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Original Article

Clinical value of miR-216a-5p and miR-34a in early screening for cervical cancer

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Abstract: Objective: To investigate the clinical value of miR-216a-5p and miR-34a in early screening for cervical cancer (CC). Methods: 99 patients were selected and classified into a cervical cancer group, a precancerous lesion group, and a chronic cervicitis group, with 33 patients in each group. The miR-216a-5p and miR-34a levels in the morning urine samples of patients in the three groups were detected. Additionally, the urine samples of CC patients were analyzed and their cervical tissues examined to confirm the presence of Human Papilloma Virus (HPV) infection. The differences in the levels of miR-216a-5p and miR-34a in CC patients exhibiting varying clinical features and the clinical values of the two biomarkers in identifying CC were analyzed. Patients in the cervical cancer group were divided into a recurrence group and a non-recurrence group, after which their levels of miR-216a-5p and miR-34a were analyzed. These patients were subsequently divided into a high-expression group and a low-expression group with the aforementioned biomarker levels in the non-recurrence group as cutoff values. The progression-free survival was compared between the low- and high-expression groups. Results: Sensitivity and specificity of the urine sample test for HPV infection were 85.19% and 93.33%, respectively. Compared to chronic cervicitis group and precancerous lesion groups, or the non-recurrence group, the levels of miR-216a-5p and miR-34a in both the cervical cancer group and recurrence group were significantly lower ($P < 0.05$). CC patients with moderately to poorly differentiated tumor cells, an infiltration depth of the muscle layer > 1/2, lymph node metastasis, parametrial infiltration, or vascular invasion had significantly lower levels of miR-216a-5p and miR-34a than those without these risk factors ($P < 0.05$). The AUC for the application of the two biomarkers in diagnosing CC individually or in combination, or in forecasting recurrence, was greater than 0.8. Additionally, the cumulative progression-free survival was shorter in the low-expression group compared to the high-expression group. Conclusion: Use of morning urine samples for testing HPV infection shows high sensitivity and specificity. Moreover, the miR-216a-5p and miR-34a levels were closely associated with the progression and recurrence of CC.

Keywords: Cervical cancer, miR-216a-5p, miR-34a

Introduction

Cervical cancer (CC) is a prevalent malignant tumor in the female reproductive system, ranking second in the incidence of malignant tumors among women, with increasing effects on younger generations.

Epidemiologic data indicated that approximately 600,000 new CC cases were reported globally in 2020, with China accounting for 18.2% of these cases. Moreover, with social development and lifestyle changes, the incidence and mortality of CC in China have been increasing annually [1, 2]. CC typically occurs and develops

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without obvious symptoms in the early stage. Most CC patients are already in the advanced or invasive stage when it is confirmed, resulting in a poor prognosis [3-5]. Hence, early diagnosis are critical for CC prognosis, particularly if the tumor is poorly-differentiated Currently, the gold standard for diagnosing CC is through pathologic examination, which involves invasive procedures. Unfortunately, these procedures are often not well-tolerated by patients, potentially leading to delays in optimal treatment [6]. Another approach for CC diagnosis and treatment is cytologic examination, which holds significant clinical value. However, this approach demands exquisite clinical techniques and state-of-the-art medical equipment. Therefore, exploring cost-effective, simple, and rapid triage method for Human Papilloma Virus (HPV)-positive women has become urgent in clinical practice. It has been reported that cervical epithelial cells can shed into the vaginal cavity through cervical secretions, accumulating near the vaginal introitus. When a patient urinates, the urine can carry these cervical epithelial cells with it, allowing urine samples yto be used as a biomarker for diagnosing CC [7]. MicroRNAs (miRNAs) are a class of short noncoding RNAs closely associated with the development and progression of solid tumors. Previous research has confirmed that miRNA plays a significant role in the development and progression of CC [8-11]. MiR-34a is a family member of miRNA, which is expressed at a low level in several solid tumors including CC [12]. Additionally, miR-216a-5p has been demonstrated to act as a tumor suppressor gene in multiple solid tumors, partially inhibiting HCP5, thereby promoting the proliferation of CC cells [13]. Based on this, the present study employed urine samples to analyze the expression levels of miR-216a-5p and miR-34a, with the aim of assessing their clinical value and providing a reference for the diagnosis and treatment of CC.

