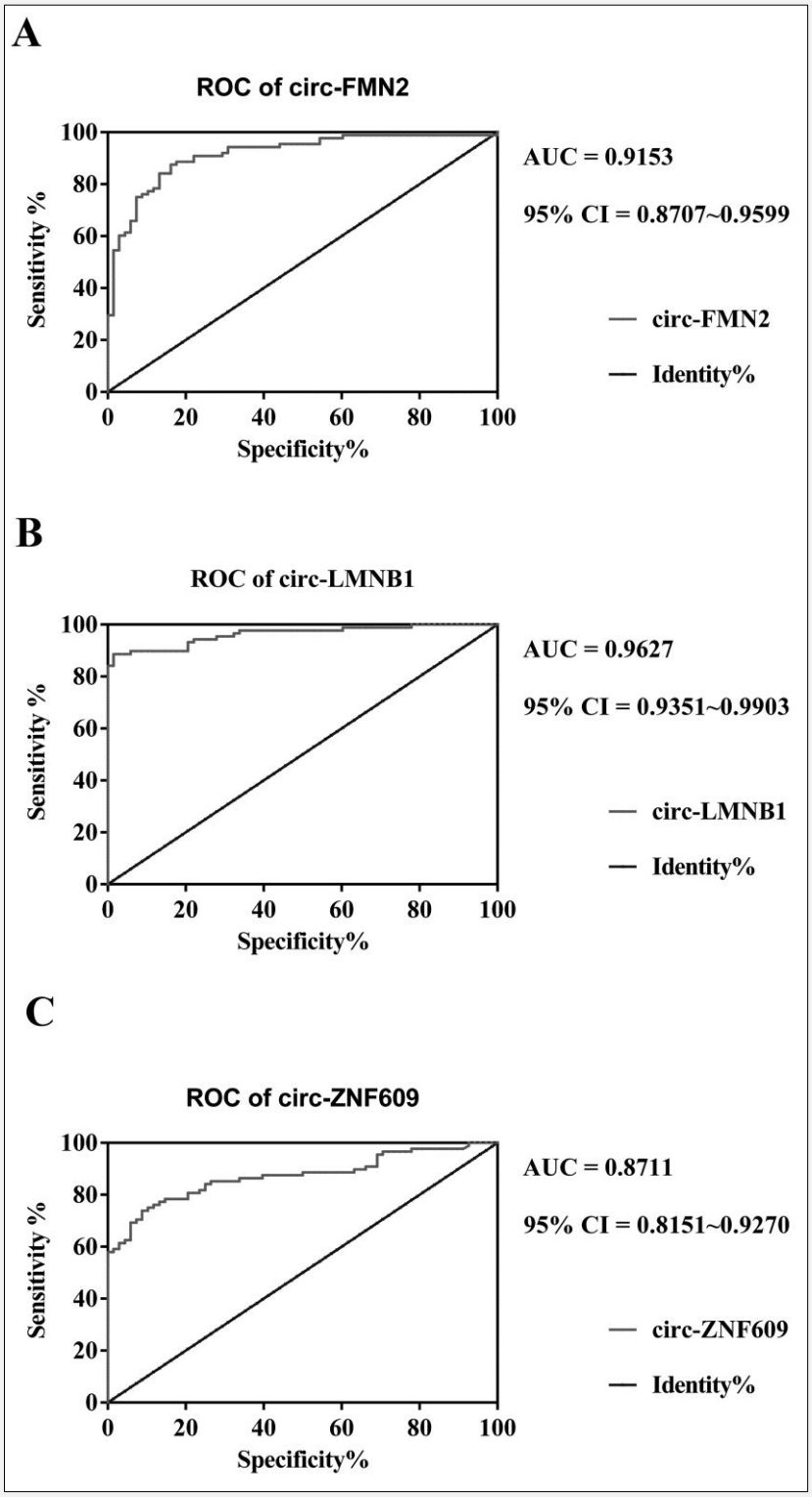

Figure 2. Receiver-operating characteristic (ROC) curve analysis of three circRNAs to discriminate CRC patients from healthy controls.

