Association of abnormal vaginal microflora and HPV infection in cervical precancerous lesions: a retrospective study

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Abstract

Introduction: The aim of this study was to analyze the correlation between abnormal vaginal microflora and different types of human papillomavirus (HPV) infection and cervical precancerous lesions during the perimenopausal period.

Methodology: This retrospective study included women patients who underwent liquid-based cytologic test (LBC), HPV test, leucorrhea routine test, or routine urine test at the China-Japan Friendship Hospital between October 2019 and January 2020. A cut-off of 45 years was used as the cut-off age for menopause. The positivity and subtypes of HPV were determined using a chip-based assay. Vaginal microflora was determined using an HB-2012a flow-through hybridization instrument.

Results: A total of 132 patients were included in this study. 97 patients were younger than 45 years of age, with a median age of 35 (8.0), and 35 patients ≥ 45 years of age, with a median age of 55 (11.0). There were no significant differences in cytology, type of cervical lesion, HPV type, and common pathogens of the reproductive tract (all p > 0.05). The multivariable analysis showed that only HPV-16 infection lesions (OR: 2.825, 95% CI: 1.121-7.120, p = 0.028), Chlamydia trachomatis infection (OR: 0.142, 95% CI: 0.024-0.855, p =0.033), and Mycoplasma infection (OR: 7.750, 95% CI: 1.603-37.474, p = 0.011) were independent risk factors for cervical precancerous lesions. The menopausal status (with age < 45 or > 45 years as its surrogate) was not associated with cervical precancerous lesions.

Conclusions: Menopause was not associated with cervical precancerous lesions. The results suggest that the prevention and treatment of HPV-16, Chlamydia trachomatis infection, and Mycoplasma infection could be significant to prevent the occurrence of cervical precancerous lesions.

Key words: Vaginal microflora; human papillomavirus; cervical cancer; risk factors.

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Introduction

Cervical cancer (CC) is a malignancy originating in the cervix’s transformation zone, most commonly in squamous cells [1]. It is the second most common cancer in women worldwide and the third most common cause of female cancer mortality [2,3]. CC results from a progression of epithelial changes from cervical intraepithelial neoplasia (CIN) 1 to CIN 2 and CIN 3, finally resulting in an invasive cervical cancer [4]. More than 90% of CIN 1, 50% of CIN 2, and 30% of CIN 3 can be self-contained by the immune system [5,6]. For CIN 1, management is usually more frequent follow-up testing. For CIN 2, CIN 3, or unspecified histologic HSIL, management typically consists of treatment except in pregnant women and patients < 25 years old without CIN 3 [7]. The treatment of CIN involves the destruction of the entire transformation zone of the cervix [7]. The treatment options include ablative and excisional techniques [7]. The choice of treatment depends on the lesion’s size and location and the type of the transformation zone [7].

Human papillomaviruses (HPV) are small, nonenveloped, double-stranded DNA viruses. Sexual infection occurs in both men and women. An estimated 79 million women aged 14–59 years are infected with HPV, with the highest prevalence in those aged 20–24 years [11]. Risk factors for HPV infection include being sexually active, increased number of different sexual partners, young age at sexual initiation, being uncircumcised (for both the male and his female partner), decreased condom use, and a history of other sexually transmitted infections [8-10]. The lifetime HPV infection rate in Chinese women is 84.6% [11]. There is a causal link between persistent infection with oncogenic types of HPV, most commonly HPV-16 and HPV-18, which are sexually transmissible pathogens. Persistent HPV infection results in squamous intraepithelial lesions graded as CIN 1, CIN 2, and CIN 3 according to how much epithelium is impacted [4].

The normal vaginal microflora plays an essential role in preventing female genital tract infections, and changes in this microflora can lead to vaginitis,
vaginosis, and cervical lesions [12]. The normal vaginal microflora includes bacteria such as *Lactobacillus*, *Bifidobacteria*, and *Bacteroides* that are synergistically and mutually constrained and coordinated with the host and the environment to maintain the vaginal microecological system [13,14]. If the balance of the vaginal microflora is lost, foreign microorganisms will have better chances of invading and causing inflammation of the vagina [15], and inflammatory stimuli can increase the risk of CC [16]. HPV-positive women have a more important vaginal microbial diversity than HPV-negative women, and the microflora plays an important role in CC development [17,18]. The vaginal microflora plays an essential role in preventing HPV infection and accelerating HPV virus clearance; therefore, vaginal microflora imbalance can favor HPV infection [19,20]. However, the microflora changes with the women’s hormonal status during the menstrual period and after menopause [21]. Whether abnormal microflora after menopause modulates the risk of HPV infection is unknown.