Materials and methods

General data

This retrospective study included patients who were admitted to Peking University First Hospital Ningxia Women and Children's Hospital (Ningxia Hui Autonomous Region Maternal and Child Health Hospital) due to abnormal changes in cervical tissues between January 2020 and January 2021.

Inclusion criteria: Patients were eligible for the study if they met the diagnostic criteria for CC, precancerous lesions, and cervicitis [14]; they had not received any related treatment prior to enrollment; their clinical data were complete; they were no less than 18 years old; they and their families had provided informed consents.

Exclusion criteria: Patients were excluded from the study if they were complicated with other gynecological diseases or malignant tumors; they had malfunctioned liver or kidney; they had neurological or psychiatric disorders; they weren't compliant; they were lactating; they had a recent history of vaginal medication use; they recently developed urinary system diseases or infectious diseases.

Sample size calculation

The study focused on assessing the differences in the miR-216-5p and miR-34a levels among CC patients, precancerous lesions, and chronic cervicitis. According to our pilot study, the expected means for the three patient categories were 0.8, 0.6, and 0.5, respectively, with standard deviations of the differences being 0.3, 0.3, and 0.2. A two-sided test was carried out with α set at 0.05 and a power (efficacy) of 90%. Using PASS 15 software, the calculated sample size was N = 78 cases. Considering a 20% loss to follow-up and refusal rate, a minimum of 99 cases were needed as study subjects, with 33 cases per group.

Grouping

The study included 33 CC patients with as the cervical cancer group, 33 with precancerous lesions as the precancerous lesion group, and 33 with chronic cervicitis as the chronic cervicitis group. The general data of the patients in the three groups are presented in **Table 1**. The study was approved by the ethics committee of Peking University First Hospital Ningxia Women and Children's Hospital (Ningxia Hui Autonomous Region Maternal and Child Health Hospital).

Observation indicators

Clinical characteristics of the enrolled CC patients were collected, including age, patho-

Table 1. Comparison of general data among the three groups

Group	Case	Age	Gravida	Parity	Menopausal status (Yes/No)
Chronic cervicitis group	33	47.1±6.2	3.03±1.07	1.94±0.97	14/19
Precancerous lesion group	33	46.8±6.7	2.94±0.97	2.09±1.04	11/22
Cervical cancer group	33	47.4±9.3	3.12±1.14	2.03±0.95	13/20
Statistical Values		0.059	0.234	0.212	0.598
P		0.943	0.792	0.809	0.742

Table 2. Primer sequences

Primer	Sequence
miR-216a-5p Upstream	5'-TGTCGCAAATCTCTGCAGGCAGAGCAGGGTCCGAGGTA-3'
miR-216a-5p Downstream	5'-CAGAGCAGGGTCCGAGGTA-3'
miR-34a Upstream	5'-TGCGCTGGCAGTGTCTTAGCT-3'
miR-34a Downstream	5'-CCAGTGCAGGGTCCGAGGTATT-3'
U6 Upstream	5'-CTCGCTTCGGCAGCACA-3'
U6 Downstream	5'-AACGCTTCACGAATTTGCGT-3'

logic type, tumor cell differentiation degree, depth of invasion, tumor diameter, lymph node metastasis, parametrial infiltration, and vascular invasion status.

Detection of HPV infection using cervical tissues and urine samples: Early morning, urine samples were collected from all study subjects for the extraction of HPV DNA using the centrifugal column method. Cervical tissue samples were also collected from all study subjects for the same purpose. PCR was used to detect the presence of 21 HPV types in all samples, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 6, 11, 42, 43, 44, and 81.

Detection of the miR-216a-5p and miR-34a levels in the urine samples of patients in each group: Early morning, urine samples were collected from all study subjects and centrifuged at 3000 *g* for 30 minutes. The samples were then centrifuged again at 13000 *g* for 5 minutes at 4°C, and the supernatant was discarded. RNA was extracted from the urine samples using an RNA cell lysis solution, followed by a reverse transcription reaction. Gene expression was detected using RT-PCR, following the instructions provided on the SYBR RT-PCR kit (Abcam, USA). The reaction system was as follows: pre-denaturation at 95°C for 30 seconds, followed by 95°C for 5 seconds, and 62°C for 30 seconds, repeated for 35 cycles. The expression levels of miR-216a-5p and miR-34a in each group were determined using the $2^{-\Delta\Delta Ct}$ method, with U6 as the internal control. The primer sequences are shown in **Table 2**.

