

A circulating serum miRNA panel as early detection biomarkers of cervical intraepithelial neoplasia

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Abstract. – OBJECTIVE: MicroRNAs (miRNAs) have been demonstrated to play critical roles in regulating the molecular process of tumorigenesis. Therefore, the purpose of this study was to establish a panel of serum miRNA signature for early detection of cervical intraepithelial neoplasia (CIN).

PATIENTS AND METHODS: One hundred and twenty-six patients with CIN and sixty healthy control subjects were recruited in this cohort study. Quantitative reverse transcript polymerase chain reaction (qRT-PCR) was conducted to detect the expression level of the panel of miRNA signature (miR-9, miR-10a, miR-20a and miR-196a) in the serum samples of all the participants. The association between HPV infection status and the expression levels of miRNAs was also evaluated. In addition, Receiver Operating Characteristic (ROC) curve was used to evaluate the diagnostic value of the combination of these four serum miRNAs.

RESULTS: The expression levels of the four miRNAs (miR-9, miR-10a, miR-20a and miR-196a) were all significantly upregulated in the serum samples derived from the CIN patients compared with those from the healthy controls ($p < 0.01$). Also, HPV infection status was significantly correlated with the expression levels of miRNAs ($p < 0.01$). The ROC analysis showed that this four-miRNA signature showed high accuracy in discriminating CIN individuals (AUC = 0.886, $p < 0.01$) from healthy controls.

CONCLUSIONS: Taken together, our findings demonstrated that the panel of four serum miRNAs (miR-9, miR-10a, miR-20a and miR-196a) are useful and novel noninvasive biomarkers for early detection of CIN.

Key Words:

Biomarker, Cervical intraepithelial neoplasia, MicroRNAs.

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Introduction

Cervical cancer is the most frequent malignancy that affecting the female genital tract world-

wide, and remains a major leading cause of death for women, with an estimated global incidence of 530 000 new cases and over 275,000 deaths per year¹. Cervical intraepithelial neoplasia (CIN) is the potentially premalignant transformation and abnormal growth (dysplasia) of squamous cells on the surface of the cervix. Human papilloma virus (HPV), especially HPV16 and HPV18, has been demonstrated to play a crucial role in the progression of CIN to cervical cancer^{2,3}. The oncoproteins of HPV can interact with tumor suppressors such as retinoblastoma (Rb) and p53 and subsequently inhibit their function to promote malignant transformation of cervical epithelial cells^{4,5}. CIN is usually curable, and therefore detecting CIN at an early stage is an effective strategy to bring down the incidence of cervical cancer as well as improve its clinical outcome.

MicroRNAs (miRNAs) are a class of small, non-coding RNAs that regulate gene expression at the posttranscriptional level. The mature miRNAs exert their function by binding to partially complementary sequences in the 3' untranslated region (3'-UTR) of target mRNAs to trigger their degradation or inhibit protein translation⁶. miRNAs have been found to be involved in multiple biological processes including, but not limited to, cell proliferation, differentiation, survival, apoptosis and organ development^{7,8}. Moreover, dysregulated expression of miRNAs has been documented in a variety of human diseases including CIN and cervical cancer⁹⁻¹¹. Lee et al¹² used TaqMan real-time quantitative PCR array methods to profile 70 significantly differentially expressed miRNAs in the early stage invasive squamous cell carcinomas compared with the normal specimens. In addition, overexpression of miR-127 was significantly associated with lymph node metastasis and in-

hibition of miR-199a could suppress the growth of cervical cancer cells. Liu et al¹³ revealed that ectopic expression of miR-7 inhibited cell viability and promoted cell apoptosis, and *vice versa*. miR-7 was also found to exert its effects by regulating its downstream target-X-linked inhibitor of apoptosis protein (XIAP).

Recently we found that a serum miRNAs panel (miR-9, miR-10a, miR-20a and miR-196a) was significantly upregulated in CIN patients in our pre-experimental study (data not shown). The purpose of this study was to evaluate the clinical value of this panel of four serum miRNAs for early detection of CIN.

