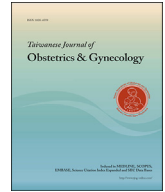




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Original Article

Correlation between HPV-negative cervical lesions and cervical microenvironment

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ABSTRACT

Objective: To investigate the correlation between high-risk human papillomavirus (HR HPV)-negative cervical lesions and cervical microenvironment in Inner Mongolia, China, and to find the pathogenic factors of HR HPV-negative cervical lesions.**Materials and methods:** 74 cases of HR HPV-negative healthy women and 80 cases of patients with cervical lesions (28 cases of LSIL, 49 cases of HSIL and 3 cases of CSCC) were selected as the study group; 26 cases of HPV-positive women and 352 cases of patients with cervical lesions (108 cases of LSIL, 214 cases of HSIL and 30 cases of CSCC) were control group. Questionnaires were collected from the study group and the control group and specimens were collected. Gram staining, hematoxylin and eosin staining microscopy, and substrate colorimetry method were used to detect vaginal micro-ecological indicators; ELISA was used to detect the concentration of SIgA, IgG, IL-2 and IL-10 in vaginal lavage fluid. Genetic testing was used to detect HPV, mycoplasma, and chlamydia infection. The changes of vaginal micro-ecology evaluation index and local immune factor concentration in healthy women and cervical lesions of all grades in the study group and the control group were compared.**Results:** Patients with cervical lesions, compared with healthy women, had a decrease in dominant lactobacilli and dysbacteriosis ($P < 0.05$), and this trend became more apparent as the disease progressed. The diversity and concentration of the flora in the HPV-negative group increased, the abnormal composition ratio decreased, and the HPV-positive group showed the opposite trend. As the lesion progressed, H_2O_2 decreased first and then increased, and the overall trend of SNa, LE, GUS, and GADP increased. The infection rate of trichomoniasis, BV and chlamydia increased and infection rate of Candida decreased. Also, compared with healthy women, patients with cervical lesions showed changes in immune factor concentration ($P < 0.05$). As the lesion progressed, IL-2 decreased, IL-10 increased, and IL-2/IL-10 decreased. However, IL-2 expression in HPV-negative group was higher than HSIL. SIgA was significantly lower in patients with cervical lesions than in healthy women. IgG had an upward trend in the HPV positive group.**Conclusion:** This study showed that vaginal micro-ecological imbalance and weakening of local cervical immune function are important reasons for the development of cervical lesions. It is expected to inhibit the development of cervical lesions by regulating the balance of vaginal micro-ecology and enhancing local immune function. By detecting *Lactobacillus vaginalis*, pre-enzyme, IL-2, IL-10, SIgA, it can guide the further diversion of HPV-positive women and predict the development direction of cervical lesions after HPV infection.© 2020 Taiwan Association of Obstetrics & Gynecology. Publishing services by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

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Introduction

Cervical lesions are an infectious disease [1], and current studies have confirmed that high-risk human papillomavirus (HR HPV) infection is the main cause of cervical lesions [2,3]. However, a large number of patients with cervical lesions had negative HPV test results [4,5]. HPV may not be involved in carcinogenesis in these

cases, and there are some other factors that may cause cervical lesions.

In 2010, Hanahan and Weinberg first proposed the theory of tumor microenvironment. The state of immune and inflammatory response in tumor microenvironment plays an important role in tumor development. The cervix is exposed to the vagina, and the micro-ecology and immunity of the vagina constitute an important micro-environment for the survival of the cervix [6]. Does the change of the cervical microenvironment affect the occurrence and development of cervical lesions? It is the focus of current research. Therefore, this study was conducted to evaluate the micro-ecology and immunological factors of vaginal and cervical secretions in patients with cervical intraepithelial neoplasia (CIN) and cervical squamous cell carcinoma (CSCC) in HR HPV-negative patients and to explore the correlation between HR HPV-negative cervical lesions and cervical microenvironment.