Follow-ups

CC patients were followed up until January 2024. Whether their CC recurred post-treatment was recorded, based on which, these CC patients were divided into a a recurrence group (11 cases) and a non-recurrence group (22 cases).

Statistical analysis

Statistical analysis was performed using SPSS 22.0. Measured data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Multiple group comparisons were conducted using analysis of variance (ANOVA), and pairwise comparisons between groups were performed using the SNK-t test. Categorical data were expressed as rates, and comparisons between groups were made using the χ^2 test. ROC curve analysis was employed to assess the clinical value of various indicators in diagnosing CC patients and evaluating their prognosis. Kappa analysis was used to evaluate the agreement of HPV infection between detecting the urine samples and cervical tissues, with the cervical tissue test for HPV infection as the reference standard. CC patients were subsequently divided into high- and low-expression groups based on the cutoff values of miR-216a-5p and miR-

Table 3. The results of HPV infection tests using urine samples and cervical tissue samples

		Cervical tissue sample test		Total
		+	-	
Urine sample test	+	46	3	49
	-	8	42	50
Total		54	45	99

Table 4. Comparison of miR-216a-5p and miR-34a levels among the three groups

Group	Case	miR-34a	miR-216a
Chronic cervicitis group	33	0.89±0.16	0.97±0.19
Precancerous lesion group	33	0.52±0.14[a]	0.62±0.13[a]
Cervical cancer group	33	0.40±0.08[a,b]	0.48±0.07[a,b]
F		129.122	96.871
P		< 0.001	< 0.001

Note: [a]compared to the chronic cervicitis group, P < 0.05; [b]compared to the precancerous lesion group, P < 0.05.

34a in the recurrence group. The K-M survival curve was used to compare the progression-free survival between the two groups. A *P*-value < 0.05 was considered significant.

Results

Comparison of consistency of HPV infection between urine sample and cervical tissue detection

A total of 54 patients were detected HPV-positive with the use of their cervical tissue samples, showing a positive detection rate of 54.55%; while 49 were positive by urine sample test, with a sensitivity of 85.19% and a specificity of 93.33%. The Kappa value was 0.818, indicating substantial agreement. Details are presented in **Table 3**.

Comparison of miR-216a-5p and miR-34a levels among the three groups

The levels of miR-216a-5p and miR-34a were significantly lower in the cervical cancer group compared to the chronic cervicitis group and the precancerous lesion group (P < 0.05); In comparison to the precancerous lesion group, the miR-216a-5p and miR-34a levels in the cervical cancer group was also lower (P < 0.05). See **Table 4**.

Comparison of miR-216a-5p and miR-34a levels among cervical cancer patients with different clinical characteristics

Patients with moderate to poor differentiation, an invasion depth of the muscle layer > 1/2, lymph node metastasis, parametrial infiltration, vascular invasion and recurrent CC had significantly lower levels of miR-216a-5p and miR-34a compared to patients with well-differentiated tumor, and an invasion depth of the muscle layer ≤ 1/2, and those without lymph node metastasis, parametrial infiltration, vascular invasion, or recurrent CC (P < 0.05). See **Table 5**.

Clinical value of miR-216a-5p and miR-34a levels in diagnosing cervical cancer

When the cutoff value for miR-216a-5p was 0.58, the AUC for diagnosing CC was 0.91; when the cutoff value for miR-34a was 0.49, the AUC was 0.89. When applied in combination, the AUC for CC diagnosis was 0.93. See **Table 6** and **Figure 1A**.

When the cutoff value for miR-216a-5p was 0.49, the AUC for predicting the recurrence of CC was 0.84; when the cutoff value for miR-34a was 0.37, the AUC for predicting the recurrence of CC was 0.96. The AUC for the same purpose was 0.96 when the two biomarkers were applied in combination. See **Table 7** and **Figure 1B**.

