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Reduced serum exosomal miR-874 expression predicts poor prognosis in colorectal cancer

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Abstract. – OBJECTIVE: Emerging evidence has indicated that serum exosomal microRNAs (miRNAs) have promising diagnostic and prognostic value for colorectal cancer (CRC). This study aimed to detect serum exosomal miR-874 expression in CRC patients and assess its potential clinical significance.

PATIENTS AND METHODS: Blood samples were collected from 125 CRC patients, 45 cases with benign adenomas (AD) and 70 healthy individuals. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to examine serum exosomal miR-874 levels.

RESULTS: The results showed that serum exosomal miR-874 levels were significantly downregulated in CRC patients compared to AD cases and healthy controls. Receiver operating characteristic (ROC) curve analysis revealed that serum exosomal miR-874 expression could discriminate CRC patients from healthy controls, as well as patients with AD. In addition, low serum exosomal miR-874 expression was associated with positive distant metastasis, positive lymph node metastasis, poor differentiation, and advanced TNM stage. Moreover, serum exosomal miR-874 expression was identified as a statistically significant independent prognostic factor for overall survival of CRC patients.

CONCLUSIONS: Collectively, serum exosomal miR-874 expression might serve as a reliable marker for CRC diagnosis and prognosis prediction.

Key Words:
Colorectal cancer, Serum exosome, MiR-874, Biomarker.

Introduction

Colorectal cancer (CRC) is one of the most prevalent malignant tumors and the third leading cause of cancer related deaths around the world[1]. In 2015, about 377,000 new cases were diagnosed with CRC and 191,100 CRC related deaths were estimated to occur in China[2]. The survival rate of CRC patients can be greatly improved if patients were diagnosed at the early stages. Unfortunately, the majority of patients are confirmed at the advanced stages due to the asymptomatic nature of CRC, leading to unfavorable prognosis[3,4]. Currently, serum carcinoembryonic antigen (CEA) is widely used for CRC early detection and prognosis prediction. However, the effectiveness of CEA as a preoperative and postoperative marker for CRC is still controversial and inconclusive[5]. Therefore, identification of reliable and non-invasive biomarkers is urgently required to improve the diagnosis and prognosis of CRC. MicroRNAs (miRNA) are a class of small non-coding RNAs (18-22 nucleotides in length) that regulate target gene expression by interfering with transcription or inhibiting translation[6,7]. MiRNAs play an important role in various biological processes including, but not limited to, cell proliferation, differentiation, organogenesis, and apoptosis. Aberrant expression of miRNAs has been shown to be closely associated with the initiation and progression of many human diseases including cancer. MiRNAs might function as either onco-miRNAs and tumor suppressive miRNAs during tumorigenesis, depending on the downstream targets they regulated. From a clinical point of view, circulating miRNAs are promising biomarkers for the detection and prognosis prediction and due to that they are highly stable in the biofluids[8-10]. Exosomes are small membranous vesicles with a maximum size of 150 nm and contain a variety of biomolecules, including lipids, proteins,

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DNA, and RNA, which play a crucial role in local and remote cell-cell communication[11,12]. Abnormal expression of exosomal miRNAs has been demonstrated to be involved in carcinogenesis of CRC. For instance, serum exosomal miR-203 expression was significantly upregulated in CRC patients and *in vitro* and *in vivo* analysis showed exosomal miR-203 overexpression greatly promoted CRC progression[13]. While low serum exosomal miR-548c-5p expression was significantly associated with aggressive clinical variables and shorter overall survival of CRC patients, indicating exosomal miR-548c-5p acted as a tumor suppressor gene in CRC[14].

MiR-874, located on chromosome 5q31.2, functioned as a tumor suppressor in some cancer types such as CRC[15,16], gastric cancer[17,18], hepatocellular carcinoma[19], breast cancer[20], and osteosarcoma[21]. However, the diagnostic and prognostic value of serum exosomal miR-874 in CRC remains poorly known. In the study, we evaluated the expression level of serum exosomal miR-874 in patients with CRC and explored its potential clinical significance as a biomarker for CRC diagnosis and prognosis.

