ORIGINAL RESEARCH

Clinical Diagnosis of Cervical Lesions Combined with Programmed Death Ligand and miR-124 Detection in Peripheral Blood

Haixia Xu, BM; Yan Jin, MM; E Hu, BM; Zhifang Huang, BM; Bixia Yi, MM; Fan Wang, MD

ABSTRACT

Objective • This study aims to analyze the expression of colposcopy combined with PD-L1 (programmed death ligand-1) and miR-124 (microRNA-124) in CC (cervical cancer) and CIN (cervical precancerous lesions), providing insights for clinical screening and diagnosis of these conditions.

Method • A total of 60 patients with suspicious cervical lesions were selected from the gynecological clinic at Jinhua People's Hospital between June 2021 and December 2021. The patients were divided into three groups: LSIL (low-grade squamous intraepithelial lesions), HSIL (high-grade squamous intraepithelial lesions), and no SIL group, with 20 cases per group. This sample distribution ensures a comprehensive representation of different lesion severities. Pathological tissues were collected from each group for immunohistochemistry analysis to assess PD-L1 expression. Peripheral blood samples were also obtained from the patients for PCR analysis to evaluate miR-124 expression. These techniques allowed us to examine the expression levels of PD-L1 and miR-124 in the samples accurately.

Result • The HSIL group exhibited a higher rate of positive PD-L1 expression compared to the LSIL and no lesion groups. Additionally, the expression level of miR-124 was lower in the HSIL group compared to the LSIL and no lesion groups ($P < .05$). Statistical measures such as means, standard deviations, and p-values were used to quantify these differences, providing a more comprehensive understanding of the results.

Conclusions • Combining colposcopy results with the expression of PD-L1 and miR-124 can effectively evaluate precancerous lesions of cervical cancer. This combined approach holds significant clinical implications by potentially enhancing early detection, diagnosis, and treatment strategies for CC and CIN. Further research in this area may lead to improved patient outcomes and contribute to the development of targeted therapies. (*Altern Ther Health Med.* [E-pub ahead of print.])

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INTRODUCTION

According to the 2020 Global Cancer Statistics Report,[1] CC(cervical cancer) is the fourth largest malignant tumor among women around the world. 604 000 women are diagnosed with CC every year, and as many as 341 000 deaths are reported. Continuous infection with high-risk types of human papillomavirus (HPV) is a well-established pathogenic factor in the development of cervical cancer (CC). HPV is a sexually transmitted virus that infects the epithelial cells of the cervix. Persistent infection with high-risk HPV types, such as HPV-16 and HPV-18, can lead to cellular changes in the cervical epithelium, promoting the development of precancerous lesions and potentially progressing to invasive cervical cancer. Immune escape plays a critical role in the development of CC, but its correlation mechanism is not yet clear.[2,3]

PD-L1 (Programmed death receptor ligand-1), called the immune card control point, plays an important role in the process of tumor immune escape. Programmed death-ligand 1 (PD-L1) expression has been implicated in tumor immune escape, a mechanism by which tumors evade immune surveillance and destruction. PD-L1 is a protein that binds to its receptor, programmed cell death protein 1 (PD-1), on the surface of immune cells, such as T cells and natural killer cells. This interaction leads to the inactivation

of these immune cells, preventing them from attacking cancer cells. Several studies have confirmed that PD-L1 is abnormally highly expressed in melanoma, lung cancer, kidney cancer and so on, mediating immune escape of tumor cells, and its immunotherapy has achieved significant efficacy in clinical trials.[4,5] Studies have found that PD-L1 on the surface of cervical cells is associated with the degree of cervical lesions. When the cervix only has HPV infection without lesions, PD-L1 expression is not high.[6]

miRNA (MicroRNA), is a kind of non-coding single-stranded RNA molecule encoded by a gene with about 22 nucleotides. Some studies have shown that miRNA has regulatory effects on cell oncogenes in the process of tumor development and development.[6,7] the expression of miR-124 gene was significantly lower, and miR-124 could significantly inhibit cell proliferation and migration, suggesting that miR-124 plays a tumor suppressor role in cervical carcinogenesis.[8] Previous studies showed the mechanism of miRNA regulating PD-L1 in various malignancies.[9] However, there are gaps in knowledge regarding the expression of PD-L1 in cervical and precervical lesions and the regulatory mechanisms between miR-124 and PD-L1.