Comparison of progression-free survival between high and low miR-216a-5p and miR-34a expression groups

In the high miR-216a-5p expression group (25 cases), 6 cases experienced recurrence by the end of the follow-up, with a progression-free survival rate of 76.00% and a progression-free survival time of 36.69 (37.14, 42.23) months. In the low miR-216a-5p expression group (8 cases), 5 cases experienced recurrence by the end of the follow-up, with a progression-free survival rate of 37.50% and a progression-free survival time of 33.79 (27.67, 39.90) months. There was a significant difference between the two groups (Log Rank = 4.050, P = 0.044). In

Table 5. Comparison of miR-216a-5p and miR-34a levels in patients with different clinical characteristics in the cervical cancer group

Indicator	case	miR-34a	*t*	*P*	miR-216a-5p	*t*	*P*
Age			0.374	0.711		1.764	0.088
< 55 years	14	0.40±0.07			0.45±0.07		
≥ 55 years	19	0.39±0.08			0.49±0.06		
Pathological type			0.706	0.485		1.192	0.242
Squamous cell carcinoma	23	0.39±0.08			0.47±0.06		
Adenocarcinoma	10	0.41±0.06			0.50±0.08		
Degree of differentiation			5.958	< 0.001		4.584	< 0.001
Moderate to poor differentiation	11	0.32±0.05			0.42±0.03		
High differentiation	22	0.43±0.05			0.50±0.07		
Depth of invasion			6.865	< 0.001		4.577	< 0.001
> 1/2 of muscle layer	18	0.34±0.05			0.44±0.05		
≤ 1/2 of muscle layer	15	0.46±0.05			0.52±0.05		
Tumor diameter			0.038	0.090		0.126	0.901
≥ 4 cm	17	0.40±0.09			0.48±0.07		
< 4 cm	16	0.40±0.06			0.48±0.06		
Lymph node metastasis			6.175	< 0.001		3.265	0.003
Present	13	0.33±0.05			0.43±0.04		
Absent	20	0.44±0.05			0.50±0.07		
Parametrial infiltration			6.696	< 0.001		4.596	< 0.001
Present	10	0.31±0.04			0.42±0.03		
Absent	23	0.43±0.05			0.50±0.07		
Vascular invasion			6.447	< 0.001		4.587	< 0.001
Present	9	0.31±0.04			0.42±0.03		
Absent	24	0.43±0.05			0.50±0.07		
Recurrence							
Yes	11	0.32±0.05	5.958	< 0.001	0.42±0.03	5.745	< 0.001
No	22	0.43±0.05			0.51±0.06		

Table 6. Clinical value of miR-216a-5p and miR-34a levels in the diagnosis of CC

Variable	Cutoff Value	AUC	95% CI	Sensitivity	Specificity	P
miR-34a	0.49	0.89	0.83, 0.96	0.87	0.94	< 0.001
miR-216a-5p	0.58	0.91	0.85, 0.97	0.88	0.97	< 0.001
Joint factor	-	0.93	0.88, 0.98	0.94	0.88	< 0.001

the high miR-34a expression group (30 cases), 8 cases experienced recurrence by the end of the follow-up, with a progression-free survival rate of 73.33% and a progression-free survival time of 38.92 (36.49, 41.35) months. In the low miR-34a expression group (3 cases), 3 cases experienced recurrence by the end of the follow-up, with a progression-free survival rate of 0.00% and a progression-free survival time of 28.33 (19.69, 36.98) months. A significant difference was noted between the two groups (Log Rank = 13.458, P < 0.001), as shown in **Figure 2**.

Discussion

Cervical carcinoma (CC) is the second most common malignant tumor in women and has been extensively studied. Due to influencing factors such as HPV infection and an increase in childbirth, the incidence of CC has been growing year on year worldwide [15, 16]. The pathogenesis of CC, is still not fully understood, is relatively complex. Clinically, it is widely believed that HPV infection is an independent risk factor for the occurrence of CC. Additionally, factors such as smoking and sexual activity at an early age also promote the occurrence and