Patients and Methods

Patients and Blood Samples

A total of 125 patients with CRC, 45 subjects with benign adenomas (AD) and 70 healthy volunteers were enrolled in this study. No patients had received any chemotherapy or radiotherapy prior to the treatment. The serum CEA levels in all CRC patients were measured by standard enzyme immunoassay as a routine clinical test. Tumor stage and histological grade were categorized according to the American Joint Committee on Cancer (AJCC) tumor/node/metastasis (TNM) staging system. Patient clinicopathological data, including age, gender, tumor size, tumor location, CEA, differentiation, lymph node metastasis, distant metastasis and TNM stage, were presented in Table I. Up to five mL of whole blood were withdrawn from all CRC patients, AD cases, and control subjects. Blood samples were centrifuged at 1200 g for 10 min. Then, the supernatant was divided into small aliquots and stored at −80°C.

Table I. Association between serum exosomal miR-874 expression and clinicopathological characteristics.

Characteristics	Patients (No. = 125)	Low miR-874	High miR-874	*p*-value
Age				0.582
<60	93	43	50	
≥60	32	13	19	
Gender				0.451
Male	76	32	44	
Female	49	24	25	
Tumor size				0.096
<5 cm	77	30	47	
≥5 cm	48	26	22	
Tumor location				0.167
Colon	71	28	43	
Rectal	54	28	26	
CEA				0.119
<5 ng/mL	61	23	38	
≥5 ng/mL	64	33	31	
Differentiation				0.002
Well/Moderate	68	22	46	
Poor	57	34	23	
Lymph node metastasis				0.007
No	59	19	40	
Yes	66	37	29	
Distant metastasis				0.011
No	92	35	57	
Yes	33	21	12	
TNM stage				<0.001
I/II	53	8	45	
III/IV	72	48	24	

Ethics Statement

The current study was approved by the Ethics Committee of Tianjin First Central Hospital. Written informed consents were collected from all participants before sample collection. All specimens were handled and made anonymous according to the ethical and legal standards.

Exosomal RNA Extraction and Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

The exosomes were isolated from serum using the total exosome isolation reagent (from serum) (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's instructions. Total RNA was extracted from exosomal samples using Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA, USA). For normalization of sample-to-sample variation during RNA isolation, 25 fmol of synthetic C. elegans miRNA-39 (cel-miR-39) was added into each sample. RNA concentrations were quantified using ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was performed using the TaqMan MicroRNA Reverse Transciption Kit (Applied Biosystems, Foster City, CA, USA). Quantitative RT-PCR was carried out with a TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). All samples were run in triplicate. The relative serum exosomal miR-874 levels were calculated using $2^{-\Delta\Delta Ct}$ method and normalized to spike-in control cel-miR-39. The primers were shown in Table II.

Statistical Analysis

The statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and MedCalc 12.0 (MedCalc Software, Ostend, Belgium). The differences between two groups or three groups were performed using the Mann-Whitney U-test or Kruskal-Wallis test, respectively. The Chi-square test was used to explore the associations between serum exosomal miR-874 expression and clinicopathologic variables. Receiver operating characteristic (ROC) curves and areas under the ROC curves values (AUC) were used to evaluate the diagnostic potential of serum exosomal miR-874 for CRC. Overall survival (OS) curves were analyzed using the Kaplan-Meier method, and differences were compared by log-rank test. Cox's proportional hazards models were performed to assess the independent predictive factors of OS. All differences were regarded as statistically significant when $p<0.05$. OS was defined as the time elapsed between treatment and death or the date of the last follow-up.

Results

Downregulation of Serum Exosomal MiR-874 In Patients With CRC

To analyze the role of serum exosomal miR-874 in CRC patients, qRT-PCR was performed to measure the levels of serum exosomal miR-874. As shown in Figure 1A, serum exosomal miR-874 levels were significantly lower in CRC patients than in AD subjects ($p<0.0001$) and healthy controls ($p<0.0001$). In addition, significantly lower serum exosomal miR-874 expression was found in AD cases compared with the controls ($p=0.0394$). Moreover, we found serum exosomal miR-874 levels in patients with distant metastasis ($p=0.0176$, Figure 1B) or lymph node metastasis ($p=0.0091$, Figure 1C) were markedly decreased compared to those without metastasis. Additionally, high serum exosomal miR-874 expression occurred more frequently in CRC patients with well/moderate differentiation ($p=0.0024$, Figure 1D) and I/II stage ($p=0.0008$, Figure 1E).