Materials and methods

Subjects

The study was approved by the Ethics Committee of the Affiliated Hospital of Inner Mongolia Medical University, and all the participants signed the informed consent form.

We selected 74 cases healthy women (HW) with HPV-negative, 80 patients with HR HPV-negative cervical lesions, including 28 cases of Low-grade Squamous Intraepithelial Lesion (LSIL), 49 cases of High-grade Squamous Intraepithelial Lesion (HSIL), and 3 cases of CSCC as the study group, which were diagnosed by pathology from November 2012 to September 2015 in China. We selected 26 cases of HPV-positive healthy women, 352 cases of HPV-positive cervical lesions, including 108 cases of LSIL, 214 cases of HSIL, and 30 cases of CSCC as the control group. Questionnaire survey was conducted to collect basic demographic information. The average age of the enrolled group was 42.22 ± 8.76 years old. The age difference between the groups was comparable and can be compared.

Sample collection

All samples were collected in non-menstrual period. There was no history of vaginal lavage and drug administration 1 week before sampling. There was no sexual life, bathing, vaginal washing and operation history within 24 h. No radiotherapy or chemotherapy was performed before the sampling.

Collection of vaginal secretions: (1) Use 3 sterile long cotton swabs to rotate the 1/3 segment of the vagina to obtain secretions for vaginal micro-ecological morphological evaluation. (2) HPV special brush was used to collect cervical secretions to detect HPV, mycoplasma and chlamydia. (3) Wash the upper third of the vagina and the cervicovaginal area with 5 ml of 0.9% NaCl, and then take the lavage solution in the test tube, separate the supernatant and store it at -20°C for immunological factor determination.

Cervical tissue material: Suspicious cervical lesions were taken through a colposcopy sterile biopsy and then sent to the pathology department for pathological diagnosis.

Vaginal microecological evaluation

Vaginal micro-ecology was evaluated according to the "Consensus of Clinical Application of Vaginal Micro-ecology Evaluation" [7]. After Gram staining, microscopic detection of bacterial population density, bacterial diversity, dominant bacteria were conducted. Pathogenic microorganisms such as clue cells, trichomoniasis, and candida were tested by hematoxylin and eosin staining microscopy. Detection of vaginal pre-incubase H_2O_2 , SNa,

LE, GUS, GADP was conducted using substrate chromogenic assay of five combinatorial assay kits.

Determination of vaginal local immune factor

The concentrations of SIgA, IgG, IL-2, and IL-10 in the supernatant were determined by enzyme-linked immunosorbent assay (ELISA) using RDElisaSIgA, IgG, IL-2, and IL-10 ELISA kits.

Detection of vaginal HPV, mycoplasma and chlamydia

After taking the material, the sample tube with the brush head was immediately sent to the laboratory for detection of HPV, mycoplasma and chlamydia by genetic testing.

Vaginal micro-ecological diagnostic criteria

1. Intensive population: $++\sim++++$ is normal, $+$, $++++$ is abnormal;
2. Flora diversity: $++\sim++++$ is normal, $+$, $++++$ is abnormal;
3. The dominant bacteria were evaluated as normal bacteria for Lactobacillus, and Bacterial Vaginosis (BV) was evaluated for clue cells ($+$), and other pathogen infections were assessed as dysbacteriosis.
4. $\text{H}_2\text{O}_2 \geq 2\mu\text{mol/L}$ is positive, $\text{SNa} \geq 7\text{U/L}$ is positive, $\text{LE} \geq 9\text{U/L}$ is positive, $\text{GUS} \geq 15\text{U/L}$ is positive, $\text{GADP} \geq 20\text{U/L}$ is positive. The positive H_2O_2 rating was normal for Lactobacillus, SNa negative, LE negative, GUS negative, and GADP negative assessment was normal.

Statistical analysis

The statistical analysis was carried out by the R3.3.1 statistical software by the School of Public Health, Tianjin Medical University, China. Qualitative data using the χ^2 test or the exact probability method. Quantitative data were tested by rank sum test. Statistical tests were performed using a two-sided test. P values less than 0.05 were considered statistically significant.