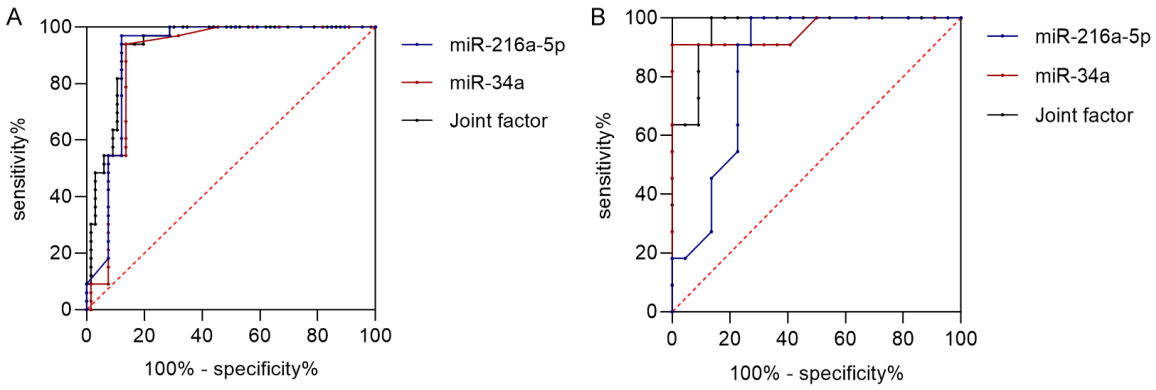

Figure 1. ROC curves of miR-216a-5p and miR-34a for CC diagnosis. A: ROC curves of miR-216a-5p and miR-34a for CC diagnosis; B: ROC curve of prognosis of CC patients assessed by miR-216a-5p and miR-34a.

Table 7. Clinical value of miR-216a-5p and miR-34a levels in assessing the prognosis of CC patients

Variable	Cutoff Value	AUC	95% CI	Sensitivity	Specificity	P
miR-34a	0.37	0.96	0.88, 1.00	1.00	0.91	< 0.001
miR-216a-5p	0.49	0.84	0.71, 0.98	0.73	1.00	0.002
Joint factor	-	0.96	0.91, 1.00	1.00	0.86	< 0.001

development of CC. Since CC patients often have no specific clinical symptoms in the early stage, most of them overlook the disease and have entered the middle or late stages once diagnosed, missing the optimal time to receive surgical treatment [17]. Therefore, it is necessary to explore an efficient detection approach for clinical practice to initiate early screening of CC and offer guidance for clinicians to implement targeted measures in order to increase diagnoses.

Currently, the detection of CC often involves invasive procedures, leading to poor compliance by patients. Therefore, exploring convenient sampling methods with high sensitivity and specificity for HPV detection is crucial for the prevention and treatment of CC. Cancer antigens such as antigens 125 and 19-9 are common serum markers for detecting cancer in patients, but they are not very efficient due to their poor sensitivity and specificity. Therefore, new biomarkers for biological samples are needed [18]. International studies have reported that, with HPV infection test using cervical tissues as the yardstick, HPV infection test with the use of urine samples has a sensitivity and specificity of 87% and 94%, respectively [19]. In this study, the sensitivity and specificity of urine sample test for HPV infection were 85.19% and 93.33%, respectively, with a Kappa value of 0.818, which are consistent with the results of previous research. These results indicated that the urine sample test had a strong agreement with the results of the cervical tissue test. When patients are in need of early CC screening but are not qualified for invasive procedures, their urine samples can be used as adjuvant diagnostic indicators.

miRNAs are a class of single-stranded non-coding RNAs that exert their regulatory effects by binding to the mRNAs of various proteins, which are in close association with the occurrence and development of several malignant tumors [20-22]. Previous studies have shown that miRNAs can act as independent tumor suppressor genes or promoters in the carcinogenesis of CC by directly acting on or influencing other tumor suppressor genes or oncogenes [23]. Since cervical epithelial cells from the cervix can shed into urine, this presents an opportunity to collect urine samples for CC diagnosis. miR-34a regulates cyclins through P53 signaling pathway, thereby promoting transition of the cell cycle from G1 phase to S phase, which allows the progression from cervicitis to precancerous lesions and ultimately to CC [24]. Research has shown that the level of miR-34a is downregulated in CC patients [25, 26]. In this study, the miR-34a level in the urine samples was significantly decreased in CC patients compared to those with cervicitis or precancerous lesions. Subgroup analysis further revealed