Patients and Methods

Study Population

This study was approved by the Ethics Committee of Baoding Second Central Hospital and written informed consent was obtained from all individual participants. In total, 126 patients with CIN, and 60 healthy controls who received therapy or had a physical examination in the Department of Obstetrics and Gynecology at the Baoding Second Central Hospital were recruited. The diagnosis of CIN was pathologically confirmed. None of the patients received any kind of treatment before the serum sample collection. The HPV infection status of CIN patients and healthy volunteers was determined using Hybrid II (HCII) assay. The clinical characteristics of the subjects in this study were summarized in Tables I and II.

Quantitative Reverse Transcription-PCR (qRT-PCR)

At least 8 mL venous blood was drawn from each participant, and then the samples were centrifuged at 3000 rpm for 5 min at 4°C to sep-

arate the serum from cellular components. The serum was immediately frozen and stored at -80°C until use. Total RNA was isolated from serum using the Qiagen miRNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The first strand cDNA was synthesized using the miScript SYBR Green PCR Kit (Qiagen). qRT-PCR was performed on the Mx3005P qPCR System (Agilent, Santa Clara, CA, USA). The PCR conditions were 94°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. The levels of four serum miRNAs (miR-9, miR-10a, miR-20a and miR-196a) were calculated and assessed using the 2^{-ΔΔCt} method. Each sample was tested in triplicate and RNU6B was used as the internal control for normalization.

Statistical Analysis

All statistical analysis was performed with the SPSS 20 software (IBM SPSS Statistics Armonk, NY, USA). The serum levels of four miRNAs (miR-9, miR-10a, miR-20a and miR-196a) in patients with CIN and healthy volunteers were compared using Mann-Whitney U test. ROC curve was used to evaluate the sensitivity and specificity of miRNA biomarkers for the diagnosis of CIN. All reported *p*-values were two-tailed and a *p*-value less than 0.05 was considered to be statistically significant.

Results

The Expression Levels Four Serum miRNAs in CIN Patients and Healthy Controls

We evaluated the levels of these four serum miRNAs (miR-9, miR-10a, miR-20a and miR-196a) in this cohort which included 126 CIN pa-

Table I. Characteristics of the subjects participating in the study.

Characteristics		CIN patients (n = 126)	Normal controls (n = 60)
Age (year)	Median ± SD	36.0 ± 5.8	37.0 ± 8.4
	Range	20-56	23-64
Smoking	Smoking	28	7
	HPV infection	71	3
	Immunosuppressive drug	13	0
CIN grade	I	38	–
	II	43	–
	III	45	–

Table II. Characteristics of the CIN patients with or without HPV infection.

Characteristics		CIN patients (n = 71) HPV positive	CIN patients (n = 55) HPV negative
Age (year)	Median ± SD	35.0 ± 4.7	37±6.5
	Range	20-54	22-56
	Smoking	19	9
	Immunosuppressive drug	8	5
CIN grade	I	18	20
	II	22	21
	III	31	14

tients and 60 control individuals. There was a significant statistical difference in the expression levels of circulating miR-9, miR-10a, miR-20a and miR-196a between the healthy controls and CIN patients ($p < 0.01$) (Figure 1).

The Association Between Serum miRNAs Expression Level and HPV Infection Status

We divided the CIN patients into 2 subgroups based on the status of HPV infection. A signifi-

