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A Novel Circulating MiRNA-Based Signature for the Diagnosis and Prognosis Prediction of Early-Stage Cervical Cancer

Huajuan Qiu, MD[1], Duoxian Liang, MD[1], Limin Liu, MD[1], Qun Xiang, MD[1], Zhijun Yi, MD[1], and Yanqin Ji, MD[1]

Abstract

Background: MicroRNAs (miRNAs) have been shown to play a key role in regulating the progression of cervical cancer (CC). This study aimed to develop a circulating miRNA-based molecular signature for the diagnosis and prognosis prediction of early-stage CC. **Methods:** This study included 112 patients diagnosed with early-stage CC, 45 patients confirmed with cervical intraepithelial neoplasia (CIN) and 90 healthy subjects. Compared to the normal controls, the expression level of miR-21 was increased, while the levels of miR-125b and miR-370 were decreased in CC in both GSE30656 and The Cancer Genome Atlas (TCGA) cohort. The expression levels and diagnostic value of these candidate miRNAs were then validated through qRT-PCR. Their diagnostic and prognostic values for early-stage CC were further explored. **Results:** Compared to the patients with CIN and healthy subjects, serum miR-21 was increased, while serum miR-125b and serum miR-370 were reduced in early-stage CC. In addition, combining these molecules yielded good performance for differentiating early-stage CC from CIN or healthy subjects. Moreover, strong association was found between serum miR-21 and lymph node metastasis (LNM) as well as recurrence-free survival of early-stage CC. Similar observations were found for serum miR-125b and serum miR-370. Patients with simultaneously high serum miR-21 + low serum miR-125b + low serum miR-370 suffered a high risk of LNM and recurrence, while those with low serum miR-21 + high serum miR-125b + high serum miR-370 had little risk for LNM and recurrence. **Conclusions:** Combining serum miR-21, miR-125b and miR-370 as a miRNA-based signature is promising for the detection and prognosis prediction of early-stage CC.

Keywords

miR-21, miR-125b, miR-370, early-stage cervical cancer, early diagnosis, prognosis prediction

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Introduction

According to the Global Cancer Statistics 2018 report, cervical cancer (CC) is the fourth most frequent cancer among female worldwide and the fourth most common cause of mortality in women.[1] Patients at the early-stages have significantly better clinical outcome following systemic treatments than those at the advanced stages. However, a large population of CC cases are diagnosed at the late stages due to its asymptomatic and non-specific nature in the early stages.[2] Papanicolaou test (pap smear) and colposcopy are the widely used methods for the detection of CC. The sensitivity of pap smear screening is heterogenous within different study populations, which might lead to the false negative results.[3] Colposcopy is an invasive procedure and might generate additional costs and risks.[4] In addition, these methodologies fail to provide the information for predicting the recurrence and prognosis of early-stage CC. Therefore, it is urgently needed to develop novel biomarkers for the detection and prognosis prediction of early-stage CC.

MicroRNAs (miRNAs) are small and highly conserved non-coding RNAs that regulate gene expression at the post-transcriptional level.[5] They bind to the 3′ untranslated region

[1] Huizhou Central People's Hospital, Huizhou, Guangdong Province, China

Corresponding Author:
Yanqin Ji, Huizhou Central People's Hospital, Huizhou 516000, Guangdong Province, China.
Email: yanqinjihzch@163.com

(3′-UTR) of target mRNAs to induce degradation or translational repression. MiRNAs regulate a number of important biological processes such as proliferation, differentiation and development. Dysregulation of miRNAs is related to many human diseases including cancer.[6-10] MiRNAs might act as either tumor suppressive miRNAs or oncomiRs in the initiation and development of cancer, depending on the tumor types and the downstream targets they regulate in the specific tumor microenvironment. Accumulative evidence has demonstrated that miRNAs are highly stable in bodily fluids.[11] Therefore, circulating miRNAs are ideal molecules as biomarkers for the early detection and prognosis of CC. For instance, the level of circulating miR-486-5p was significantly upregulated in CC patients, indicating it might serve as a diagnostic biomarker tor CC.[12]