Results

Vaginal micro-ecology evaluation of healthy women and cervical lesions in all groups in the study group and control group

In HPV-negative and HPV-positive groups, compared with respective controls, patients with cervical lesions (including the cases of LSIL, HSIL, and CSCC) showed a decrease in dominant lactobacilli and dysbacteriosis, and the difference was statistically significant ($P < 0.05$) (Table 1, Fig. 1). As the lesion progressed, this trend became more apparent, and the dysbacteriosis of the CSCC group was most significant. The diversity and concentration of the flora increased gradually in the normal composition ratio of the study group, and the abnormal composition ratio gradually decreased, while the opposite trend appeared in the control group. The vaginally pre-formed enzyme H_2O_2 increased in the study group, and the CSCC rebounded after the control group decreased. The overall trend of SNa, LE, GUS and GADP positive rates were all increasing (Table 1, Fig. 2). Trichomonas, BV and chlamydia infection rate increased, candida infection rate decreased, and mycoplasma only infected in LSIL, HSIL group (Table 2, Fig. 3).

Table 1
Comparison of dominant bacteria, bacterial diversity, bacterial density, and positive pre-enzymes in all patients.

Indicator			HW	LSIL	HSIL	CSCC	χ^2	p
HPV(+)	Dominant bacteria	Normal flora	65.4	48.1	41.6	20.0		
		Flora imbalance	0	6.5	16.8	60.0	*	<0.001
		BV	34.6	45.4	41.6	20.0		
	Bacterial diversity	1+	0	4.6	14.0	36.7		
		2/3+	96.2	88.9	78.5	53.3	*	<0.001
		4+	3.8	6.5	7.5	10.0		
	Bacterial density	1+	0	4.6	14.0	36.7		
		2/3+	96.2	88.9	78.5	53.3	*	<0.001
		4+	3.8	6.5	7.5	10.0		
	Pre-enzymes	H ₂ O ₂ (+)	23.1	36.1	29.0	50.0	7.11	0.068
		SNA(+)	11.5	28.7	15.4	23.3	9.40	0.024
		LE(+)	30.8	39.8	46.3	53.3	4.12	0.249
		GUS(+)	0	1.9	1.9	6.7	*	0.301
		GADP(+)	3.8	13.0	19.2	26.7	7.15	0.067
		Normal Flora	67.6	53.6	57.1	33.3		
		Flora Imbalance	8.1	10.7	8.2	66.7	*	0.030
HPV(-)	Dominant bacteria	BV	24.3	35.7	34.7	0		
	Bacterial diversity	1+	5.4	10.7	6.1	0		
		2/3+	86.5	82.1	89.8	100.0	*	0.897
		4+	8.1	7.1	4.1	0		
	Bacterial density	1+	5.4	10.7	6.1	0		
		2/3+	86.5	82.1	89.8	100.0	*	0.897
		4+	8.1	7.1	4.1	0		
	Pre-enzymes	H ₂ O ₂ (+)	29.7	21.4	32.7	66.7	3.07	0.380
		SNA(+)	10.8	14.3	14.3	33.3	1.52	0.677
		LE(+)	31.1	21.4	40.8	66.7	4.70	0.195
		GUS(+)	2.7	0	4.1	33.3	*	0.021
		GADP(+)	6.8	17.9	10.2	33.3	4.42	0.219
		Normal Flora						
		Flora Imbalance						

The percentage (%) of patients in each subgroup of HPV (+) and HPV (-) categories were shown.

Asterisk represent that statistical method we used is Fisher's exact method.

Vaginal local immune factors in healthy women and patients with cervical lesions in the study group and the control group

In HPV-negative and HPV-positive patients, the concentration of immune factors appeared in patients with cervical lesions ($P < 0.05$) compared with the respective healthy controls. With the increase of the degree of disease, IL-2 expression was the lowest in HSIL, IL-10 was elevated, and Th1/Th2 was lowest in HSIL. In the control group, IL-2 decreased, IL-10 increased, and Th1/Th2 decreased. SIgA was significantly lower in patients with cervical lesions than in healthy women. As the degree of disease increased, SIgA decreased in the study group, and the opposite trend appeared in the control group. There was no significant change in IgG in the study group and an upward trend in the control group (Table 3, Fig. 4).

