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Clinical significance of serum miR-196a in cervical intraepithelial neoplasia and cervical cancer

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ABSTRACT. Previous studies have reported that miR-196a is upregulated in cervical cancer tissues and cell lines. However, whether serum miR-196a is increased in patients with cervical cancer or cervical intraepithelial neoplasia (CIN), and its potential clinical value remained unknown. In total, 105 cervical cancer patients, 86 CIN patients, and 50 healthy volunteers were recruited. Quantitative reverse transcription-polymerase chain reaction was performed to compare the serum levels miR-196a in all participants. The associations between serum miR-196a and CIN grade/clinicopathological parameters of cervical cancer were also examined. A survival analysis was performed using the Kaplan-Meier method. Univariate and multivariate analyses were conducted to explore the independent risk factors for cervical cancer. Our results revealed that serum miR-196a levels were higher in patients with cervical cancer ($P < 0.01$) and CIN ($P < 0.05$) compared to those in healthy controls. Serum miR-196a was associated with CIN grade and various cervical cancer parameters including tumor size ($P = 0.031$), lymph node metastasis ($P = 0.018$),

FIGO stage (P = 0.004), and grade (P = 0.011). Cervical cancer patients with higher serum miR-196a levels had a poorer overall survival rate (P = 0.004). Multivariate analysis revealed that high serum miR-196a was an independent predictor for poor survival of cervical cancer (HR = 3.510; 95%CI = 1.961-6.874; P = 0.025). In conclusion, our findings suggest that serum miR-196a overexpression is associated with CIN grade and cervical cancer progression. Therefore, serum miR-196a may be a reliable biomarker for early detection and prognosis of cervical cancer.

Key words: Cervical intraepithelial neoplasia; Cervical cancer; miR-196a; Prognosis; Biomarker

INTRODUCATION

Cervical cancer remains the second most common female malignancy after breast cancer, accounting for approximately 529,800 new cases and 275,100 deaths each year worldwide (Ferlay et al., 2010). Cervical cancer progresses slowly from pre-invasive cervical intraepithelial neoplasia (CIN; scaled 1-3) to invasive stages. Approximately 20% of CIN2 lesions progress to CIN3, and 10-40% of CIN3 develop into cervical cancer (McCredie et al., 2008; Castle et al., 2009). Human papillomavirus (HPV) infection is the number one risk factor for cervical cancer, and additional risk factors include smoking, parity, and oral contraceptives. Currently, surgery, radiation, and chemotherapy are first-line treatments for cervical cancer. As treatment outcomes are closely related to the clinical stage, screening cervical cancer at an early stage of CIN is crucial to reduce mortality rates. Biomarkers with high specificity and sensitivity for cervical cancer and/or CIN are still lacking, thus the identification of novel biomarkers for cervical cancer/CIN screening is important.

miRNAs are generally 21-25-nucleotide, non-coding-RNAs that regulate gene expression at the post-transcriptional level. miRNAs have been shown to participate in almost every cellular process investigated to date, and alterations in miRNA expression are associated with many human diseases including cancer (Wijnhoven et al., 2007; Melo et al., 2011). The regulatory role of miRNAs is complex as they may function either as oncogenes or tumor suppressors, depending on the effects of their targeted genes. Currently three miR-196 members (miR-196a-1, miR-196a-2 and miR-196b) have been identified, and miR-196a genes are located within the HOX gene clusters (Chen et al., 2011). miR-196a has been reported to promote tumorigenesis in multiple types of cancers such as pancreatic cancer, glioma, lung cancer, and ovarian cancer (Kong et al., 2011; Liu et al., 2012; Yang et al., 2014; Fan et al., 2015). Furthermore, serum levels of miR-196a were significantly increased in patients with pancreatic cancer, and higher serum levels were associated with advanced clinical stage and shorter survival time (Kong et al., 2011). The miR-196a gene polymorphism has also been shown to contribute to cancer risk and susceptibility (Dou et al., 2010; Lee et al., 2014), suggesting it has an oncogenic role during the development of cancer.

It has previously been shown that miR-196a is overexpressed in both cervical cancer tissues and cervical cancer cell lines (Villegas-Ruiz et al., 2014). However, whether serum miR-196a is upregulated in patients with CIN or cervical cancer remained unknown. Moreover, the potential clinical value of serum miR-196a had not yet been investigation. Thus, the aim of this study was to determine whether there is an association between serum miR-196a levels and CIN/cervical cancer.

MATERIAL AND METHODS

Study design and population

This study was approved by the Ethics Committee of Baoding Second Central Hospital and written informed consent was obtained from all the participants. In total, 105 cervical cancer patients, 86 CIN patients, and 50 healthy controls who received therapy or had a physical examination in the Department of Obstetrics & Gynecology at the Baoding Second Central Hospital were recruited. The diagnoses of both cervical cancer and CIN were pathologically confirmed. The clinical staging of cervical cancer was done according to the International Federation of Gynecology and Obstetrics (FIGO) grading system. None of the patients received any kind of therapy before serum sample collection. The clinical characteristics of the patients are summarized in Table 1.