In this study, we first profiled the commonly changed miRNAs in 2 accessible datasets GSE30656 and The Cancer Genome Atlas (TCGA) CESC cohort. Three commonly altered miRNAs including miR-21, miR-125b and miR-370 were identified. Then the levels of circulating miR-21, miR-125b and miR-370 were determined in early-stage CC patients, patients with cervical intraepithelial neoplasia (CIN) and healthy controls. The early diagnostic value and prognosis prediction potential of this circulating miRNA-based signature for the CC was further investigated.

Materials and Methods

Study Population

The study enrolled 112 patients diagnosed with early-stage CC and 45 patients confirmed with CIN. Ninety healthy subjects were recruited as controls. All the participants were Han ethnicity and the samples were collected in our hospital. Only CC patients with no prior therapy and with available clinical follow-up data were included. The CC patients were staged based on the FIGO (International Federation of Gynecology and Obstetrics) staging system. The inclusion criteria of early-stage CC were: i) Pathological diagnosis of CC; ii) The CC was at the stage I-IIA based on FIGO. The exclusion criteria were active infections, chronic inflammatory diseases, secondary malignancy and undetermined cervical abnormalities. The inclusion criteria of CIN: histologically confirmed CIN1 or CIN2 or CIN3 lesion. The exclusion criteria were active infections, chronic inflammatory diseases, other malignancy or precancerous lesions and undetermined cervical abnormalities. Healthy controls were free of any malignancy or precancerous lesions or any chronic disease. The clinical characteristics of this study cohort was listed in Table 1.

Table 1. The Clinical Characteristics of the Study Cohort.

Clinical characteristics	Early-stage CC, n = 112	CIN, n = 45	Healthy subjects, n = 90
Age			
≥50	53	20	41
<50	59	25	49
Menopause			
Yes	50	22	42
No	62	23	48
Histological subtype		/	/
SCC	107		
AC	5		
Tumor size (cm)		/	/
<4	87		
≥4	25		
Tumor grade		/	/
G1	95		
G2	17		
G3	0		
FIGO		/	/
I	90		
IIA	22		
Recurrence		/	/
Yes	19		
No	93		
Lymph node metastasis		/	/
Positive	20		
Negative	92		

SCC: squamous cell carcinoma, AC: adenocarcinoma.

Serum Samples

At least 6 mL fasting venous blood samples were collected, and none of the patients received any treatment prior to collection. Following centrifugation at 4000 rpm for 10 min at 4°C, serum samples were obtained and stored at −80°C until further analysis.

RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from 200 μL serum samples using miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The quality and concentration of total RNA were determined by a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The first-strand cDNA was obtained by reverse transcription with the SuperScript III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). The amplification and quantification of cDNA were performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) and SYBR Premix Ex Taq (Takara, Dalian, China). The amplification program was initiated with denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The exogenous synthetic miRNA cel-miR-39 was used as the external control. All the above-mentioned steps were conducted based on the manufacturers' protocols. The relative expression of serum miRNAs was quantified using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

The normality test was performed first to assess the data distribution. As the data was not normally distributed, the nonparametric Kruskal-Wallis test was used to compare the differences of circulating miRNAs among different groups.

Then the area under the curve (AUC) by conducting receiver operating characteristic (ROC) analysis was calculated to evaluate the early diagnostic values of circulating miR-21, miR-125b and miR-370. Recurrence-free survival (RFS) was defined as the time duration from randomization until occurrence of local, regional or distant recurrence. The differences in RFS between different groups were plotted by the Kaplan-Meier method and compared using the log-rank test. Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA) and Medcalc statistical software (Medcalc Software, Ostend, Belgium). All statistical tests were 2 tailed, and $P < 0.05$ was considered to be statistically significant.