A: circ-FMN2; B: circ-LMNB1; C: circ-ZNF609. AUC - areas under the curve.

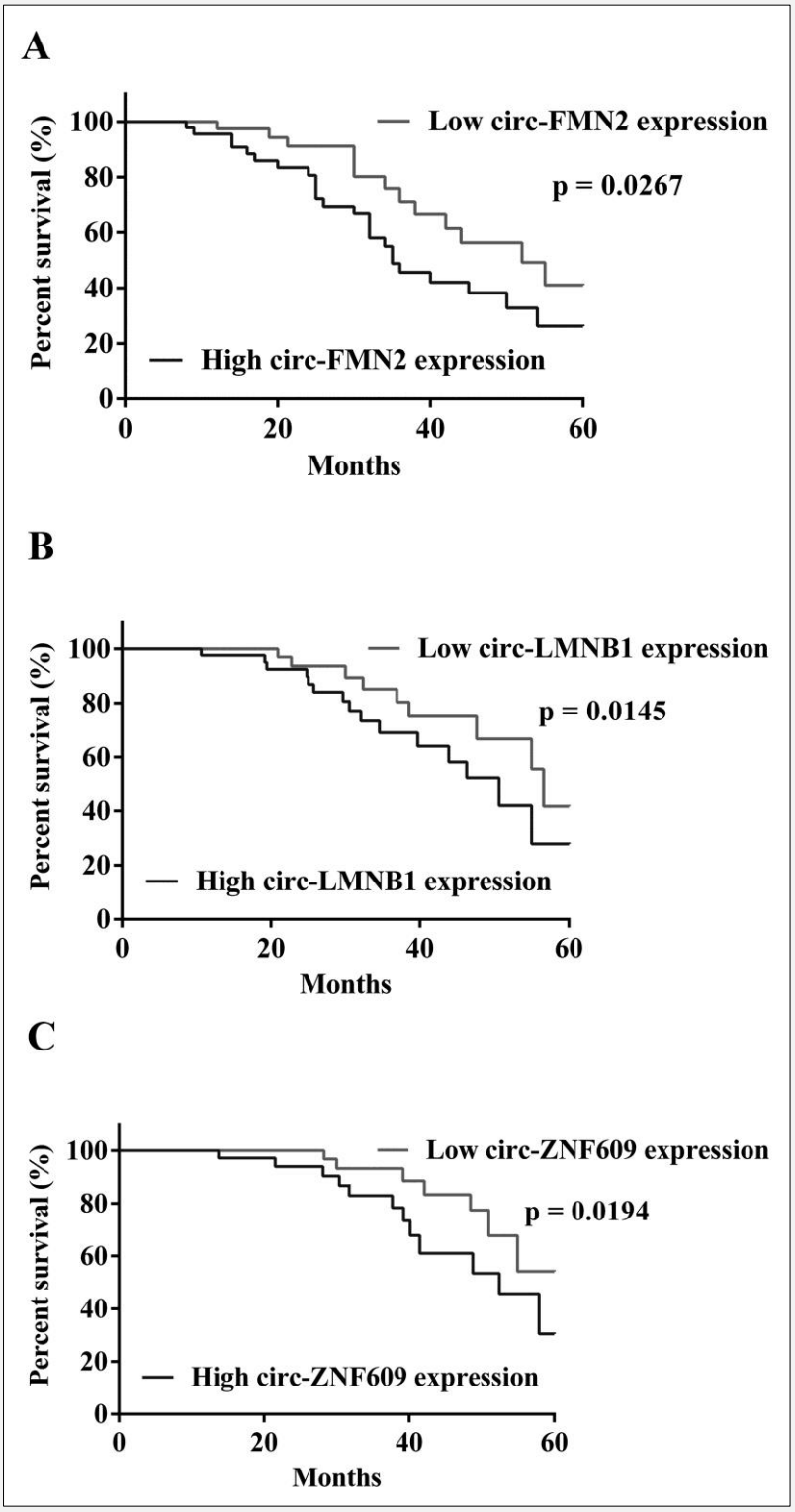

Figure 3. Kaplan-Meier survival analysis of the association between three circRNA expression levels and overall survival rate of 88 CRC patients.