Table 1. Serum miR-196a levels and clinicopathological characteristics of cervical cancer patients.

Parameters	Group	Total (N)	Serum miR-196a (N)		P value
			Low	High	
Age (years)	≤50	64	38	26	0.299
	>50	41	29	12	
Tumor size (cm)	≤4	72	51	21	0.031
	>4	33	16	17	
Lymph node metastasis	No	68	49	19	0.018
	Yes	37	18	19	
Cell type	SCC	84	56	28	0.310
	Others	21	11	10	
Parametrial infiltration	No	70	47	23	0.390
	Yes	35	20	15	
FIGO stage	I-II	74	54	20	0.004
	III-IV	31	13	18	
Grade	Well/Moderate	77	55	22	0.011
	Poor	28	12	16	
HPV infection	No	13	6	7	0.218
	Yes	92	61	31	

Quantitative reverse transcription-PCR (qRT-PCR)

Approximately 8 mL venous blood was drawn from study participants, and then the samples were centrifuged at 3000 rpm for 5 min at 4°C in order to separate 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 protocol. cDNA was synthesized using the miScript SYBR Green PCR Kit (Qiagen). qRT-PCR was conducted 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 level of serum miR-196a was calculated and assessed using the $2^{-\Delta\Delta Ct}$ method. Each sample was assayed in triplicate and RNU6B was used as the internal control for normalization.

Statistical analysis

All statistical analyses were performed with the SPSS 20 software (IBM SPSS Statistics,

Armonk, NY, USA). The serum levels of miR-196a in cervical cancer patients, CIN patients, and healthy volunteers were compared using one-way ANOVA. Chi-square tests were performed to examine the associations between serum miR-196a levels and CIN grade/clinicopathological parameters of cervical cancer. The correlation between serum miR-196a levels and a 5-year overall survival was evaluated using the Kaplan-Meier method. Univariate and multivariate analyses were used to explore independent prognostic factors for cervical cancer. All reported P values were two-tailed and a P value less than 0.05 was considered to be statistically significant.

RESULTS

Serum miR-196a levels in patients with cervical cancer and CIN

qRT-PCR was performed to examine the levels of serum miR-196a in cervical cancer patients, CIN patients, and healthy volunteers. The results show that patients with cervical cancer had a significantly higher level of serum miR-196a than those of CIN patients ($P < 0.05$) and controls ($P < 0.01$). Similarly, serum miR-196a was significantly upregulated in CIN patients compared to that of the healthy volunteers ($P < 0.05$; Figure 1).

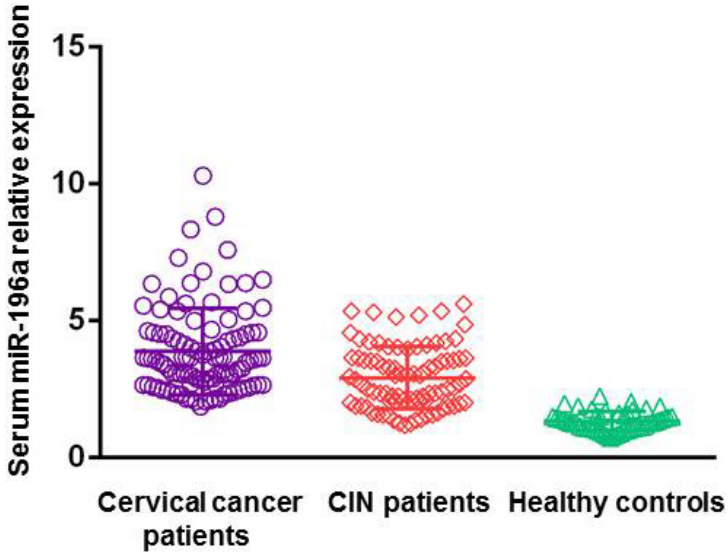

Figure 1. Expression level of serum miR-196a in patients with cervical cancer and CIN. The cervical cancer patients had a significantly higher level of serum miR-196a than CIN patients ($P < 0.05$) and controls ($P < 0.01$). Similarly, the expression level of serum miR-196a was significantly increased in CIN patients compared to that of the healthy volunteers ($P < 0.05$).

Association between serum miR-196a levels and the clinicopathological parameters of cervical cancer

The 105 cervical cancer patients were split into two groups (low or high) based on the mean serum level of miR-196a (higher or lower than the mean value of 3.88). The results show that serum miR-196a levels are associated with various clinical parameters including tumor size (P =

0.031), LN metastasis (P = 0.018), FIGO stage (P = 0.004), and grade (P = 0.011). However, miR-196a levels were not correlated with age (P = 0.299), cell type (P = 0.310), parametrial infiltration (P = 0.390), or HPV infection (P = 0.218; Table 1).