Results

The Diagnostic Values of Serum miR-21, miR-125b and miR-370 in Early-Stage CC

We first identified the commonly and significantly changed miRNAs between cervical cancer and normal cervical epithelium through the dbDEMC 2.0 (https://www.picb.ac.cn/dbDEMC/). Two studies namely GSE30656 and TCGA cohort were included. Then the commonly and significantly changed miRNAs between cervical cancer and normal cervical epithelium were screened and identified. The cut-off was set as fold change$\geq$2 and $P < 0.05$. Compared to the normal controls, the expression level of miR-21 was increased, while the levels of miR-125b and miR-370 were decreased in CC in both GSE30656 and TCGA cohort. Therefore, these candidate miRNAs were selected for further validation. For the number of included patients, there were 10 CC cases and 10 controls in GSE30656, as well as 299 CC cases and 3 controls in the TCGA cohort.

As shown Figure 1A, the expression level of serum miR-21 was significantly higher in early-stage CC patients than in patients with CIN and healthy subjects ($^{**}P < 0.01$, $^{***}P < 0.001$). The AUC values for discriminating early-stage CC from healthy controls or CIN were 0.783 and 0.689, respectively. On the contrary, the circulating miR-125b was markedly reduced in early-stage CC patients compared to patients with CIN and healthy controls ($^{**}P < 0.01$, $^{***}P < 0.001$). The AUC values of serum miR-125b for differentiating early-stage CC from healthy subjects or CIN were 0.642 and 0.735, respectively (Figure 1B). Similarly, the level of serum miR-370 was significantly lower in early-stage CC patients than in patients with CIN and healthy subjects ($^{***}P < 0.001$). For serum miR-370, the AUC values serum for identifying early-stage CC from healthy controls or CIN were 0.822 and 0.821, respectively (Figure 1C).

The Early Diagnostic Value of the Circulating miRNA-Based Signature Including Serum miR-21, miR-125b and miR-370

Then serum miR-21, miR-125b and miR-370 was combined as a miRNA signature to evaluate its diagnostic accuracy for early-stage CC. Interestingly, the newly developed circulating miRNA signature was able to discriminate early-stage CC from healthy controls with an AUC value of 0.912 (Figure 2A). Similarly, the AUC value for the circulating miRNA signature identifying early-stage CC from CIN was 0.897 (Figure 2B).

The Association Between Serum miR-21/miR-125b/miR-370 and Lymph Node Metastasis (LNM) of Early-Stage CC

The median level of serum miR-21 in the early-stage CC was used as the cut-off to split the early-stage CC patients into high serum miR-21 group and low serum miR-21 group. As shown in Figure 3, a higher percentage of CC patients suffering LNM was observed in the high serum miR-21 group (25.00%) than in the low serum miR-21 group (10.71%). Similarly, a higher percentage of LNM was observed in the low serum miR-125b group (23.21%) than in the high serum miR-125b group (12.50%). Also, the patients in the serum miR-370 group (28.57%) had a significantly higher change of LNM than those in the high serum miR-370 group (7.14%). Then we explored the association between the combined serum miRNA signature and LNM of early-stage CC. A total of 17 patients were with simultaneously high serum miR-21 + low serum miR-125b + low serum miR-370, and 21 patients were with simultaneously low serum miR-21 + high serum miR-125b + high serum miR-370. Surprisingly, up to 52.94% patients in the high serum miR-21 + low serum miR-125b + low serum miR-370 group suffered LNM, while none of the patients in the low serum miR-21 + high serum miR-125b + high serum miR-370 group had LNM.