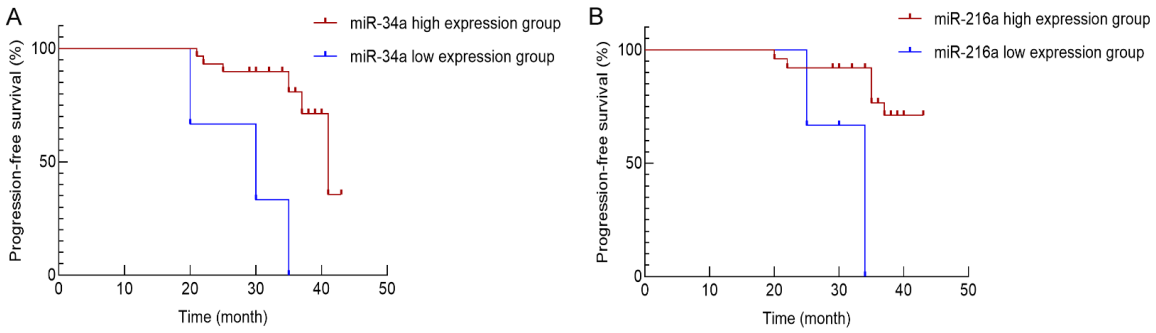

Figure 2. K-M curve diagram. A: K-M curves of progression-free overall survival comparing miR-34a high-expression and low-expression groups; B: K-M curves of total progression-free survival in the high-expression and low-expression miR-216a-5p groups.

that patients with moderate to poor cell differentiation, an infiltration depth of the muscle layer greater than 1/2, lymph node metastasis, parametrial infiltration, or vascular invasion had even lower levels of miR-34a, which is in line with the results from previous research on the expression level of miR-34a in the serum of CC patients, confirming the presence of lowly expressed miR-34a in the urine samples of CC patients. In addition, this finding indicated that the severer the condition of CC patients, the lower the expression level of miR-34a in the urine sample, which further suggests that miR-34a participates in the occurrence and development of CC throughout the route of cervicitis - precancerous lesion - cervical cancer.

MiR-216a-5p has been established as a tumor suppressor gene in various cancers [27]. Studies have shown that miR-216a-5p can inhibit tumor growth by targeting TPT1 in pancreatic cancer [28]. Previous foundational research has also demonstrated that miR-216a-5p can accelerate tumor regression by promoting the expression of apoptotic family proteins in breast cancer [29]. However, clinical research on the expression level of miR-216a-5p in CC patients is minimal. The results of this study indicated that the miR-216a-5p level in the urine samples was significantly reduced in CC patients compared to those with cervicitis or precancerous lesions. Subgroup analysis suggested that reduced urinary miR-216a-5p was closely associated with the severity of the disease in CC patients, demonstrating that miR-216a-5p participated in the development of CC.

The study also explored the clinical value of miR-216a-5p and miR-34a levels in the urine samples for the diagnosis of CC. The results indicated that the AUC for miR-216a-5p and miR-34a for diagnosing CC exceeded 0.9 when the two biomarkers were applied in combination. This indicates that the detection of miR-216a-5p and miR-34a in the urine samples has high clinical value for the diagnosis of CC. Moreover, as obtaining urine samples for clinical use is relatively easy, this offers a convenient method for clinical diagnosis of CC.

Furthermore, the study also performed an in-depth analysis on the value of miR-216a-5p and miR-34a in assessing the prognosis of patients. Recurrent patients had significantly lower levels of miR-216a-5p and miR-34a compared to non-recurrent patients. The AUC of miR-216a-5p alone in evaluating the prognosis of patients was greater than 0.8, while the AUC of miR-34a combined for evaluating the prognosis of patients was greater than 0.9, which was consistent with the value of the combined diagnosis with miR-34a and miR-261a-5p. This suggests that the combined detection of these two biomarkers can serve as an auxiliary indicator to assess prognosis. The results of the K-M survival curve analysis revealed that patients with lower miR-216a-5p and miR-34a expression levels had a shorter cumulative progression-free survival period. Furthermore, the results demonstrated as well that miR-216a-5p and miR-34a levels could be used as indicators for evaluating the prognosis of CC patients.

However, this study has some limitations. First, it did not further explore the differences in miR-216a-5p and miR-34a expression levels across different types of biological samples, such as plasma and serum, making it difficult to obtain

specific results for urine samples. Second, the samples were from the same center and the sample size was relatively small. A larger sample size from multiple centers would be needed to supplement the study.

In conclusion, the detection of HPV infection using morning urine samples produces highly consistent results with that from cervical tissue samples. The expression levels of miR-216a-5p and miR-34a may be involved in the progression from cervicitis to precancerous lesions and ultimately to CC, and can serve as auxiliary post-treatment indicators for prognosis of C.

Disclosure of conflict of interest

None.

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