Discussion

HPV infection has been considered as the main cause of cervical cancer formation [8], and the research focus is mainly on the related pathogenic mechanism of HPV. However, it has been reported in the literature that about 42% of patients with cervical lesions have a negative HPV test [9]. For HR HPV-negative cervical cancer and precancerous lesions, the tumor development mechanism, biological characteristics and potential clinical detection indicators are rarely studied.

In 1975, Mintz proposed the theory that the tumor microenvironment determined the direction of tumor cell development, and predicted that tumor cells as "seeds" must cooperate with the surrounding environment "soil" to occur and develop [10]. Cervical lesions are complex biological behaviors involved in the regulation of multiple factors, and their development is actually a process in which tumor cells interact with the body environment. Cervical immune microenvironment includes vaginal micro-ecology and vaginal local immune system [11]. It is the external environment of

the cervix that depends on survival and lip-tooth dependence. It plays a key role in the occurrence and development of cervical lesions [12].

The main components of vaginal micro-ecology are *Lactobacillus vaginalis* and microbial flora. Under normal circumstances, vaginal micro-ecology maintains a dynamic balance [13]. When the balance breaks, the number of lactobacilli is reduced or the function is inhibited, the vaginal acid environment is destroyed, hypoxia, local immune function is reduced, the anti-tumor effect is weakened, and cervical lesions are prone to occur. KYRGIUO believed that genital tract infections and changes in the vaginal micro-ecological environment played an important role in the development of cervical cancer [14]. In this study, patients with HPV-positive and HPV-negative cervical lesions showed a decrease in dominant lactobacilli and dysbacteriosis compared with healthy women. HPV-positive healthy women, although infected with HPV, maintained a normal micro-ecological environment without cervical lesions. The patients with HPV-negative cervical lesions, although not infected with HPV, had a serious micro-ecological imbalance, and cervical lesions occurred. Therefore, the occurrence of cervical lesions is closely related to the local micro-ecology of the vagina. The level of cervical lesions is positively correlated with the degree of dysbacteriosis. Can we believe that the vaginal micro-ecological imbalance leads to a weakening of the local anti-tumor effect of the vagina to cause cervical lesions.

In this study, with the development of cervical lesions, vaginal flora imbalance caused pre-formed enzyme H₂O₂ decreased, SNA, GUS, GADP, LE increased. The flora diversity and concentration ratio were imbalanced, causing increased chances of infection of trichomoniasis, BV, chlamydia and other pathogenic bacteria, destroying the inherent defense mechanism of the vaginal micro-environment, and producing some harmful metabolites and carcinogens directly causing damage to the genital tract mucosal epithelial cells. Chronic inflammation caused local wounds to be difficult to heal, requiring constantly renewed cells, increasing the