The Association Between Serum miR-21/miR-125b/ miR-370 and RFS of Early-Stage CC

As shown in Figure 4A, the patients in the high serum miR-21 group had worse RFS than those in the low serum miR-21 group ($P = 0.027$). Although no statistical significance was observed for serum miR-125b between high and low serum miR-125b group ($P = 0.078$), the trend that the patients in the low serum miR-125b had shorter RFS could be observed (Figure 4B). The patients in the low serum miR-370 group suffered worse RFS compared to those in the high serum miR-370 group ($P = 0.001$) (Figure 4C). Interestingly, the patients in the high serum miR-21 + low serum miR-125b + low serum miR-370 group suffered significantly shorter RFS than those in the low serum miR-21 + high serum miR-125b + high serum miR-370 group ($P < 0.001$). None of the patients in the low serum miR-21 + high serum miR-125b + high serum miR-370 group had recurrence following treatments (Figure 4D).

Discussion

In this study, compared to the CIN patients and healthy subjects, the level of circulating miR-21 is significantly increased

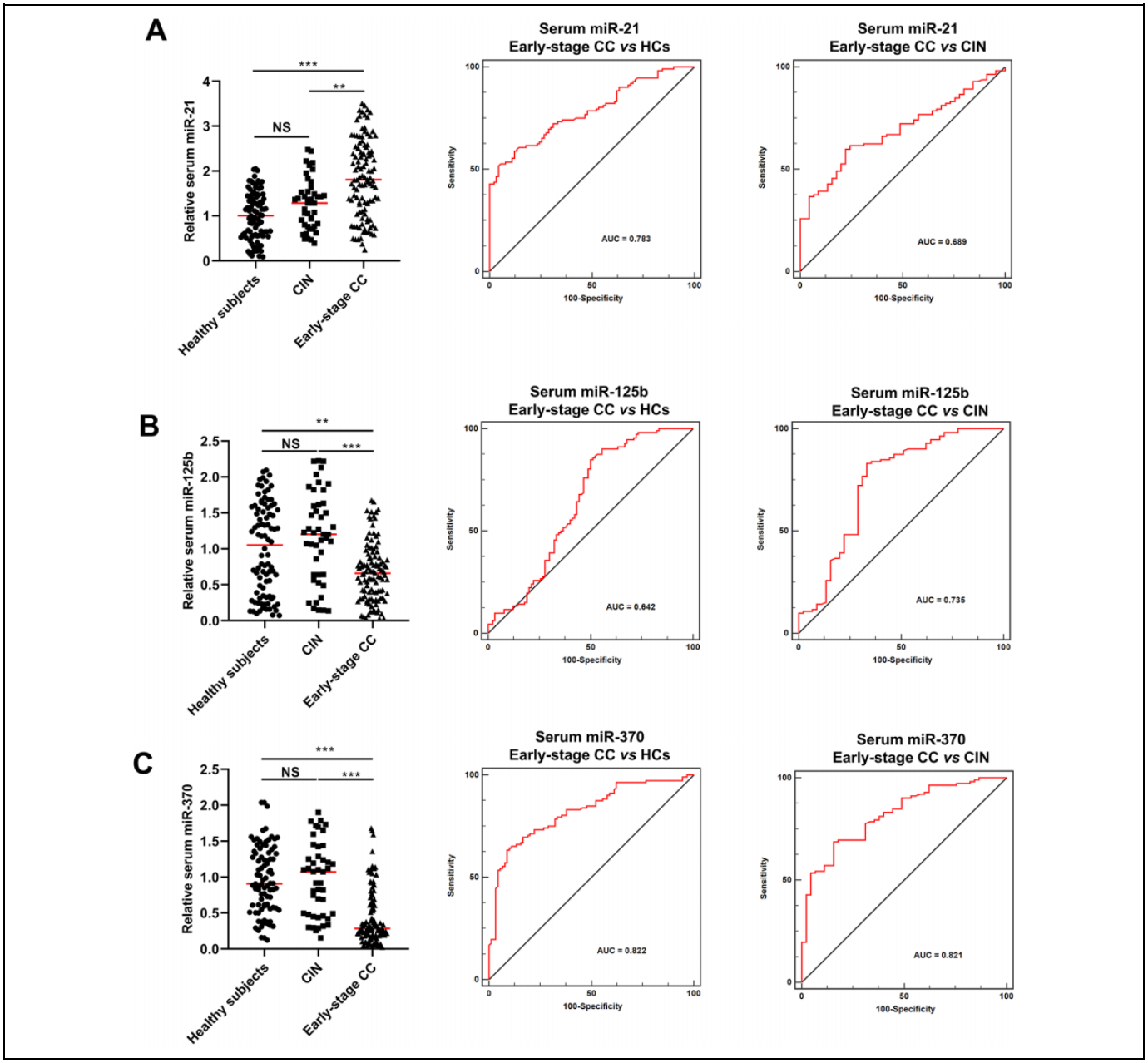

Figure 1. The diagnostic value of serum miR-21, serum miR-125b and serum miR-370 for early-stage CC. A, The expression level of serum miR-21 was increased in early-stage CC patients compared to patients with CIN and healthy subjects, and the diagnostic accuracy of serum miR-21 was revealed by ROC analysis. B, Serum miR-125b was reduced in early-stage CC and its diagnostic accuracy. C, Serum miR-370 was reduced in early-stage CC and its diagnostic accuracy.