A: circ-FMN2; B: circ-LMNB1; C: circ-ZNF609.

ences with gender, age, tumor size, and local invasion.

Diagnostic values of three circRNAs in CRC

After investigating the dysregulated expressions of three circRNA serum samples from CRC patients and healthy controls, we further carried out ROC curve analysis in order to provide more accurate predictions concerning the diagnostic value. As shown in Figure 2A - 2C, the ROC curve analysis verified that the diagnostic accuracy of circ-FMN2, circ-LMNB1, and circ-ZNF609 with AUC of 0.9153 (95% CI = 0.8707 ~ 0.9599), 0.9627 (95% CI = 0.9351 ~ 0.9903), and 0.8711 (95% CI = 0.8151 ~ 0.9270), respectively.

Prognostic values of three circRNAs in CRC

The Kaplan-Meier survival analysis was applied to measure the prognostic values of circ-FMN2, circ-LMNB1, and circ-ZNF609 in CRC patients. As shown in Figure 3A - 3C, CRC patients with lower circ-FMN2, circ-LMNB1, and circ-ZNF609 expressions had prominently better overall survival rates compared to those with higher expressions (log-rank test, p = 0.0267, 0.0145, and 0.0194).

DISCUSSION

CircRNAs have been shown to participate in the regulation of various tumor progressions, including CRC. For instance, circRNA-100290 level was markedly increased in CRC tissues and cell lines which may promote CRC progression via miR-516b/FZD4 signaling pathway [18]. In addition, some circRNAs were confirmed to be associated with poor prognosis in patients with CRC. Wang et al. found that low expression level of hsa_circ_001988 was correlated with favorable outcome of CRC patients along with the area under ROC curve of 0.788 [19]. Jin et al. showed that increased hsa_circ_0005075 predicts a poor prognosis and acts as an oncogene in CRC [20]. Owing to the importance and stability of circRNAs in CRC, accumulating studies focused on the possibility of circRNAs as new biomarkers concerning detecting CRC and predicting outcome. According to a previous study circFMN2 was significantly increased in CRC tissues and cell lines, while knockdown of cirFMN2 could inhibit cell proliferation and migration indicating circFMN2 may be an oncogene in CRC [15]. Furthermore, the up-regulation of circ-LMNB1 could enhance the malignant characteristics and progression in CRC [16]. Circ-ZNF609 was known for to dysregulate various tumors and function as an oncogene in breast cancer [21], gastric cancer [22], nasopharyngeal carcinoma [23], and CRC [17]. In this manuscript, we investigated the clinical significance of three circRNAs in CRC patients. From the results, circ-FMN2, circ-LMNB1, and circ-ZNF609 were remarkably increased in serum samples of CRC patients as compared to healthy controls. After analyzing the correlation between these cirRNAs and clinicopathological parameters, circ-FMN2, circ-LMNB1, and circ-ZNF609 up-regulation was correlated with histological grade, lymph node metastasis, and TNM stage. Notably, the ROC curve analysis confirmed that circ-FMN2, circ-LMNB1, and circ-ZNF609 may function as biomarkers for CRC diagnosis with high specificity and sensitivity. Notably, by Kaplan-Meier analysis, we further verified that higher circ-FMN2, circ-LMNB1, and circ-ZNF609 expressions were associated with worse overall survival.

CONCLUSION

In the present study, we firstly evaluated circ-FMN2, circ-LMNB1, and circ-ZNF609 as diagnostic and prognostic indicators for CRC which may contribute to clinical diagnosis and treatment.

Declaration of Interest:
None.

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