In the current literature, there is a lack of comprehensive understanding regarding the regulatory role of miRNA in PD-L1 expression in cervical intraepithelial neoplasia (CIN). This study aims to address this research gap by investigating the expression levels of miR-124 and PD-L1 in cervical tissues of patients with CIN. The specific objectives of this study are to assess the correlation between miR-124 expression and PD-L1 levels, and to explore the potential prognostic and therapeutic implications of these findings in cervical cancer (CC). To achieve our objectives, we employed colonoscopy as a screening method to identify patients with CIN. We then utilized immunohistochemistry to detect PD-L1 expression in cervical tissues. Additionally, we measured the expression levels of miR-124 using peripheral blood samples obtained from the patients. This study holds significant implications for cervical cancer diagnosis, prognosis, and treatment. By investigating the relationship between miR-124 expression and PD-L1 levels in cervical lesions, we aim to shed light on the regulatory mechanisms underlying PD-L1 expression in CIN. The findings of this study may contribute to the development of novel prognostic markers and therapeutic targets for CC. Understanding the role of miRNAs in modulating PD-L1 expression could potentially guide the design of targeted therapies aimed at restoring immune responses against cervical tumors. Ultimately, this research has the potential to improve patient outcomes and advance precision medicine approaches in the management of cervical cancer.

METHODS

Human subjects

60 patients with suspected cervical lesions (increased vaginal discharge, vaginal fluid and contact bleeding) who visited the gynecological outpatient of Jinhua People's Hospital from June 2021 to December 2021 were selected as the study population. All study subjects were aware and voluntarily signed an informed consent form, and the ethics committee approved all relevant operations. All patients underwent vaginal colposcopy and histopathological examination. According to the results of the examination, the patients were divided into three groups in accordance with the degree of lesion: LSIL (low-grade squamous intraepithelial lesions) group (CIN I), HSIL (highly squamous intraepithelial lesions) group (CIN II-III), and non-lesion group. All participants provided informed consent, and the study was approved by the ethics committee at Jinhua People's Hospital.

Some patients were excluded because at least one of the following criteria was present: glandular abnormalities, sexually transmitted disease, other infectious disease, immunological disorder and pregnancy.

Vaginal colposcopy

The patient is placed in the lithotomy position, and a speculum is used to expose the vagina and cervix fully. The structure, shape and color of the cervix are initially observed, and the focus of the colposcope is adjusted. A large cotton ball soaked in 3% acetic acid is applied to the surface of the cervix for 1 minute, and the original squamous epithelial zone, transformation zone, and columnar epithelial zone are carefully observed for any abnormal images such as acetowhite epithelium, columnar epithelial edema, and irregular blood vessels. The surface contour and border are also examined. Finally, 5% iodine solution is applied to observe the iodine staining changes in cervical lesions, and multiple biopsies are taken in areas where there is no staining or abnormal images under the colposcope. If no suspicious lesions are found under the colposcope, biopsies are taken in the transformation zone. All tissue samples are immediately fixed in 10% formalin and sent for examination.

The pathological diagnostic criteria referred to the 2014 WHO Classification of Tumours of Female Reproductive Organs.[10]

TCT (Thinprep cytologic test) detection

Automatic processing, production, staining, sealing and reading by the programmed ultrathin cell detection system. The TBS (The Bethesda System) classification results were divided into no intraepithelial cells and malignant cells (NILM), atypical squamous cells (ASC-US), atypical squamous cells (ASC-H), LSIL, HSIL, squamous cell carcinoma (SCC), atypical gland cells (AGC), and atypical glands Cell-prone neoplasia (AGC-FN), cervical carcinoma in situ, and adenocarcinoma. Positive lesions were ASC-US or above; otherwise negative.

Immunohistochemical staining

Tissue sections were subjected to routine deparaffinization and hydration and then subjected to high-pressure repair in PBS buffer. Next, they were immersed in 3% hydrogen peroxide at 25°C, followed by blocking and incubation with

appropriately diluted antibodies overnight. After 12 h, the sections were diluted with secondary antibodies 30 min, followed by PBS washing. The sections were then incubated with an appropriate amount of DAB. The sections were counterstained with hematoxylin for 5 min, dehydrated with different concentrations of ethanol gradients for 2 h, and any remaining liquid on the surface was absorbed with sterile filter paper. Xylene was used for transparency, and neutral gum was used for mounting. Tissue morphology was observed and photographed under a light microscope.