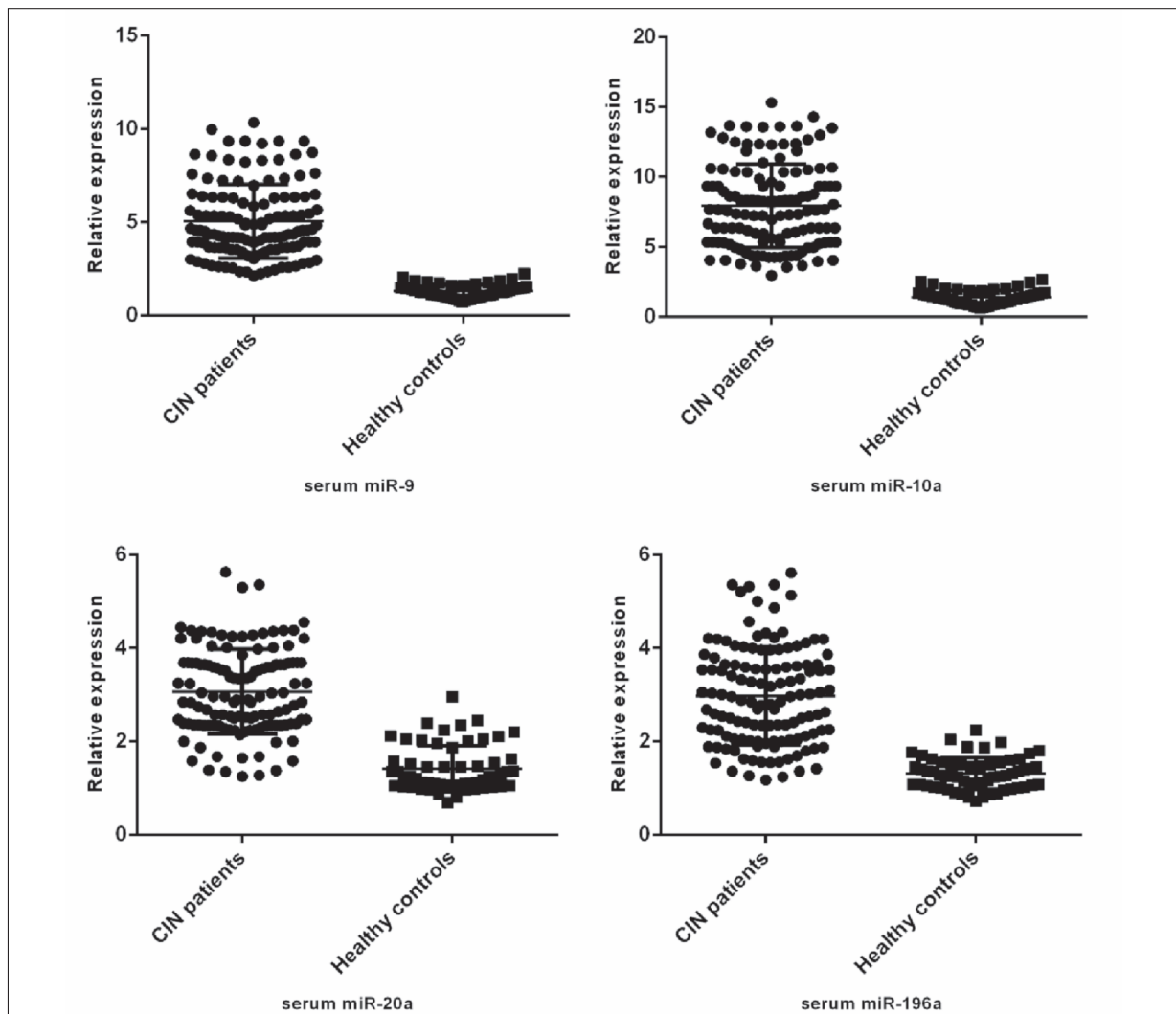


Figure 1.

