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Association of microRNA-10b expression and P16 with pathological features in various stages of cervical precancerous lesions

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ABSTRACT

Cervical cancer is the fourth most prevalent cancer for females with 14,100 new cases each year globally. Efficient screening and intervention at the precancerous stage is the key point to the prevention and treatment of cervical cancer. However, no widely recognized biomarkers have been discovered yet. We investigated the expression of miR-10b in cervical cells and its correlation with clinicopathological features in different pathological grades of cervical precancerous lesions. The expression of miR-10b in cervical cytology samples from 20 cases of LSIL, 22 cases of HSIL, 18 cases of early-stage cervical cancer, and 20 cases of cervicitis controls were assessed using qPCR. From the same cervical cytology samples, the human papillomavirus (HPV) load was assessed using semi-PCR and the lesion size, and gland involvement levels from the same subjects were assessed during the cervical examination. The correlation between miR-10b expression and different pathological grades of cervical lesions was analyzed. We also calculated the correlation between HPV load, lesion size, gland involvement, P16 expression, and different pathological grades. The expression of miR-10b exhibited a step-decreasing manner from cervicitis control (4.23(4.00,4.71)) to LSIL (2.67(2.52,2.90)), HSIL (1.49(1.30,1.80)) and cervical cancer group (0.65(0.55,0.80)). There is a significant difference ($P<0.001$) between cervicitis and HSIL, cervicitis and cervical cancer, ISIL and HSIL, as well as ISIL and cervical cancer but not between the cervicitis group and the LSIL group. In addition, more severe pathological grades were correlated with a bigger rate of gland involvement ($P<0.001$). We also found that different pathological grades were correlated with the intensity of P16 expression ($P=0.001$), and the intensity of P16 expression is positively correlated with different pathological grades ($P<0.05$). Repressed expression of miR-10b is related to the progression of cervical precancerous lesions. Increased gland involvement rate and increased intensity of P16 expression are risk factors for developing cervical cancers. Our result showed that miR-10b may be a potential biomarker for the screening and ranking of cervical precancerous lesions.

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Introduction

Cervical cancer is characterized by disruption of cell cycle progression, and abnormal proliferation of cervical epithelial cells accompanied by an irregular expression of tumor suppressor genes and oncogenes. It is the fourth most prevalent cancer for females with 14,100 new cases each year globally. Cervical intraepithelial neoplasia (CIN) is the early lesion of invasive cervical cancer. It is divided into two main types: low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL) (1).

Persistent human papillomavirus (HPV) infection, is recognized as one of the main risk factors related to the occurrence and development of CIN. The severity of HPV infection is often correlated to a load of HPV virus in the cervical tissue samples which is an important parameter for the progression of CIN in clinical diagnosis (2). Glandular involvement indicates the disease-involved area in the cervical canal and the depth of invasion. Previous studies were mainly focused on the correlation of glandular involvement with HSIL postoperative residual/recurrence. It is believed that glandular involvement increases the chance of recurrence after HSIL surgery by 1.33 times. P16INK4A gene (P16) is a tumor suppressor gene encoded by cyclin-dependent protein kinase inhibitor 4a (INK4A). The dysfunction of P16 caused excessive cell proliferation due to altered cell cycle regulation, which promoted the underdeveloped cells from the G1 phase to enter the S phase earlier, leading to the formation of tumors. P16 staining has been used increasingly as an adjunct assessment to cervical pathological examination for CIN progression. Although many pathological features and disease-related genes have been found and used in medical practice, more efforts are still urgently needed to establish a more comprehensive and accurate model for the diagnosis or control of CIN. Thus, exploring the expression alteration pattern of CIN-related genes is highly essential to improve early diagnosis and intervention (3,4).

With continual research, multiple markers have been identified for CIN. Of interest, one of the markers is microRNA-10b (miR-10b), a member of the microRNA-10 family. Previous studies showed that repressed expression of miR-10b is correlated ted with the proliferation, migration, and invasion of cervical cancer cells, and the inhibition of apoptotic cell death (5,6). However, the role

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of miR-10b in the progression of CIN is still not fully studied. Therefore in this study, we investigated the expression pattern of miR-10b in different pathological grades of cervical precancerous lesions, as well as the correlation between clinicopathological features and the progression of cervical cancers (7,8).