The Diagnostic Value of Serum Exosomal MiR-874 for CRC

ROC curve revealed that serum exosomal miR-874 had good diagnostic potential to discriminate CRC subjects from controls with the AUC of 0.818, the specificity and sensitivity were 78.6% and 80.8%, respectively (Figure 2A). More im-

Table II. Primers for qRT-PCR.

Gene	Forward primer	Reverse primer
miR-874	GGCCCTGAGGAAGAACTGAG	TGAG ATCCAACAGGCCTTGAC
cel-miR-39	CAGAGTCACCGGGTGTAAAT	CCAGTGCGTGTCGTGGAGTC

portantly, combining CEA and serum exosomal miR-874 could well differentiate CRC patients from control subjects with the AUC of 0.894, and the sensitivity was 85.6% and specificity was 81.4%, indicating CEA/miR-874 combination had better diagnostic power than either marker alone (Figure 2B). ROC curve was also performed on CRC patients and AD subjects, and the AUC, specificity and sensitivity for serum exosomal miR-874 were 0.729, 62.2%, and 76.8%, respectively (Figure 2C).

Serum Exosomal MiR-874 Expression and Clinical Variables

Based on the median value of serum exosomal miR-874 expression, we divided all CRC patients into high serum exosomal miR-874 expression group (n=69) and low serum exosomal miR-874 expression group (n=56). The association between serum exosomal miR-874 expression and clinical characteristics was then investigated. As presented in Table I, low serum exosomal miR-874 expression was strongly correlated with differentiation (p=0.002), lymph node metastasis (p=0.007), distant metastasis (p=0.011), and TNM stage (p<0.001). In contrast, there was no association of serum exosomal miR-874 expression with age (p=0.582), gender (p=0.451), tumor size (p=0.096), tumor location (p=0.167), and CEA (p=0.119).

Serum Exosomal MiR-874 Expression and Survival in CRC Patients

The association between serum exosomal miR-874 expression and prognosis of CRC was investigated. Patients with high serum exosomal miR-874 expression levels had significantly longer OS than those with low serum exosomal miR-874 expression (p=0.0085, Figure 3A). In addition, patients with distant metastasis (p=0.0236, Figure 3B) and lymph node metastasis (p=0.0171, Figure 3C) had shorter OS than those without. Moreover, patients with poor differentiation (p=0.0133, Figure 3D) or at the advanced TNM stage had short-