Therefore, this study aimed to analyze the correlation between abnormal vaginal flora and different types of HPV infection and cervical precancerous lesions during the perimenopausal period. The results could provide a theoretical basis for the involvement of abnormal vaginal flora and HPV infection in cervical precancerous lesions in older women.

Methodology

Patients

This retrospective study included women patients admitted to the Gynecology Clinic of China-Japan Friendship Hospital between October 2019 and January 2020. The inclusion criteria were 1) patients with or without self-reported symptoms or discomfort who underwent liquid-based cytologic test (LBC), HPV test, leucorrhea routine test, or routine urine test within 6 months, and 2) were positive for any of the above tests at the time of visit. The exclusion criterion: patients with a confirmed sexually transmitted disease (such as AIDS, syphilis, and gonorrhea). This study was approved by the Ethics Committee of China-Japan Friendship Hospital (approval number: 2021-GZR-116). The requirement for the patient’s informed consent was waived by the committee.

Grouping

The transitional period of menopause usually begins in the middle and late 40s and lasts for about 4 years, with menopause occurring at a median age of 51 [22,23]. Because the menopausal status was not consistently included in the patient charts, a cut-off of 45 years was used as the cut-off age for menopause.

Data collection

The age and clinical data of the patients, including cytology, type of cervical lesion, HPV type, and common pathogens of the reproductive tract, were collected from the electronic medical record system of the hospital. The HPV typing and vaginal microflora data were collected from the reports of Beijing Kaipu Medical Laboratory.

Pathology screening

The cervix was exposed using a vaginal endoscope. The outer opening of the cervix was cleaned with a cotton ball with 0.9% sodium chloride saline. The cervix was observed with naked eyes. Then, 3% acetic acid was applied to the cervix’s surface to observe whether there were abnormal areas such as leukoplakia, acetic white epithelium, columnar epithelial edema, mosaic, punctate blood vessels, various abnormal blood vessels, and true local cervical erosions. Biopsy and endocervical curettage (ECC) were performed at abnormal sites of acetic acid white test and iodine test or randomly at 3, 6, 9, and 12 o’clock and were sent to the pathology department for routine examination. The histopathological results were reported as a low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and squamous cell carcinoma (SCC).

| Characteristics          | Total (n = 132) | < 45 (n = 97) | ≥ 45 (n = 35) | P        |
|--------------------------|----------------|--------------|--------------|----------|
| Age (years), median (IQR)| 37 (14.5)      | 35 (8.0)     | 55 (11.0)    | < 0.001* |
| Cytology (n, %)          |                |              |              | 0.637    |
| Normal + inflammation    | 43 (32.6%)     | 34 (35.1%)   | 9 (25.7%)    |          |
| ASCUS                    | 20 (15.2%)     | 14 (14.4%)   | 6 (17.1%)    |          |
| LSIL                     | 51 (38.6%)     | 38 (39.2%)   | 13 (37.1%)   |          |
| ASC-H                    | 9 (6.8%)       | 5 (5.2%)     | 4 (11.4%)    |          |
| HSIL                     | 9 (6.8%)       | 6 (6.2%)     | 2 (8.6%)     |          |
Table 1 (continued). Characteristics of the patients.