in early-stage CC patients, while serum miR-125b and serum miR-370 are markedly reduced. In addition, combining serum miR-21, serum miR-125b and serum miR-370 as a miRNA signature exhibits good performance for identifying the early-stage CC from CIN or healthy controls. Moreover, high serum miR-21, low serum miR-125b and low serum miR-370 are strongly associated with LNM and recurrence. The patients with simultaneously high serum miR-21 + low serum miR-125b + low serum miR-370 suffer a higher chance of LNM and recurrence, while those with simultaneously low serum miR-21 + high serum miR-125b + high serum miR-370 experience no LNM and recurrence. Collectively, this novel circulating miRNA-based signature which includes serum miR-21, serum miR-125b and serum miR-370 shows great promise for the detection and prognosis prediction for early-stage CC.

Consistent with our results, various independent studies have indicated that the expression level of circulating miR-21 was significantly increased in patients with CC compared to the healthy controls.[13,14] In addition, increased serum miR-21 level was strongly associated with lymph node metastasis of CC.[15]

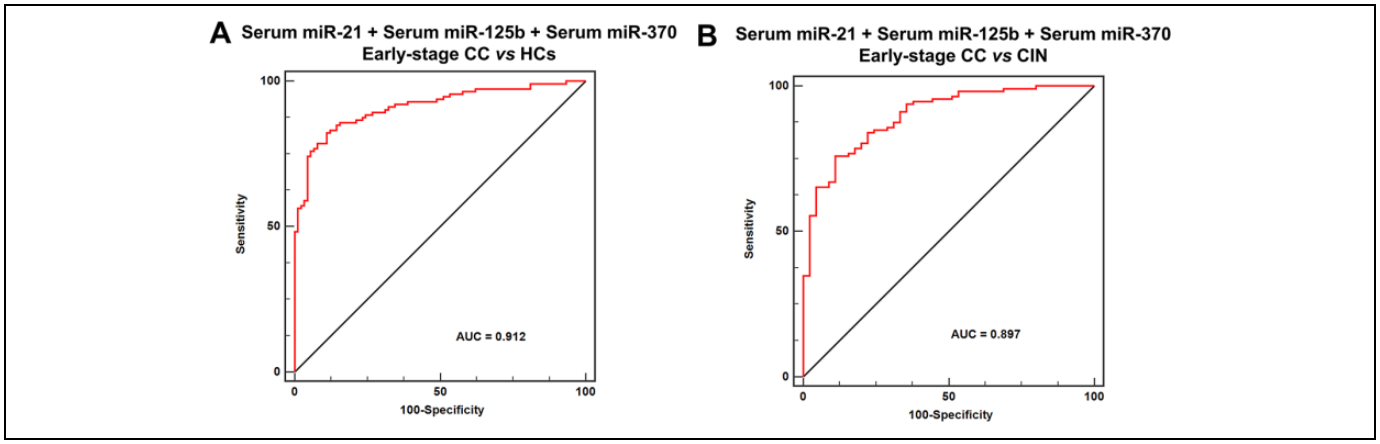

Figure 2. The diagnostic accuracy for combining serum miR-21, serum miR-125b and serum miR-370 as a miRNA signature for the discriminating early-stage CC from healthy controls (A) or patients with CIN (B).