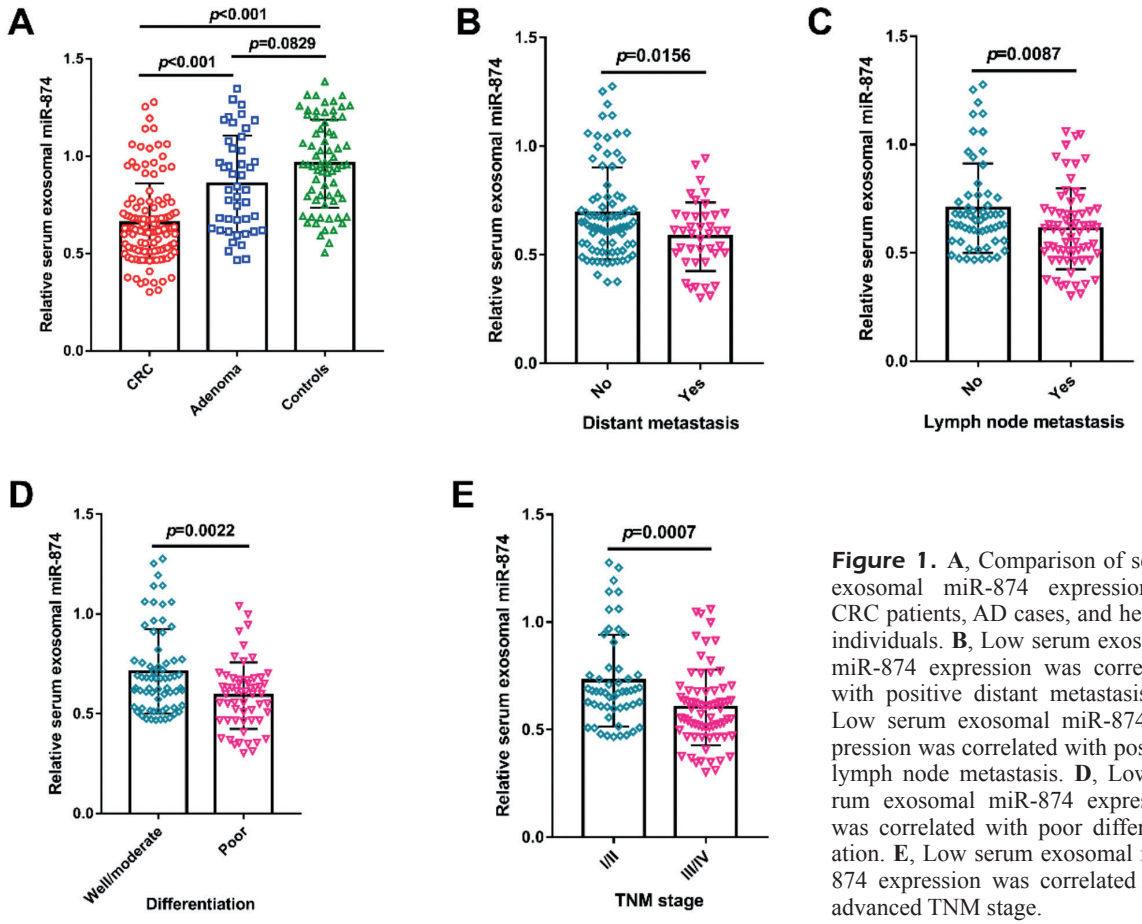

Figure 1. **A**, Comparison of serum exosomal miR-874 expression in CRC patients, AD cases, and healthy individuals. **B**, Low serum exosomal miR-874 expression was correlated with positive distant metastasis. **C**, Low serum exosomal miR-874 expression was correlated with positive lymph node metastasis. **D**, Low serum exosomal miR-874 expression was correlated with poor differentiation. **E**, Low serum exosomal miR-874 expression was correlated with advanced TNM stage.