Association between serum miR-196a levels and CIN grade

The mean level of serum miR-196a in CIN patients (mean relative expression of 2.92) was used as cut-off point to divide the 86 patients with CIN into a high serum miR-196a group (N = 40) and low serum miR-196a group (N = 46). 67% of CIN3 patients were in the high serum miR-196a group, which was significantly more than that of CIN2 patients (32%; P = 0.010) and CIN1 patients (21%; P = 0.001). No significant difference was detected between CIN1 and CIN2 patients regarding the serum miR-196a distribution pattern (P = 0.507; Table 2).

Table 2. Correlation between serum miR-196a expression and CIN grade.

Serum miR-196a	CIN1 (N)	CIN2 (N)	CIN3 (N)
High expression	4	8	28
Low expression	15	17	14
P value	0.507 (CIN1 *vs* CIN 2)	0.010 (CIN2 *vs* CIN3)	0.001 (CIN3 *vs* CIN1)

Serum miR-196a levels and cervical cancer prognosis

The association between serum miR-196a levels and the overall survival of cervical cancer was evaluated using the Kaplan-Meier method. The five-year overall survival rate of patients in the high serum miR-196a group was 39.47%, which was significantly lower than that of patients (73.13%) in the low serum miR-196a group (P = 0.004; Figure 2).

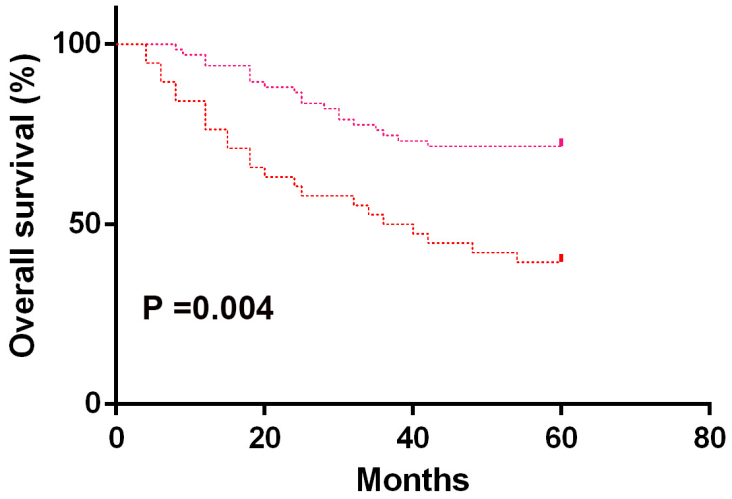

Figure 2. Association between serum miR-196a levels and overall survival. The five-year overall survival rate of cervical cancer patients in the high serum miR-196a group was significantly lower than that of the low serum miR-196a group (P = 0.004).

The univariate analysis showed that LN metastasis (hazard ratio (HR) = 2.342; 95% confidence interval (CI) = 1.524-4.325; P = 0.038), FIGO stage (HR = 3.814; 95%CI = 2.374-6.964; P = 0.006), grade (HR = 2.747; 95%CI = 1.867-5.214; P = 0.024), and serum miR-196a levels (HR = 3.102; 95%CI = 2.031-5.775; P = 0.013) were prognostic factors for cervical cancer. The multivariate analysis revealed that FIGO stage (HR = 4.254; 95%CI = 2.552-8.365; P = 0.009), grade (HR = 3.173; 95%CI = 1.633-5.306; P = 0.036), and serum miR-196a levels (HR = 3.510; 95%CI = 1.961-6.874; P = 0.025) were independent factors associated with survival in cervical cancer (Table 3).

Table 3. Univariate and multivariate analyses of the association between clinical parameters and overall survival.

Parameters	Univariate analysis			Multivariate analysis		
	HR	95%CI	P value	HR	95%CI	P value
Age	1.125	0.436-1.541	0.547			
Tumor size	1.734	0.917-2.457	0.116			
Lymph node metastasis	2.342	1.524-4.325	0.038	1.568	0.917-2.684	0.338
Cell type	1.057	0.637-1.263	0.745			
Parametrial infiltration	1.463	0.865-2.191	0.217			
FIGO stage	3.814	2.374-6.964	0.006	4.254	2.552-8.365	0.009
Grade	2.747	1.867-5.214	0.024	3.173	1.633-5.306	0.036
HPV infection	1.278	0.748-1.819	0.385			
Serum miR-196a	3.102	2.031-5.775	0.013	3.510	1.961-6.874	0.025