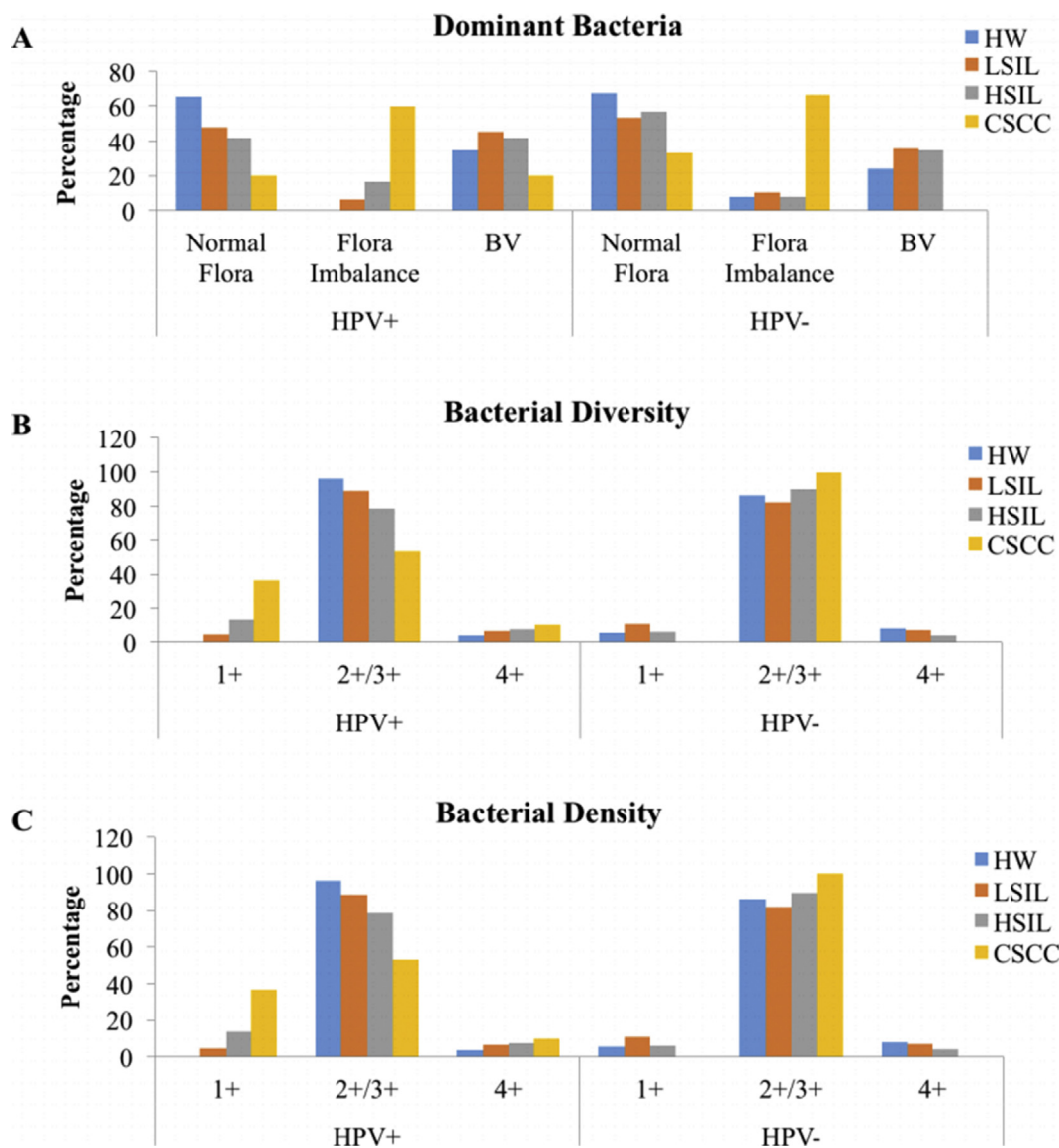


Fig. 1. Composition of vaginal dominant bacteria, diversity, concentration and pre-enzyme in healthy women and cervical cancer patients in the study group and control group (%).

risk of cell mutation and tumorigenesis, leading to cervical lesions [14,15]. Li Qing reported that vaginal pathogens were closely related to high-grade CIN lesions, and the structure and diversity of vaginal strains in patients with high-grade CIN were obvious [16]. Both domestic and foreign studies have shown a significant correlation between BV and cervical lesions [17]. Changes in related factors in the vaginal microenvironment suggest that there is a micro-environmental flora imbalance in patients with cervical lesions, causing local pH changes, harmful substances, affecting cell viability, metabolic patterns and biological behavior, inhibiting cell differentiation and immune response, leading to tumorigenesis.