Materials and Methods

Participants and clinical information collection

60 patients were enrolled in the Department of Cervical Cancer Early Prevention and Treatment at Second People's Hospital of Fujian University of Traditional Chinese Medicine (from January 2020 to July 2021). The patients were diagnosed with CIN and early cervical cancer by HPV test, three-step cervical screening program, and cervical pathological examination. The study was approved by the Ethics Committee of the Second People's Hospital Affiliated with the Fujian University of Traditional Chinese Medicine. All patients have given their signed informed consent for the use of their cervical samples and clinical information.

Inclusion and exclusion criteria

Subjects' inclusion criteria were as follows in the present study:1)Confirmed with biopsy and pathological examination after surgery; 2)No history of radiation therapy or immunotherapy; 3)Signed informed consent. And the exclusion of the subject criteria was as follows**: 1)** Patients with malignant tumors in other parts of the body; 2) Patients with cervical lesions and cervical cancer recurrence after surgery; 3) Patients with thyroid dysfunction; Patients with other major diseases in the cervix and reproductive system

Diagnostic criteria for cervical intraepithelial neoplasia and cervical cancer

The cervical intraepithelial neoplasia diagnostic system is based on tumors of the uterine cervix in the 2014 WHO Classification Of Tumors Of Female Reproductive Organs. LSIL is classified by the proliferation of squamous epithelium basal/parabasal cells, irregular nuclear polarity and cytonuclear atypia, and fewer mitotic figures present in the lower one-third layer of the epithelium. The P16 immunohistochemical staining in cervical biopsies is negative or shows patchy staining of epithelial cells. HSIL is classified by loss of nuclear polarity, increased nuclear-to-cytoplasmic (N : C) ratios, and numerous mitotic figures. The cytonuclear abnormalities are present in the lower and middle-third layer of the epithelium or even the whole layer. The P16 staining is positive in an intense and diffuse pattern that extends from basal layers upward to at least two-thirds of the epithelium.

The diagnostic criteria for cervical cancer followed "Guidelines for the Diagnosis and Treatment of Cervical Cancer" (2021 edition, Gynecological Oncology Committee of the Chinese Anti-Cancer Association)where the FIGO staging of early cervical cancer is defined as Ia2-IIa 2.

miR-10b Detection by Using Real-time Fluorescence-based Quantitative PCR

The total RNA of the cervical cytology samples was extracted using TRIzol® Reagent according to the manufacturer's protocol. Briefly, 0.2 ml chloroform was added to each 1 ml TRIZOL reagent with lysed homogenate sample for two-phase separation. RNA solutions were obtained by performing RNA precipitation-washing-drying-dissolution steps. RNA concentration and purity were determined by NanoDrop ND-1000, based on UV absorbance (concentration formula: A260×40ng/ul; purity: A260/A280 ratio). The miR-10b forward primer wasAGCTGTTCAGTGCACTACAGA and the miR-10b reverse primer was GTGCTACCCTGTAGAAC. The real-time fluorescence-based quantitative PCR of miR-10b was carried out using ViiATM 7 Real-Time PCR System. Primers were designed using Primer 5.0 and ViiATM 7 Real-time PCR System from Applied Biosystems and synthesized by Yingjun Biotechnology Company.

HPV16 detection

Semi-quantitative detection of HPV16 load was tested by Human Papillomavirus (HPV) nucleic acid detection kit from Hangzhou Detong Biotechnology using hybrid capture-chemiluminescence technology according to the manufacturer's protocol. Following the protocol of the HPV detection kit, the brief steps were denaturation, hybridization, capturing, washing, luminescence reaction & reading and results analysis.

Statistical analysis

SPSS 22.0 software was used for data analysis. Non-normal distribution data were compared by a non-parametric test, and the correspondence between continuous variables was analyzed by Kendall'stau-b and Spearman correlation analysis. P value ≤0.05 was considered to be statistically significant.