Image-pro plus measures the cumulative optical density (IOD), which calculates the integration of all positive signals in the observed section under the light microscope, including the intensity and area of the positive signal.

Blood sample collection

All study subjects were asked to fast for 8-12 hours and 2 mL of fasting venous blood was collected from the elbow vein between 7:00-8:00 AM the following day. The blood was immediately treated with 2% ethylenediaminetetraacetic acid (EDTA) anticoagulant and stored at 4°C.

PCR

Total RNA was extracted from samples, and the PCR reaction system consisted of 2 μL of cDNA, 5.8 μL of PCR mix, 4.8 μL of primers (miR-124 and U6), and 7.4 μL of ddH_2O. U6 RNA was used as an endogenous control for data analysis.

Real-time PCR was performed on a Life Technologies QuantStudio 6 Flex System PCR instrument and performed using SYBR Green PCR kit (ELK Biotechnology, EQ010). The relative expression level was characterized by $2^{-\Delta\Delta CT}$. Each experiment was repeated three times. The primers for miR-124 and U6 were purchased from a third-party biological company (Table 1).

Statistical analysis

Data were analyzed by Statistic Package for Social Science (SPSS) 20.0 statistical software (IBM, Armonk, NY, USA). Quantitative data were expressed as ($\bar{x} \pm s$), and a non-paired two-sample t-test was used for intergroup comparison. $P < .05$ indicated statistical significance.

RESULTS

Description of the Study Groups

All patients were diagnosed by colposcopy biopsy, and according to the diagnosis of increased leucorrhea, vaginal fluid and contact bleeding, the result was patients with cervical cancer without obvious lesions, LSIL and HSIL.

The baseline features of all the groups are shown in Table 2. Among the 20 patients with HPV positive but no positive colposcopy results, the highest infection rates of HPV16 and HPV52 were approximately 30% (6 / 20) and 25% (5 / 20), respectively. The proportion of patients with only one type was 75% (15 / 20), while 25% (5 / 20) were infected with multiple types. Of the 20 LSIL patients, HPV52 and HPV58 types detected had the highest infection rates of approximately 30% (6 / 20), followed by HPV16 and HPV E6E7, both 10% (2 / 20). Of the 20 patients with HSIL, the highest infections were HPV16 and HPV52, at approximately 40% (8 / 20), with 75% (15 / 20), and 25% (5 / 20) with multiple types.

Table 1. Primer Sequences

Primer		Gene information	Primer Sequences (5 ` - 3 `)
H-U6	RT	NR_004394.1	AACGCTTCACGAATTTGCGT
	Forward		CTCGCTTCGGCAGCACAT
	Reverse		AACGCTTCACGAATTTGCGT
hsa-miR-124-5p	RT	MIMAT0004591	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGATCAAGGT
	Forward		GGCGTTCACAGCGGACC
	Reverse		CTCAACTGGTGTCGTGGAGTC

Table 2. Baseline characteristics and pathological features of patients (n = 20/group)

Subjects infected with HPV types (n)	No lesion	LSIL(CIN I)	HSIL(CIN II-III)
11	1	0	1
16	6	2	4
18	1	0	0
31	0	1	1
33	0	1	1
51	1	0	2
52	5	3	4
58	3	3	3
61	0	1	0
68	2	1	0
E6E7+	1	2	3
Other	1	6	2
Multiple	5	3	2
TCT			
NILM	7	3	5
ASC-US	5	3	4
ASC-H	2	1	1
LSIL	2	12	3
HSIL	1	1	5
Pathological features			
Gland	1	2	10
involvement	14	0	0
inflammation	0	2	0
Intermediate differentiation	1	0	1
Highly differentiated	0	0	2
Condyloma plana	1	11	0

Abbreviations: CIN, cervical intraepithelial neoplasia; LSIL, Low-grade squamous intraepithelial lesions; Highly squamous intraepithelial lesions; TCT, Thinprep cytologic test

PD-L1 Immunohistochemical staining

PD-L1 positive signal optical density IOD in LSIL and HSIL tissues were 2221, 4978, 7103, respectively, and pairwise comparisons were statistically significant ($P < .05$, $P < .01$) (Figure 2A). The immunohistochemistry results directly showed that PD-L1 has different shades of PD-L1 in no SIL group, LSIL and HSIL tissues, that is, PD-L1 tan positive expression varies.