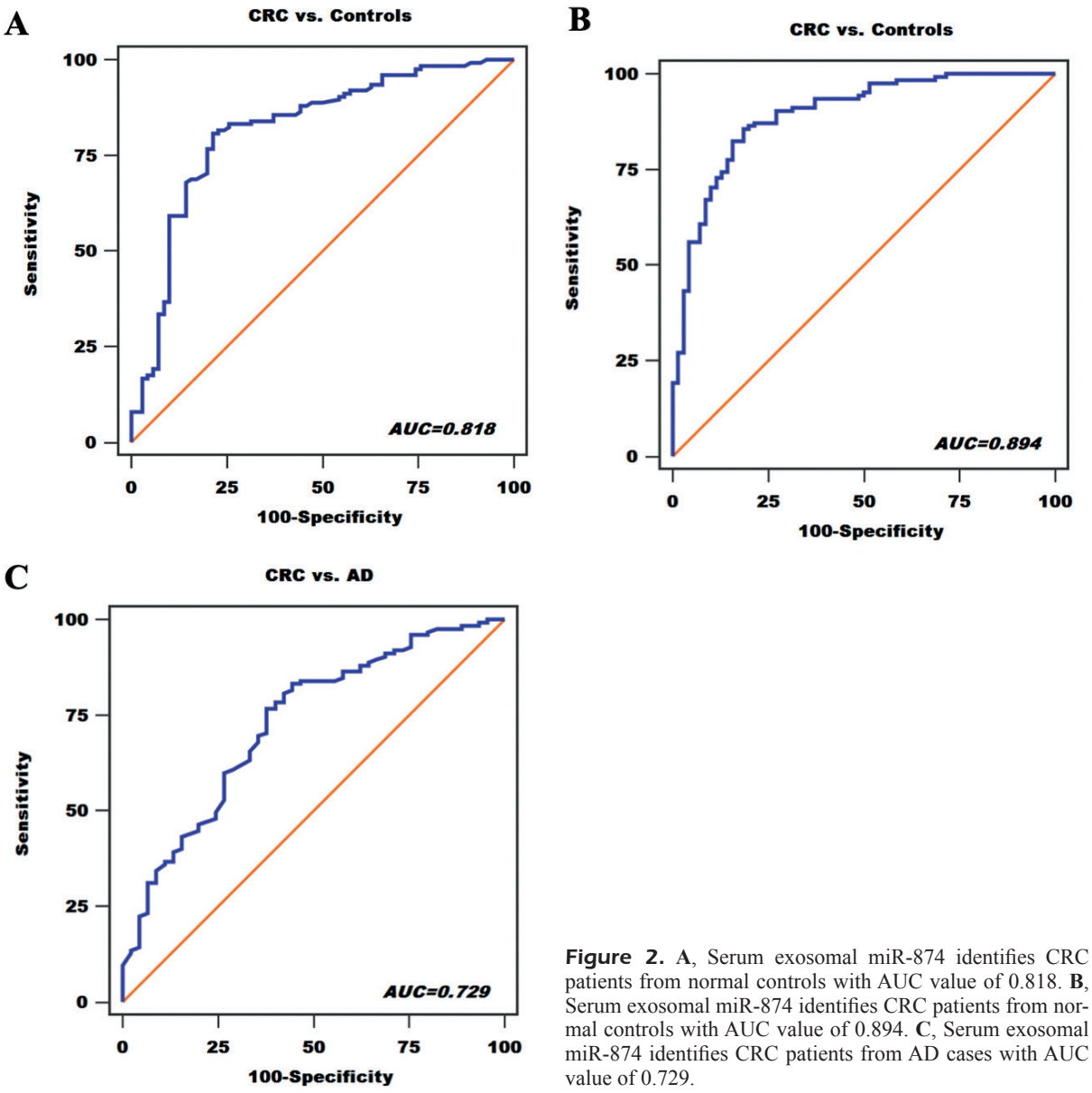

Figure 2. **A**, Serum exosomal miR-874 identifies CRC patients from normal controls with AUC value of 0.818. **B**, Serum exosomal miR-874 identifies CRC patients from normal controls with AUC value of 0.894. **C**, Serum exosomal miR-874 identifies CRC patients from AD cases with AUC value of 0.729.

er OS (p=0.0010, Figure 3E). Univariate analysis for OS showed that distant metastasis (HR=2.32; 95% CI=1.54-3.28; p=0.024), lymph node metastasis (HR=2.85; 95% CI=1.78-3.96; p=0.017), differentiation (HR=3.12; 95% CI=1.92-4.41; p=0.013), TNM stage (HR=3.67; 95% CI=2.46-5.17; p=0.002), and serum exosomal miR-874 (HR=3.34; 95% CI=2.15-4.74; p=0.008) were indicators of poor prognosis of CRC patients. In multivariate analysis, five parameters including distant metastasis (HR=2.75; 95% CI=1.62-3.91; p=0.020), lymph node metastasis (HR=3.18; 95% CI=1.94-4.52; p=0.012), differentiation (HR=3.23; 95% CI=2.13-4.52; p=0.009), TNM stage (HR=3.51; 95% CI=2.28-4.94; p=0.003), and serum exosomal miR-874 (HR=3.07; 95% CI=1.84-4.32; p=0.014) were strongly associated with worse OS (Table III).

Discussion

In this study, first, we demonstrated that serum exosomal miR-874 levels in CRC patients were significantly lower than those in AD subjects and healthy individuals, low serum exosomal miR-874 expression was strongly associated with aggressive clinical features. Second, ROC