Results

Paitents description

Among the 60 patients, there were 20 cases of LSIL, aged from 32 to 61 years, with an average age of 45.4±7.5 years old. In terms of pathological features, there were 13 cases with a lesion diameter ≥1cm, 7 cases with a lesion diameter <1cm, and no cases with the accumulation of glands. One case out of 20 was positive (+) for P16, and 19 cases were negative. 22 cases were included in the HSIL group, aged from 35 to 60 years, with an average age of 47.4±9.5 years. In terms of pathological features, there were 13 cases with lesion diameters ≥1cm, and 9 cases with lesions less than 1cm. There were 19 cases without accumulation of glands, and 3 cases with gland involvement.19 cases were negative for P16 and 3 cases were positive. According to the intensity of the P16 expression, they were defined as 1 case of positive (+), 1 case of positive (++), and 1 case of positive (+++).

In addition, there were 18 patients included in the early cervical cancer group, aged from 37 to 59 years, with an average age of 48.6±7.4 years. In terms of pathological features, there were 10 cases with lesion diameter ≥1cm, and 8 cases with less than 1cm. There were 10 cases without gland accumulation, and 8 cases with gland involvement. 10 cases were tested with P16 negative. No cases were positive (+), 4 cases were positive (++), and 4 cases were positive (+++).

Twenty cervicitis cases were included as the control group after being examed with cervical examination to rule out any sign of cervical malignant changes during the

same period when the patient groups were enrolled. The age range of the control group is 33-59 years old, with an average of 43.9±8.5 years old. There was no significant difference in the age of the 4 groups of patients (P>0.05).

Expression analysis of miR-10b in different pathological grades

The relative expression of miR-10b exhibited a step-decreasing manner from cervicitis control (4.23(4.00,4.71)) to LSIL (2.67(2.52,2.90)), HSIL (1.49(1.30,1.80)) and cervical cancer group (0.65(0.55,0.80)) (Figure 1 and Table 1). With pairwise comparisons, there was a statistically significant difference in the expression of miR-10b between cervicitis versus HSIL, cervicitis versus cervical cancer, ISIL versus HSIL, and ISIL versus cervical cancer. Although not significant, there is a trend (p=0.071) between the miR-10b expression of the cervicitis group and the LSIL group (Table 1). The lower level of miR-10b was significantly associated with the higher stage of cervical cancer.

HPV virals load in different pathological grades of CIN

There was a statistically significant difference in HPV load between different pathological grades of cervical precancerous lesions (P<0.001). By doing pairwise comparisons, we found no significant difference in HPV load between the HSIL group and the cervical cancer group (P>0.05). While there was a significant difference between the LSIL group and the HSIL Group, as well as the LSIL group and the cervical cancer group (P<0.05) (Table 2).

Lesion size in different pathological grades of CIN

There was no correlation between different pathological grades and lesion sizes (P>0.05), nor between individual groups (P>0.05) as shown in table 3.

Gland involvement rate in different pathological grades of CIN

The rates of endocervical gland involvement were positively associated with an increased pathological grade of cervical precancerous lesions (P<0.001). With more intense grades, the rate of glandular involvement was higher as shown in Table 4.

Expression of P16 in different pathological grades of CIN

The analysis showed that the intensity of P16 expression was correlated with more severe pathological grades

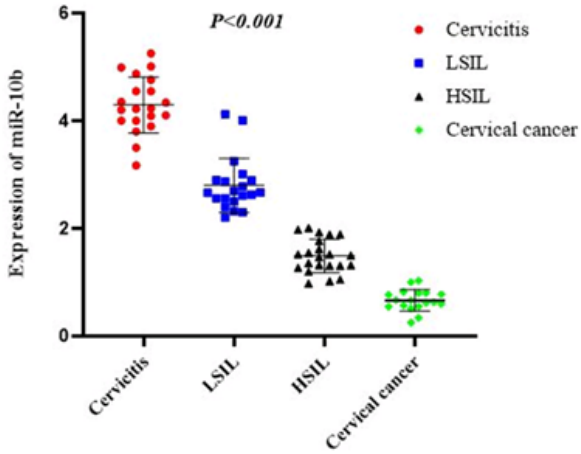

Figure 1. Pathological grades of cervical lesions.