The lowest immune scores for PD-L1 ($P < .05$) expression in non-SCC tissues, low and weakly positive for PD-L1 in LSIL tissues, and strongly positive in HSIL tissues ($P < .01$). The PD-L1 positive cells were mainly distributed in the interstitial space between the cervical squamous epithelial cells. Therefore, it can be seen that the positive expression of PD-L1 is gradually increased no SIL group, LSIL and HSIL groups, indicating that PD-1 not only suppresses the activity of lymphocytes and participates in tumor immunity through immunosuppression but also promotes the occurrence and development of CC. As shown in Figure 1.

Figure 1. PD-L1 immunohistochemistry results of HSIL patients without lesions, LSIL groups and HSIL groups

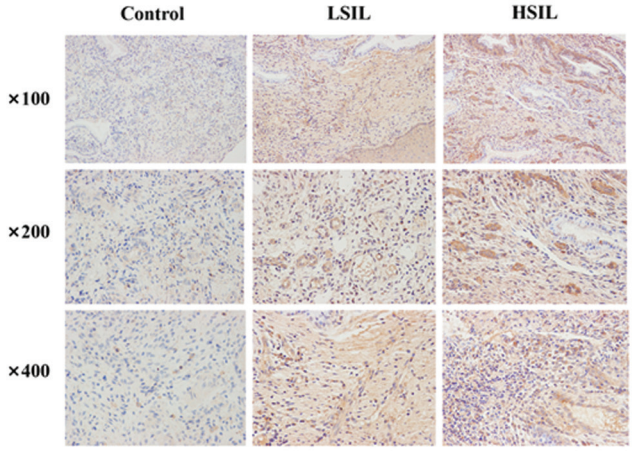
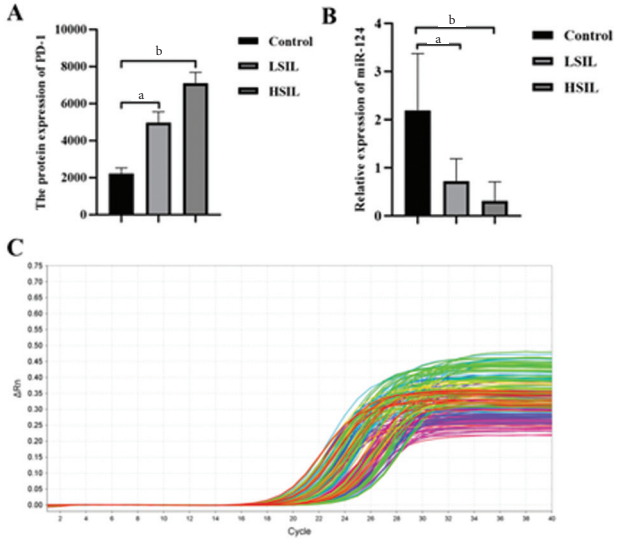


Figure 2. (A) Quantification of PD-L1 immunohistochemistry in patients in each group. (B) PCR results of miR-124 in each group. (C) The amplification map of the PCR experiments.



[a]$P < .01$
[b]$P < .001$)

PCR results of miR-124

The study showed that the expression of miR-124 in the LSIL group was significantly lower than that in the group without lesions, and the difference was statistically significant ($P < .05$). The expression of miR-124 in the HSIL group was significantly lower than that in the group without lesions, which was statistically significant ($P < .01$; Figure 2B).

DISCUSSION

According to clinical data, the incidence of cervical cancer in young women is increasing in recent years, and the incidence of cervical cancer also shows a trend of younger development.[11] If the differential diagnosis of lesions is not timely in the early stage, the life safety of patients will be seriously threatened. Previous clinical practice shows that through early surgery, the 5-year cure rate of cervical cancer can reach 80%~90%,[12] so the early clear diagnosis of cervical cancer and precancerous lesions is of key value for the disease treatment and prognosis recovery of patients. But before, cervical cancer lesions generally did not have specific performance, and after the onset of clinical manifestations similar to the cervical columnar epithelial ectopic cervical disease, and in the traditional cytology examination of specimen satisfaction is not ideal, sensitivity to cervical cancer and precancerous lesions, so the difficulty of early diagnosis of cervical cancer is higher, diagnostic accuracy is not ideal.[13,14] In this situation, it is very important to explore more screening methods with high accuracy and satisfaction for cervical cancer examinations.