Immunization is another core element in the tumor microenvironment and plays a key role in tumorigenesis. IL-2 and IL-10 are representative of cellular immune Th1 and Th2, respectively, and can enhance cellular immunity and suppress immune response [18]. Secretory Immunoglobulin A (SIgA) and Immunoglobulin G (IgG) are the main components of vaginal humoral immunity. Sheng Lei [19] reported that the expression level of IL-2 in cervical cancer and precancerous lesions of Uygur women was significantly decreased, and the expression level of IL-10 was significantly

increased. Liu Jing [20] reported a decrease in the concentration of immune factors such as SIgA and IgG in vaginal infections. In this study, HPV-positive and HPV-negative cervical lesions showed more changes in immune factors than healthy women. As the lesion progressed, IL-2 decreased, IL-10 increased, Th1/Th2 decreased, Th1 shifted to Th2, immunosuppression occurred, and cervical lesions developed. In the HPV-negative study group, IL-2 has the lowest expression of HSIL, which may indicate early tumor, strong immune response and weak inflammation. In the late stage of the tumor, the immune response is weak and the inflammation is strong [21]. Patients with HPV-positive and HPV-negative cervical lesions had significantly lower SIgA than healthy women; HPV-positive healthy women, although infected with HPV, maintained a high level of local immunity without cervical lesions; in patients with HPV-negative cervical lesions, although not infected with HPV, local immunoreduction occurred to cause cervical lesions. It was suggested that with the decrease of vaginal mucosal immune function, cervical lesions developed and SIgA was also expected to be an effective indicator of local immunity. The expression of IgG in HPV-negative healthy women and the patients

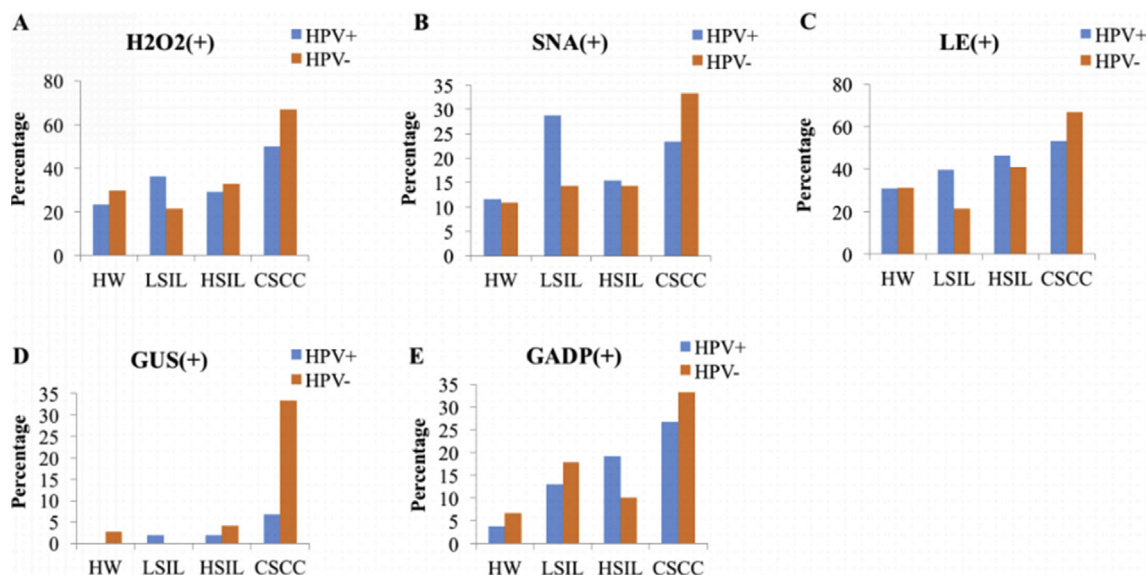


Fig. 2. Pathogenic microbial infections in healthy women and cervical cancer patients in the study group and control group (%).

Table 2

Comparison of the pathogenic microbial infections in all patients.