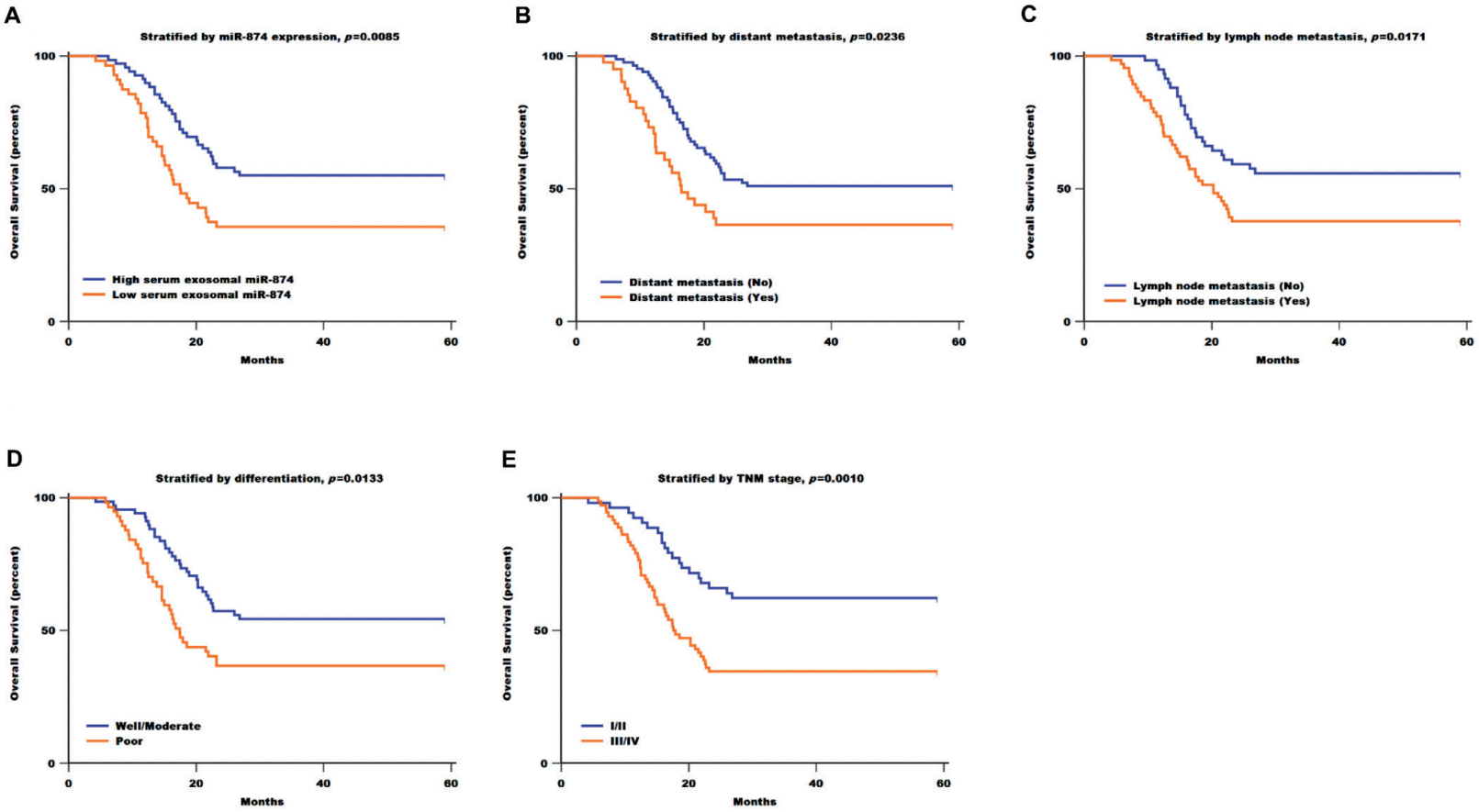

Figure 3. **A**, Overall survival curves of CRC patients stratified by serum exosomal miR-874 expression. **B**, Overall survival curves of CRC patients stratified by distant metastasis. **C**, Overall survival curves of CRC patients stratified by lymph node metastasis. **D**, Overall survival curves of CRC patients stratified by differentiation. **E**, Overall survival curves of CRC patients stratified by TNM stage.

Table III. Univariate and multivariate analyses of prognostic factors for OS of CRC patients.

		Univariate analysis		Multivariate analysis	
Features		**HR (95% CI)**	***p***	**HR (95% CI)**	***p***
Age (years)	≥60 *vs.* <60	1.17 (0.85-1.51)	0.257	-	-
Gender	Male *vs.* Female	1.21 (0.91-1.57)	0.215	-	-
Tumor size (cm)	≥5 *vs.* <5	1.45 (1.07-1.92)	0.147	-	-
CEA (ng/mL)	≥5 *vs.* <5	1.56 (1.14-2.03)	0.118	-	-
Differentiation	Poor *vs.* Well/Moderate	3.12 (1.92-4.41)	0.013	3.23 (2.13-4.52)	0.009
Lymph node metastasis	Yes *vs.* No	2.85 (1.78-3.96)	0.017	3.18 (1.94-4.52)	0.012
Distant metastasis	Yes *vs.* No	2.32 (1.54-3.28)	0.024	2.75 (1.62-3.91)	0.020
TNM stage	III/IV *vs.* I/II	3.67 (2.46-5.17)	0.002	3.51 (2.28-4.94)	0.003
Serum exosomal miR-874	Low *vs.* High	3.34 (2.15-4.74)	0.008	3.07 (1.84-4.32)	0.014