Table 1. Expression levels in different cervical lesions groups.

Pathology	MiR-10b Median (P_{25}, P_{75})	Z value	P value	Pairwise comparisons	Pairwise comparisons P value
Cervicitis	4.23(4.00,4.71)	72.718	<0.001	Cervicitis-LSIL	0.071
				Cervicitis-HSIL	<0.001
LSIL	2.67(2.52,2.90)			Cervicitis - Cervical cancer	<0.001
HSIL	1.49(1.30,1.80)			LSIL-HSIL	0.014
Cervical cancer	0.65(0.55,0.80)			LSIL- Cervical cancer	<0.001

LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; P25, xx; P75, xx.

Table 2. HPV virals load in different pathological grades of CIN.

Pathology	HPV virals load (P_{25}, P_{75})	Z value	P value	Pairwise comparisons	Pairwise comparisons P value
LSIL	243.00 (147.25,289.00)	20.903	<0.001	LSIL-HSIL	0.005
HSIL	334.50 (272.50,401.00)			LSIL- Cervical cancer	<0.001
Cervical cancer	375.00 (334.75,401.25)			HSIL- Cervical cancer	0.406

Table 3. Lesion size in different pathological grades of CIN.

Pathology	Cases	Lesion size (%)		Total
		≥1cm	<1cm	
LSIL	20	65.00%	35.00%	33.33%
HSIL	22	59.10%	40.90%	36.67%
Cervical cancer	18	55.60%	44.40%	30.00%
Total	60	60.00%	40.00%	100.0%

Spearman r=0.077, P<0.05; Kendall's tau-b =0.073, P<0.05.

Table 4. Gland involvement rate in different pathological grades of CIN.

Pathology	Cases	gland involvement (%)		Total
		None	Yes	
LSIL	20	100.00%	0.00%	33.33%
HSIL	22	86.40%	13.60%	36.67%
Cervical cancer	18	55.60%	44.40%	30.00%
Total	60	81.70%	18.30%	100.0%

Spearman r=0.452, P<0.001; Kendall's tau-b =0.426, P<0.001.

Table 5. Correlation of P16 expression and different cervical lesions.

Pathology	Cases	P16 expression (%)				Total
		Negative	+	++	+++	
LSIL	20	95.00%	5.00%	0.00%	0.00%	33.33%
HSIL	22	86.40%	4.50%	4.50%	4.50%	36.67%
Cervical cancer	18	55.60%	0.00%	22.20%	22.20%	30.00%
Total	60	80.00%	3.30%	8.30%	8.30%	100.0%

Spearman r=0.405, P=0.001<0.05; Kendall's tau-b =0.371, P=0.002<0.05.

of cervical precancerous lesions (P<0.05). When the pathological grade of the lesions increased, the expression of P16 also showed an increasing trend (See Table 5).

Discussion

The main cause of cervical cancer is persistent HR-HPV infection. Although there were plenty of studies on the occurrence and development of cervical cancer at the molecular level, there is not yet a substantial achievement in the medication treatment for cervical cancer (9,10). The major treatments for cervical cancer are still surgery, radiotherapy, and chemotherapy. Early diagnosis and intervention are still the main methods for preventing, controlling, and treating cervical precancerous lesions. It is a progressive process from cervical precancerous lesions to cervical cancer. Early diagnosis of CIN through cervical cancer screening and prompt treatment of HSIL is the focal point for the secondary prevention and control of cervical cancer. Therefore, it has always been an important scientific issue to find the key biological indicators for predicting the progression of cervical precancerous lesions in current cervical cancer research (11,12).