DISCUSSION

Cervical cancer, a malignant disease of the cervix, remains one of the leading causes of cancer deaths globally. The clinical outcome of patients with cervical cancer depends mainly on the clinical stage at which therapy is initiated. Although the prognosis of cervical cancer has substantially improved over the past decades, patients with advanced stage cervical cancer have poor overall survival. To improve outcomes of this deadly disease, identifying new molecular biomarkers for early detection, diagnosis, and prognosis is an effective strategy. CIN is a precancerous lesion that can develop into cervical cancer. If CIN is detected and treated effectively, the incidence of cervical cancer is significantly reduced. Unfortunately, there are currently no known specific markers for cervical cancer and/or CIN, thus regular screening is extremely important.

miRNAs may function as oncogenes or tumor suppressors in cancer development. Alteration in miRNA expression is closely correlated with tumor initiation, promotion, and progression via its effects on downstream target genes. Therefore, the identification of miRNAs associated with clinical features and prognosis of cancer may be valuable as biomarkers for clinical use. Deregulation of miR-196a is a frequent event in cancer, indicating its crucial role in tumorigenesis. In addition to cancer progression, miR-196a was shown to be involved in several important biological functions related to development, cell differentiation, immunology, inflammation, and viral defense (Chen et al., 2011). For example, the misexpression of miR-196a in the anterior of Xenopus laevis embryos caused eye anomalies (Qiu et al., 2009). Moreover, Kim et al. (2009) showed that miR-196a was an important regulator for the proliferation and osteogenic differentiation of human adipose tissue-derived mesenchymal stem cells.

In the present study, our results show that serum miR-196a levels were upregulated in patients with cervical cancer or CIN compared to those of healthy controls. Moreover, serum miR-196a levels were associated with CIN grade and various important clinical parameters of cervical

cancer. Additionally, cervical cancer patients with higher serum miR-196a levels had a poorer overall survival rate. Multivariate analysis revealed that serum miR-196a was an independent risk factor for cervical cancer, indicating that serum miR-196a may be a promising prognostic biomarker. Similar to our results, previous studies have found that miR-196a is overexpressed in cervical cancer tissues, CIN tissues, and cervical cancer cell lines (Gocze et al., 2013; Zhang et al., 2013; Villegas-Ruiz et al., 2014; Hou et al., 2014). Specifically, Hou et al. (2014) reported that upregulation of tissue miR-196a in patients with cervical cancer was associated with poor clinical outcome, indicating that miR-196a may function as an oncogene in cervical cancer. Moreover, miR-196a was shown to promote the proliferation of cervical cancer cell lines via regulation of FOXO1 and p27Kip1 directly (Hou et al., 2014). Netrin 4 (NTN4) is a direct target of miR-196a and its expression is inversely correlated with miR-196a expression. Ectopic expression of miR-196a promotes the proliferation and migration of cervical cancer cell lines through downregulation of NTN4, whereas the opposite was observed upon miR-196a suppression (Zhang et al., 2013). miR-196a has also been reported to play oncogenic roles in a number of types of cancers. Overexpression of miR-196a increased cell proliferation, migration, invasion, and induced epithelial to mesenchymal transition in head and neck cancer, whereas reduced oncogenic behavior was observed with miR-196a inhibition (Suh et al., 2014). Huang et al. (2014) showed that miR-196a was upregulated in pancreatic cancer cell lines, and may play an important role in the regulation of cell proliferation and migration by interacting with its metastasis-related target gene nuclear factor-kappa-B-inhibitor alpha. Higher expression of tissue miR-196a was detected in patients with gastric cancer and was associated with poor prognosis. Additionally, miR-196a has been shown to promote gastric carcinogenesis both *in vitro* and *in vivo* (Sun et al., 2012; Tsai et al., 2014).

While most studies have suggested that miR-196a enhances tumorigenesis in cancer, its role in tumor suppression cannot be ignored. For example, the expression level of miR-196a was shown to be reduced in melanoma cells compared to that in healthy melanocytes. miR-196a downregulation can lead to HOX-B7 and BMP-4 overexpression, which may be responsible for the early steps of melanoma development (Braig et al., 2010). Li et al. (2010) showed that overexpression of miR-196a or miR-196b inhibited the invasion and metastatic capacity of breast cancer cells both *in vitro* and *in vivo*, suggesting miR-196a may play a role in tumor suppression in breast cancer. This contradictory role of miR-196a in regulating cancer development is poorly understood. It is possible that the function of miR-196a depends on the cancer cell type and on the specific tumor microenvironment in which the cells reside. Hence, further investigations are needed to reveal the complex role miR-196a in different cancers.

In conclusion, increased serum miR-196a was associated with higher CIN grade, and correlated with poorer prognosis in cervical cancer patients. Therefore, serum miR-196a may be a promising biomarker for both CIN and cervical cancer.

Conflicts of interest

The authors declare no conflict of interest.

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