Through a deep understanding of the mechanism of tumor immune escape, scholars have found that the relevant signaling pathways can be greatly reversed by inhibiting the mechanism of tumor immune escape. PD-L1 signaling pathway, as one of the important pathways mediating tumor immune escape, has attracted much attention.[15,16] Therefore, PD-L1 plays an important role in the immune escape of tumors[17,] which not only participates in the occurrence and development of tumors but is also associated with the invasion and metastasis of tumors.[18] Our results showed that PD-L1 expression increased with CIN grade. Namely, there is a direct correlation between PD-L1 expression and HPV-positive, indicate that this pathway contributes to immune evasion of HPV-associated CIN and CC.

CC is one of the most common gynecological malignancies, which has the characteristics of high incidence, easy metastasis and easy to relapse, and tends to be younger. Etiological analysis suggests that the occurrence of CC is not only the result of HPV (human papillomavirus) infection but also the result of the joint action of various environmental carcinogenic factors and genetic factors.[19,20] Research shows that miRNAs act as an important part in the cancerous process of cervical epithelial cells, among which miR-124 is closely associated with the development of cervical cancer.[21,22] In our study, the miR-124 gene in HSIL group was significantly lower than that in lesion-free group, suggesting that the expression of miR-124 can significantly inhibit cell proliferation and migration and miR-124 plays a tumor suppressor role in cervical carcinogenesis.[23]

Our results showed that PD-L1 expression increased with CIN grade. Namely, there is a direct relevance between PD-L1 expression and CIN, indicating that this pathway damages cervical anti-HPV responses and contributes to immune evasion of HPV-associated CIN and CC.[3]

The findings of our study have important clinical implications for the early screening and treatment of cervical lesions. The increased expression of PD-L1 and down-regulation of miR-124 were found to be positively associated with cervical HPV positivity and tumor metastasis. These molecular markers, PD-L1 and miR-124, can serve as valuable biomarkers in the clinical setting. Firstly, the expression levels

of PD-L1 and miR-124 can be utilized as diagnostic biomarkers for cervical intraepithelial neoplasia (CIN) and cervical cancer (CC). By assessing the expression of PD-L1 and miR-124, healthcare professionals can identify individuals who are at a higher risk of developing cervical lesions. This enables early detection and intervention, leading to improved patient outcomes. Secondly, these biomarkers hold potential therapeutic implications. Our findings suggest that targeting the PD-L1 pathway may be an effective strategy for immunotherapy in cervical cancer. PD-L1 inhibitors have shown promise in the treatment of various cancers by enhancing the immune system's ability to recognize and eliminate tumor cells. Therefore, our study suggests a potential avenue for targeted immunotherapy in CC patients with elevated PD-L1 expression. While our study provides valuable insights, it is essential to acknowledge its limitations. Firstly, we did not explore the relationship between PD-L1 and miR-124 expression in cancer tissues of CC patients. Investigating this relationship could help elucidate any potential synergistic role in the occurrence and development of cervical cancer. Additionally, the sample size limitation of our study may affect the generalizability of the results. Further research with larger cohorts is warranted to validate our findings. In future research, it would be worthwhile to investigate the relationship between PD-L1 and miR-124 expression in cancer tissues of CC patients. Understanding the interplay between these two factors could provide a more comprehensive understanding of their roles in CC development and progression. Furthermore, exploring potential therapeutic strategies that target the PD-L1 pathway in combination with miR-124 modulation could be a promising approach for improving immunotherapy outcomes in cervical cancer patients. Continued research in these areas will contribute to advancing personalized medicine in the field of cervical cancer.

ETHICAL COMPLIANCE

The ethics committee of The Second Clinical School of Medicine, Wenzhou Medical University approved this study. Signed written informed consents were obtained from the patients and/or guardians.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to report relevant to this article.

AUTHOR CONTRIBUTIONS

HX, YJ and FW designed the study and performed the experiments, EH and ZH collected the data, EH, ZH and BY analyzed the data, HX, YJ and FW prepared the manuscript. All authors read and approved the final manuscript. HX and YJ contributed equally to this work.

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