MicroRNAs are important non-coding regulatory molecules, which play key roles in the post-transcriptional regulation of gene expression in multicellular organisms. It has been proved that miRNAs are involved in the occurrence and development of a wide range of tumors, including cervical cancer. Therefore, exploring the correlation between miRNA and tumor occurrence and development will be a novel and unique research topic, which has great potential to become a target for tumor diagnosis and treatment (13,14).

Studies have shown that the expression of miR-10b was significantly lower in cervical cancer specimens compared with normal cervical tissues. Furthermore, overexpression of miR-10b inhibited the proliferation and migration of multiple cervical cancer cell lines, such as Hela and SiHa cells. Moreover, miR-10b is known to play a protective role in the treatment of cervical cancer due to its function of promoting apoptosis and autophagy of cervical cancer cells. In this study, we further analyzed the expression of miR-10b in different pathological grades of cervical precancerous lesions and investigated the potential role of miR-10b in the occurrence and development of cervical lesions (15,16).

In this study, we found that the relative expression of miR-10b in different pathological grades of cervical precancerous lesions was statistically significant. In line with previous studies, we found that the expression of miR-10b in the cervical cancer group was significantly lower than that of the cervicitis group, (refer to the previous study), indicating that miR-10b may function as a tumor suppressor in cervical cancer. Aberrant expression of miR-10b can contribute to the onset of cervical cancers by disrupting the balance between oncogenes and tumor suppressor genes. In addition, with the development of cervical precancerous lesions, from cervicitis /LSIL to HSIL group and then early cervical cancer stage, the expression of miR-10b showed a significant downward trend, suggesting that the expression of miR-10b was suppressed in different pathological stages of cervical precancerous lesions, and its expression showed a negative correlation with the progress of pathology (17,18). It is plausible that miR-10b played a role as a tumor suppressor gene in cervical cancer. The progression of cervical cancer is highly related to persistent HR-HPV infection, cell cycle abnormalities, and irregular proliferation of cervical cells. As a potential tumor suppressor microRNA, lower expression of miR-10b may lead to abnormal expression of its target mRNA, subsequently participating in the occurrence and proliferation of cervical cancer. Therefore, it may be used as a biomarker that reflects the progression of cervical precancerous lesions to cancer (19,20).

Moreover, we investigated the clinicopathological characteristics of cervical precancerous lesions in different pathological grades. It was found that the high-risk HPV (HR-HPV) load was significantly lower in the LSIL group compared to the HSIL group and cervical cancer group, while there was no difference between the HSIL group and the cervical cancer group probably due to the virals load was already very high at HSIL stage and will not further increase. Although persistent HR-HPV infection is the cause of cervical cancer, HR-HPV viral load does not

always precisely reflect the progression of cervical precancerous lesions. The different pathological grades and lesion sizes of cervical precancerous lesions also showed no correlation, suggesting that measurement of lesion size cannot demonstrate the progression of cervical precancerous lesions. One of the reasons might be that cervical lesions showed a pattern of multiple lesions and discontinuities and in this study, only the largest lesion on the surface of the cervix was included (21,22).

Here, we also found that endocervical glandular involvement was associated with a higher chance of high-grade cervical precancerous lesions, suggesting that glandular involvement may play a role in the progression of CIN. Our result showed the expression intensity of tumor repressor gene P16 was positively correlated with different pathological grades of cervical precancerous lesions ($P<0.05$), validating the reliability of P16 to be used as the indicator for CIN progression.

In summary, the expression of miR-10b gradually decreased with the progression of cervical precancerous lesions, which can be used as a molecular marker to evaluate the prognosis and progress of CIN. The next step would be increasing the sample size and further exploring the interaction between miR-10b and other markers that reflect the progression of cervical precancerous lesions, providing more accurate prognostic factors for clinical diagnosis and treatment of cervical precancerous lesions with a multi-marker prediction model.

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Interest conflict
The authors declare that they have no conflict of interest.

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Author's contribution
Conception and design: Yong Deng; Methodology: Yong Deng, Hongmei Jiang; Data Collection: Yong Deng, Hongmei Jiang, Dongmei Wu; Data analysis and interpretation: Dongmei Wu; Manuscript writing: All authors; Final approval of manuscript: All authors.

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