Indicator		HW	LSIL	HSIL	CSCC	χ^2	<i>p</i>
HPV(+)	Trichomonad(+)	3.8	8.3	15.4	16.7	5.55	0.136
	Candida(+)	15.4	1.9	1.9	0	*	0.010
	Clue cell(+)	3.8	19.4	26.2	26.7	7.64	0.054
	Mycoplasma(+)	0	3.7	4.7	0	*	0.740
	M.urealyticum(+)	23.1	60.2	69.6	76.7	24.68	<0.001
HPV(-)	Trichomonad(+)	9.5	7.1	8.2	66.7	11.42	0.010
	Candida(+)	5.4	0	2.0	0	*	0.624
	Clue cell(+)	13.5	14.3	24.5	33.3	3.19	0.363
	Mycoplasma(+)	0	10.7	2.0	0	*	0.023
	M.urealyticum(+)	18.9	57.1	40.8	66.7	16.68	0.01

The percentage (%) of patients in each subgroup of HPV (+) and HPV (–) categories were shown.

Asterisk represent that statistical method we used is Fisher's exact method.

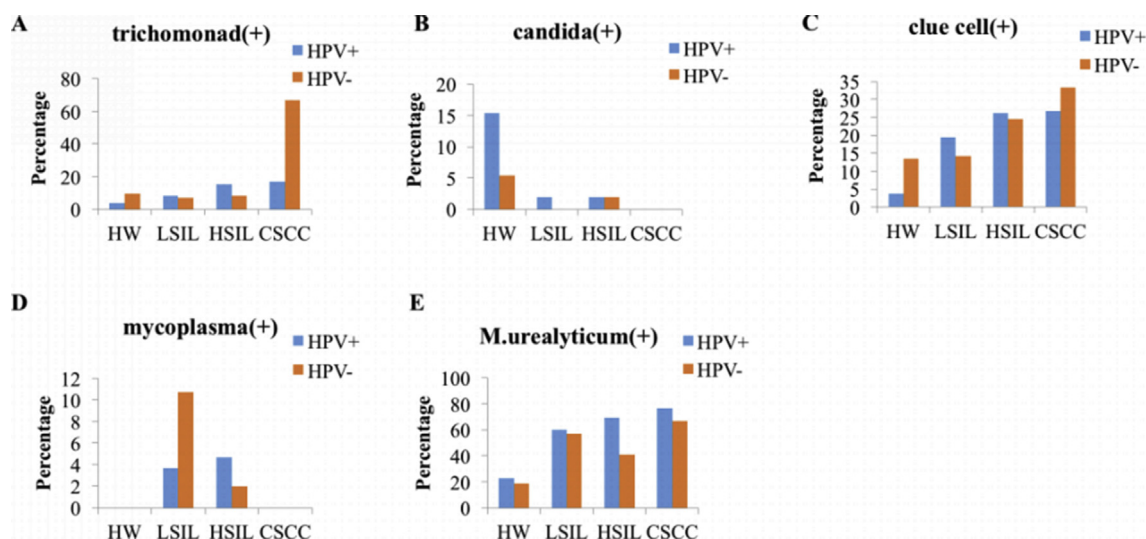


Fig. 3. Comparison of vaginal immune indicators between healthy women and cervical lesions at all levels (%) in the study group and control group.

Table 3
Comparison of vaginal immune indicators in all patients.

	Indicator	HW	LSIL	HSIL	CSCC	z	p
HPV(+)	IL-2	58.93 (27.20)	72.83 (17.32)	47.91 (17.52)	47.17 (41.42)	84.98	<0.001
	IL-10	34.44 (40.19)	13.47 (8.68)	19.20 (11.24)	49.14 (18.48)	91.67	<0.001
	IL-2/IL-10	1.27 (6.10)	4.92 (3.65)	2.49 (1.98)	0.97 (1.82)	57.70	<0.001
	IgA	1.29 (0.88)	0.66 (0.71)	0.85 (1.41)	0.96 (1.6)	20.77	<0.001
	IgG	2.58 (1.24)	1.55 (3.75)	3.83 (6.95)	3.25 (2.81)	16.69	0.001
HPV(-)	IL-2	66.56 (26.53)	68.62 (15.72)	47.95 (17.69)	55.84 (42.4)	39.84	<0.001
	IL-10	29.29 (35.34)	11.97 (9.7)	19.54 (12.58)	25.76 (24.21)	19.07	<0.001
	IL-2/IL-10	2.60 (4.41)	5.43 (6.08)	2.44 (1.81)	4.66 (2.66)	14.76	0.002
	IgA	1.49 (0.91)	0.62 (1.98)	0.94 (1.56)	0.77 (0.5)	11.48	0.009
	IgG	2.56 (1.10)	3.52 (4.85)	2.86 (4.55)	3.60 (8.18)	1.71	0.636