analysis showed that serum exosomal miR-874 had good diagnostic power in identifying CRC patients from normal controls. Third, CRC patients with lower serum exosomal miR-874 expression had shorter OS. Finally, serum exosomal miR-874 emerged as an independent prognostic marker for OS of CRC patients. Previously it was also reported that miR-874 played a tumor suppressive role in CRC. Zhao et al[15] showed that miR-874 expression was markedly lower in CRC tissues, and miR-874 upregulation significantly restrained cancer cell growth and stimulated cell apoptosis by degrading signal transducer and activator of transcription 3 (STAT3). Similarly, downregulation of miR-874 expression was found in CRC tissues and cell lines, miR-874 overexpression or X-linked inhibitor of apoptosis protein (XIAP) inhibition significantly attenuated the tumorigenesis and enhanced chemosensitivity in CRC cells[16]. Besides CRC, the role of miR-874 has also been reported in various types of cancer. For instance, miR-874 expression was significantly decreased in GC tissues. MiR-874 upregulation greatly suppressed the growth, migration, invasion, and tumor angiogenesis of GC cells by inversely regulating STAT3[17] and aquaporin 3 (AQP3)[18]. Jiang et al[19] showed that miR-874 expression was markedly decreased both in cancer tissues and cell lines of hepatocellular carcinoma (HCC), miR-874 overexpression or SRY-related high-mobility-group box 12 (SOX12) inhibition repressed invasion and epithelial-mesenchymal transition and *vice versa*. In breast cancer, miR-874 downregulation was observed in cancerous tissues and ectopic miR-874 expression remarkably inhibited the tumorigenesis by regulating cyclin-dependent kinase 9 (CDK9)[20]. Dong et al[21] revealed miR-874 expression was frequently reduced in osteosarcoma cell lines and tissues, miR-874 upregulation and E2F transcription factor 3 (E2F3) knockdown exhibited inhibitory effects in cancer cells, including reduced proliferation, migration, and invasion *in vitro*. Nohata et al[22] reported that miR-874 downregulation occurred more frequently in head and neck squamous cell carcinoma tissues, and restoration of miR-874 significantly restrained the carcinogenesis by degrading histone deacetylase 1 (HDAC1). In esophageal squamous cell carcinoma (ESCC), miR-874 was underexpressed in ESCC tissues and cell lines, and reduced miR-874 expression was strongly associated with multiple aggressive phenotypes of ESCC[23]. Zhang et al[24] showed that miR-874 expression was remarkably downregulated in both retinoblastoma tissues and cell lines, miR874 upregulation could inhibit cancer cell proliferative and invasive abilities by targeting metadherin. In addition, miR-874 downregulation was found in cervical cancer tissues and cell lines, miR-874 overexpression could contribute to decreased cancer cell proliferation, migration, and invasion[25]. In pancreatic ductal adenocarcinoma (PDAC), miR-874 expression was significantly decreased in PDAC tissues and cell lines. Upregulation of miR-874 attenuated cell proliferation and invasiveness by negatively regulating the expressions of paired box 6 (PAX6)[26]. In rhabdomyosarcoma, *in vitro* analysis revealed that miR-874 overexpression or guanine nucleotide exchange factor (GEFT) inhibition significantly suppressed cancer cell proliferation, invasion, migration, and promoted apoptosis[27]. Moreover, *in vitro* and *in vivo* evidence showed that[28] miR-874 upregulation remarkably inhibited the tumorigenicity in non-small cell lung cancer. These data suggested that miR-874 might exert a tumor suppressive function in the initiation and progression of many tumor types.

Conclusions

To the best of our knowledge, this is the first study to explore the role of serum exosomal miR-874 in CRC. We found serum exosomal miR-874 was markedly under expressed in CRC patients. In addition, lower serum exosomal miR-874 level was associated with shorter survival and poorer prognosis. Taken together, serum exosomal miR-874 is a reliable and novel biomarker for CRC detection and prognosis prediction.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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