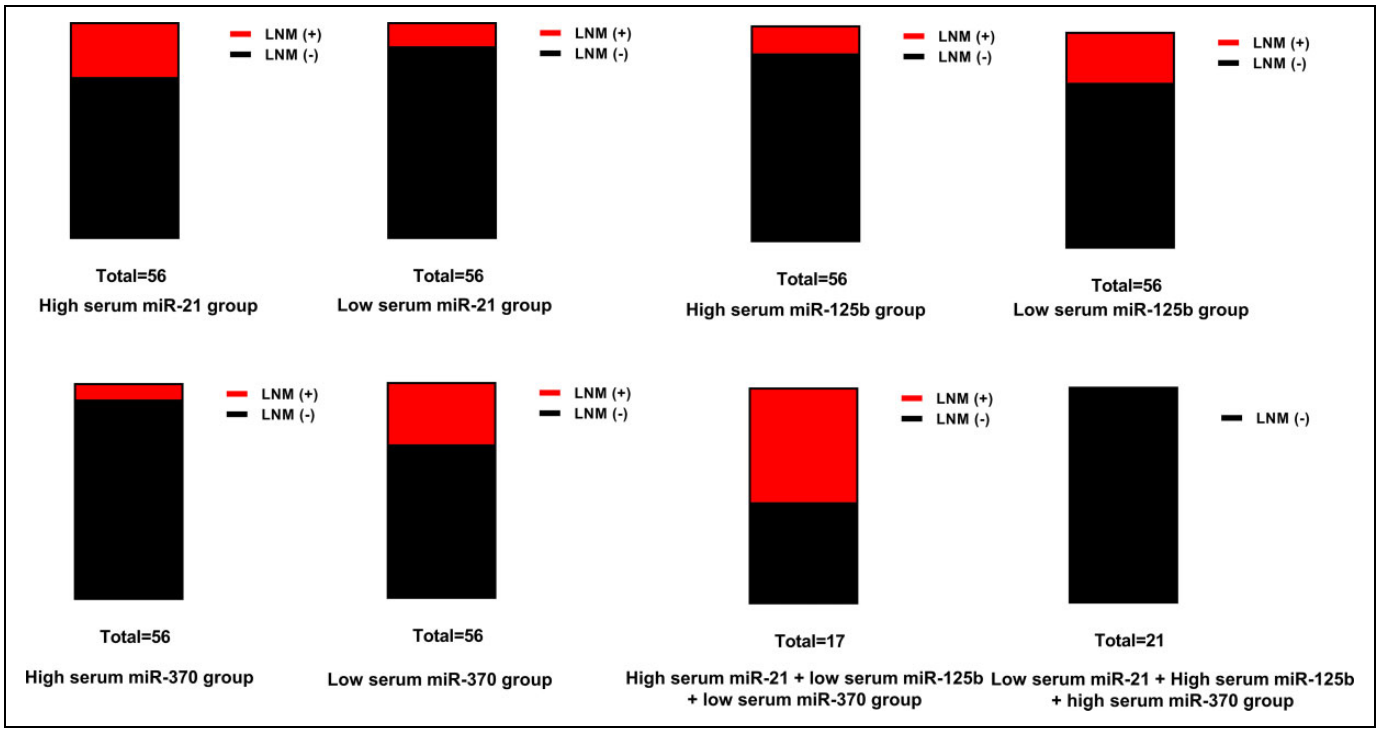

Figure 3. The association between serum miR-21/serum miR-125b/serum miR-370 and the lymph node metastasis of early-stage CC.