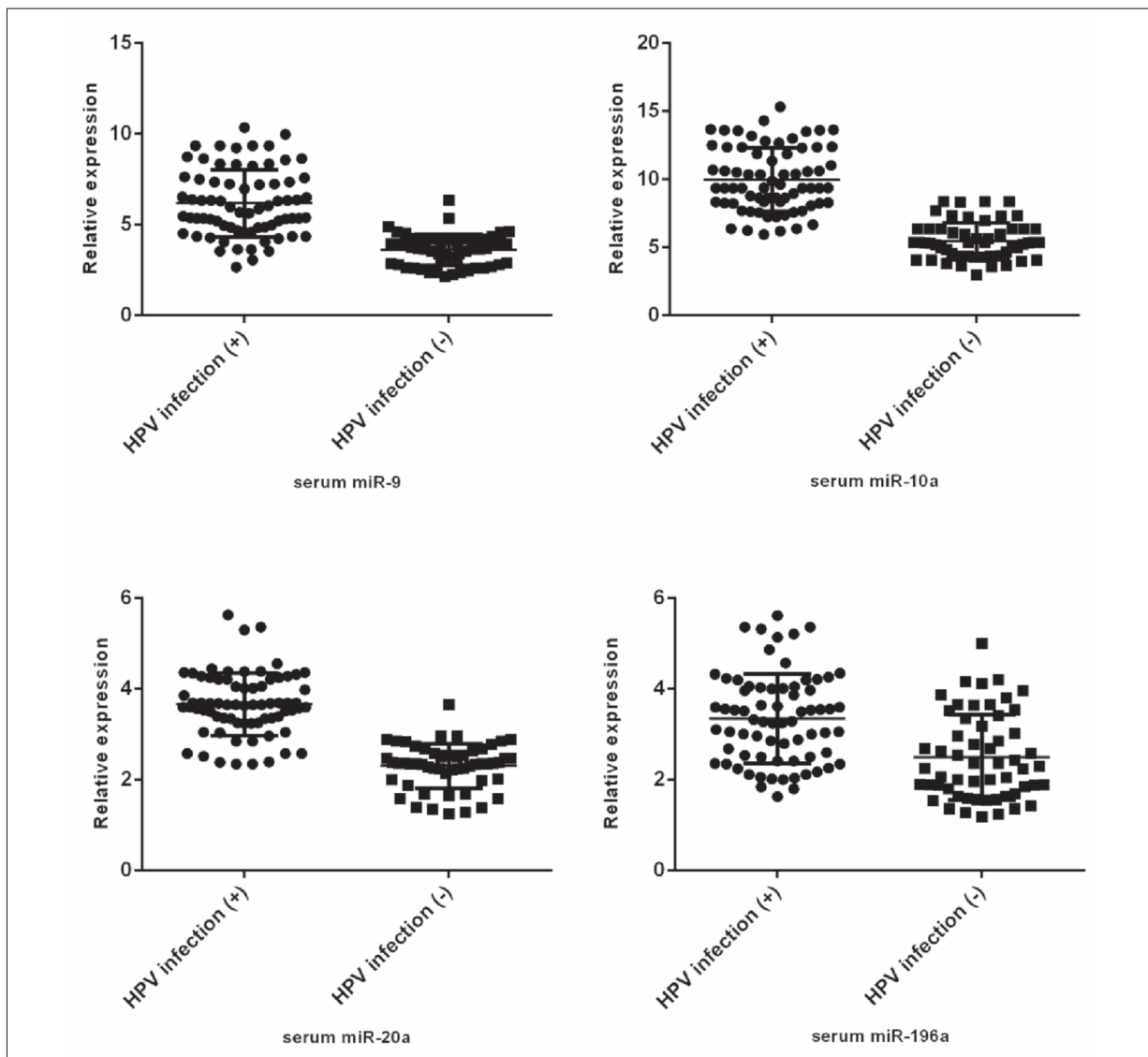


Figure 2.

cantly increased expression of serum miR-9, miR-10a, miR-20a and miR-196a ($p < 0.01$) was detected in CIN patients with HPV infection compared with those without HPV infection, indicating overexpression of these miRNAs might be closely associated with HPV infection, and their expression changes might occur even before the morphological changes of cervical epithelium.

The Four-miRNA Panel Signature Discriminates CIN Patients From Healthy Subjects

With ROC analysis, we evaluated the diagnostic value of this four-miRNA in distinguishing

CIN patients from healthy volunteers. It exhibited high accuracy in discriminating CIN from normal controls when serum miR-9, miR-10a, miR-20a and miR-196a were combined to form a panel (AUC = 0.886, $p < 0.01$).

Discussion

Cervical cancer is a largely preventable disease due to the wide application of HPV vaccine. However, it is still one of the leading causes of cancer-related deaths among women worldwide especially in low- to middle-income countries.