The mean and median values of each subgroup in both HPV (+) and HPV (-) categories were shown (mean, median [IQR]).

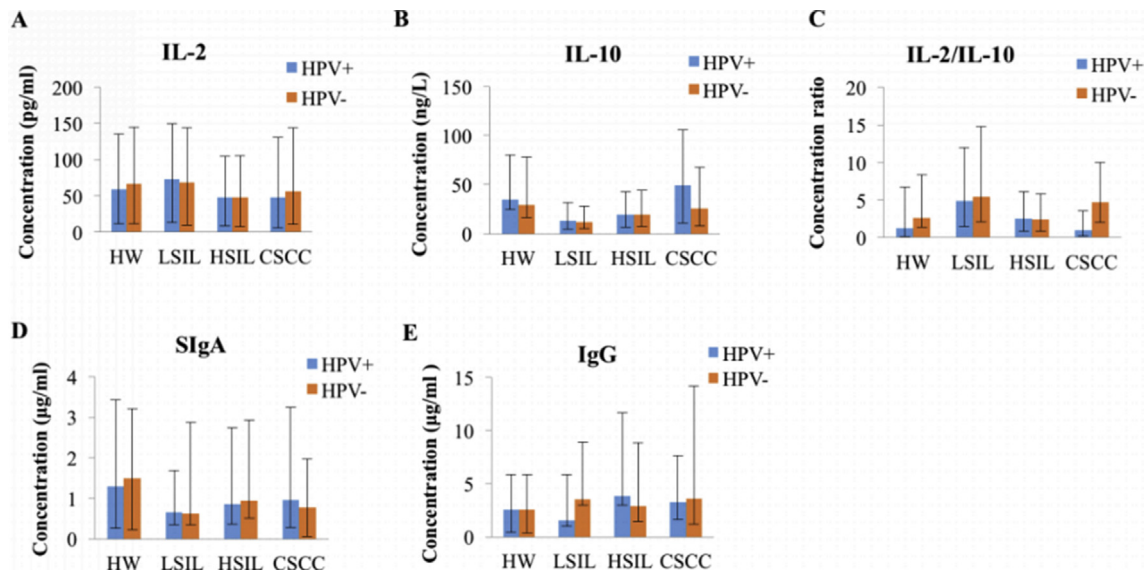


Fig. 4. Comparison of vaginal immune indicators in all patients.

with cervical lesions was not statistically significant, indicating that this group of patients had no previous and current HPV infection, further indicating that vaginal microenvironment changes in patients with HPV-negative cervical lesions were the main cause of cervical lesions.

There are some limitations in this study. First, the number of HPV-positive patients with cervical lesions was more than that of HPV-positive women without lesions. Second, this is a cross-sectional study, which only suggested the correlation between cervical lesions and vaginal microenvironment. Further longitudinal studies are necessary to determine whether vaginal microenvironment and immunity are the cause or consequence of HPV infection.

Conclusion

In summary, our data showed that micro-ecology and immunity is associated with cervical lesions in women. Maintaining vaginal micro-ecological balance, timely treatment of vaginal infections, and improving local vaginal immunity may prevent the occurrence of cervical lesions. These findings contribute to a better understanding of cervical microenvironment, micro-ecology, immunity, and cervical lesions, which may become a new entry point for cancer prevention and treatment.

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Declaration of competing interest

The authors declare that they have no competing interests.

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