One possible explanation for the increased miR-21 in the blood of CC patients is that the cancer cells might synthesize and secrete more miR-21 into the extracellular environment compared to the normal cells. In addition, miR-21 has been demonstrated to be play a tumor promoting role in CC development. For instance, miR-21 regulated many oncogenic behaviors of cervical squamous cells by regulating its downstream target CCL20.[16] In addition, abnormal expression of miR-21 was also closely with the cisplatin resistance of CC cells,[17] indicating miR-21 might serve as a potential target for the treatment of CC.

The reduced expression of serum miR-125b and miR-370 in early-stage CC suggests that these 2 molecules might function as tumor suppressive miRNAs in CC. Cui et al showed that ectopic expression of miR-125b inhibited proliferation and induced apoptosis by targeting PI3K/Akt pathway.[18] It is well recognized that human papillomavirus (HPV) infection is a major risk factor for CC.[19] A negative correlation was found between miR-125b expression level and human papillomavirus DNA synthesis,[20] which strongly supports the tumor suppressive role in miR-125b in the initiation and development of CC. Loss of miR-125b might be a key factor for the tumorigenesis

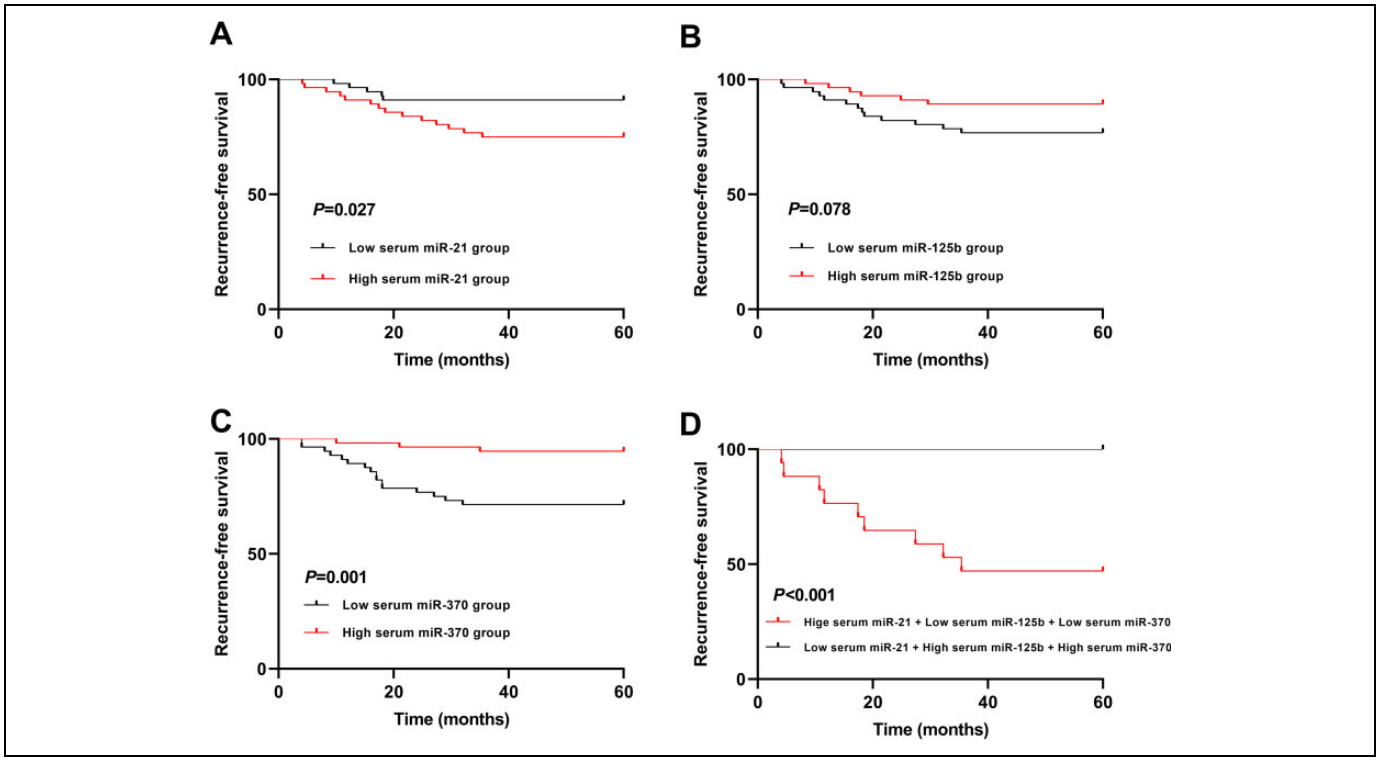

Figure 4. The association between serum miR-21/serum miR-125b/serum miR-370 and RFS of early-stage CC. A, Patients in the high serum miR-21 group had worse RFS. B, No significant correlation was found between serum miR-125b and RFS. C, Patients in the low serum miR-370 group had worse RFS. D, Patients with simultaneously high serum miR-21 + low serum miR-125b + low serum miR-370 suffered significantly shorter RFS than those with low serum miR-21 + high serum miR-125b + high serum miR-370.

of CC at the early stages. Therefore, it is reasonable to observe that the level of circulating miR-125b was significantly reduced in the early-stage CC. Currently, little information is available for the role of miR-370 in the progression of CC. Wu and Zhou reported that circAGFG played an oncogenic role in CC by absorbing miR-370. In addition, the expression of miR-370 was significantly reduced in GC cells,[21] suggesting that miR-370 might act as a tumor suppressive miRNA in CC.