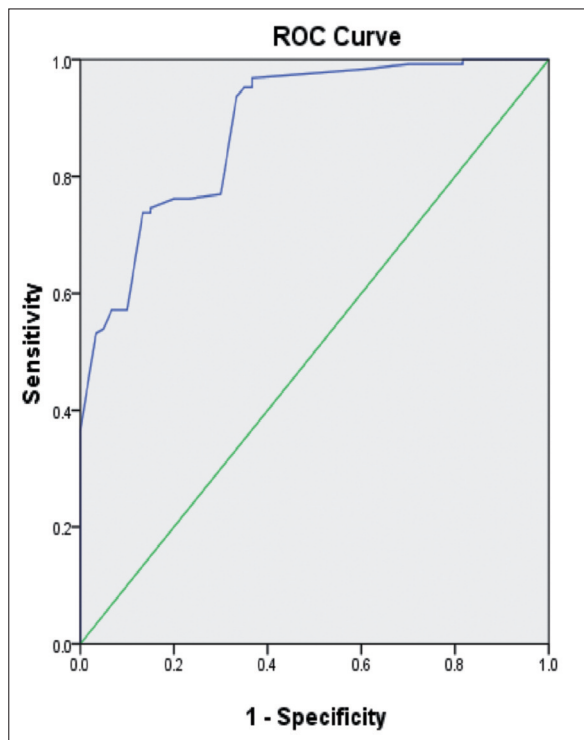


Figure 3.

CIN is a precancerous lesion that might progress into cervical cancer if it is not treated appropriately. Therefore, exploring the biomarkers that can distinguish the CIN patients from healthy subjects help early detection of CIN.

miRNAs have been proved to play a variety of regulatory roles in the development of cancer. Also, they are very stable and present in the body fluids such as urine, serum, plasma and saliva¹⁴. Therefore, miRNAs are considered to be a class of promising non-invasive biomarkers for identifying cancer at an early stage. In this study, our results showed that the expression levels of serum miR-9, miR-10a, miR-20a and miR-196a were all significantly upregulated in CIN patients in comparison with the healthy subjects. In addition, the CIN patients with positive HPV infection had a higher expression level of these four miRNAs compared with those without HPV infection. Moreover, this four-miRNA signature could discriminate CIN individuals from healthy controls with high accuracy. Therefore, we propose to establish this panel as a novel non-invasive biomarkers for detecting CIN at an early stage. The clinical value of this panel is needed to be further confirmed in large-scale cohort studies.

Consistent with our findings, previous studies have shown that these four miRNAs might promote the progression of cervical cancer. miR-9 was the most activated miRNA by HPV E6 in a p53-independent manner, and tissue miR-9 expression level was effective in distinguishing high-grade CIN specimens from normal cervical epithelium, indicating miR-9 played a central role in the pathogenesis of CIN^{15,16}. Long et al¹⁷ showed that the expression level of miR-10a was increased in human cervical cancer and miR-10a could promote the oncogenic behaviors of cervical cancer cells by targeting CHL1. Safari et al¹⁸ demonstrated that upregulation of miR-10a and miR-20a were associated with aggressive progression and poor prognosis in human cervical cancer, suggesting that both miR-10a and miR-20a might act as oncogenes during the development of this malignant disease. Chen et al¹⁹ reported that the comprehensive set of serum miRNAs (miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p and miR-4484) were good biomarkers for predicting the lymph node metastasis in patients with early-stage cervical squamous cell carcinoma. As regards to miR-196a, we have demonstrated that serum miR-196a was overexpressed in CIN patients and cervical patients. In addition, higher serum miR-196a expression level was associated with higher CIN grade and poorer clinical outcome of cervical cancer²⁰. Similarly, Hou et al²¹ also showed that miR-196a could promote the proliferation of cervical cancer cells through the regulation of FOXO1 and p27Kip1. Taken together, miR-9, miR-10a, miR-20a and miR-196a all function as oncogenes in cervical cancer and their secretion in the serum might be closely correlated with the progression of CIN as well as cervical cancer.

Conclusions

We have established a serum miRNAs panel (miR-9, miR-10a, miR-20a and miR-196a) that could effectively distinguish CIN patients from healthy controls with high accuracy. These circulating miRNAs might have great clinical value for screening CIN at an early stage.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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