Interestingly, combining serum miR-21, miR-125b and miR-370 improves the accuracy for the diagnostic and prognostic efficacies for early-stage CC. In the clinical setting, the early-stage with simultaneously low serum miR-21 + high serum miR-125b + high serum miR-370 might have low risk for developing LNM and recurrence. However, special attention or aggressive treatments might be given to those early-stage CC patients simultaneously high serum miR-21 + low serum miR-125b + low serum miR-370. This finding might provide important guidance for improving the prognosis of early-stage CC. However, larger cohort studies are needed to perform to confirm our findings. Both GSE30656 and TCGA cohort for CC are mainly constituted of Caucasian women. Our results showed that the expression patterns of serum miR-21, miR-125b, and miR-370 in Han Chinese women with CC were consistently with the results of GSE30656 and TCGA cohort, indicating that this miRNA-based molecular signature is very robustly deregulated in CC regardless of ethnicity and sample types.

Circulating miRNAs have demonstrated good performance for the diagnosis and prognosis prediction of CC. For instance, various circulating miRNAs including miRNA-20a, miR-1246, miR-2392, miR-3147, miR-3162-5p and miR-4484 are potential biomarkers for detecting the status of lymph node metastasis in patients with CC.[22,23] Jia et al showed that a serum miRNA-based signature (miR-21, miR-29a, miR-25, miR-200a and miR-486-5p) well differentiated CC patients from healthy controls. In addition, serum miR-29a and miR-200a was strongly associated with unfavorable clinicopathological parameters of CC.[4]

In conclusion, the level of serum miR-21 is increased, while the levels of serum miR-125b and miR-370 are decreased in early-stage CC. In addition, these 3 molecules are closely associated with the LNM and recurrence of early-stage CC. Combining serum miR-21, miR-125b and miR-370 as a miRNA-based signature show great promise for the detection diagnosis and prognosis prediction of early-stage CC.

Authors' Note

This study was approved by the Ethics Committee of Huizhou Central People's Hospital (F2018-027). Written informed consent was obtained from all participants.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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ORCID iD

Yanqin Ji https://orcid.org/0000-0003-3261-3912

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