| Characteristics                                      | Total (n = 132) | < 45 (n = 97) | ≥ 45 (n = 35) | P    |
|------------------------------------------------------|-----------------|---------------|---------------|------|
| Cancer typing (n, %)                                 |                 |               |               |      |
| Histological inflammation + koilocytic cell          | 25 (18.9%)      | 16 (16.5%)    | 9 (25.7%)     |      |
| LSIL                                                 | 67 (50.8%)      | 49 (50.5%)    | 18 (51.4%)    |      |
| HSIL                                                 | 39 (29.5%)      | 32 (33%)      | 7 (17.9%)     |      |
| SCC                                                  | 1 (2.9%)        | 0             | 1 (2.9%)      |      |
| HPV type (n, %)                                      |                 |               |               |      |
| High-risk type                                       | 104 (78.8%)     | 77 (79.4%)    | 27 (77.1%)    |      |
| 16                                                   | 34 (25.8%)      | 28 (28.9%)    | 6 (17.1%)     |      |
| 18                                                   | 15 (11.4%)      | 14 (14.4%)    | 1 (2.9%)      |      |
| Both 16 and 18                                       | 1 (2.1%)        | 1 (2.9%)      | 0             |      |
| No 16/18                                             | 29 (20.4%)      | 18 (18.6%)    | 11 (31.4%)    |      |
| 31                                                   | 4 (3.0%)        | 3 (3.1%)      | 1 (2.9%)      |      |
| 33                                                   | 4 (3.0%)        | 2 (2.1%)      | 2 (5.7%)      |      |
| 35                                                   | 3 (2.3%)        | 3 (3.1%)      | 0             |      |
| 39                                                   | 6 (4.5%)        | 6 (6.2%)      | 0             |      |
| 45                                                   | 2 (1.5%)        | 2 (2.1%)      | 0             |      |
| 51                                                   | 10 (7.6%)       | 8 (8.2%)      | 2 (5.7%)      |      |
| 52                                                   | 14 (10.6%)      | 10 (10.3%)    | 4 (11.4%)     |      |
| 56                                                   | 7 (5.3%)        | 3 (3.1%)      | 4 (11.4%)     |      |
| 58                                                   | 13 (9.8%)       | 10 (10.3%)    | 3 (8.6%)      |      |
| 59                                                   | 3 (2.3%)        | 2 (2.1%)      | 1 (2.9%)      |      |
| 68                                                   | 5 (3.8%)        | 5 (5.2%)      | 0             |      |
| Medium-risk type                                     |                 |               |               |      |
| Age (years)                                          | 14 (10.6%)      | 8 (8.2%)      | 6 (17.1%)     |      |
| ≥ 45 (n = 35)                                        | 0               | 0             | 0             |      |
| 66                                                   | 4 (3.0%)        | 1 (1.0%)      | 3 (8.6%)      |      |
| 73                                                   | 7 (5.3%)        | 4 (4.1%)      | 3 (8.6%)      |      |
| 82                                                   | 2 (1.5%)        | 2 (2.1%)      | 0             |      |
| Low-risk type                                        |                 |               |               |      |
| Age (years)                                          | 39 (29.5%)      | 32 (33.0%)    | 7 (20.0%)     |      |
| ≥ 45 (n = 35)                                        | 0               | 0             | 0             |      |
| 66                                                   | 4 (3.0%)        | 1 (1.0%)      | 3 (8.6%)      |      |
| 73                                                   | 7 (5.3%)        | 4 (4.1%)      | 3 (8.6%)      |      |
| 82                                                   | 2 (1.5%)        | 2 (2.1%)      | 0             |      |
| Infection                                            |                 |               |               |      |
| Neisseria gonorrhoeae                                | 60 (45.5%)      | 45 (46.4%)    | 15 (42.9%)    | 0.719|
| Chlamydia trachomatis                                | 14 (10.6%)      | 13 (13.4%)    | 1 (2.9%)      | 0.112|
| Ureaplasma parvum                                    | 48 (36.4%)      | 35 (36.1%)    | 13 (37.1%)    | 0.911|
| Ureaplasma parvum 1                                  | 5 (3.8%)        | 3 (3.1%)      | 2 (5.7%)      | 0.608|
| Ureaplasma parvum 3                                  | 26 (19.7%)      | 18 (18.6%)    | 8 (22.9%)     | 0.583|
| Ureaplasma parvum 6                                  | 20 (15.2%)      | 15 (15.5%)    | 5 (14.3%)     | 0.368|
| Ureaplasma parvum 14                                 | 0               | 0             | 0             |      |
| Mycoplasma                                           | 13 (9.8%)       | 9 (9.3%)      | 4 (11.4%)     | 0.744|
| Mycoplasma hominis                                   | 11 (8.3%)       | 7 (7.2%)      | 4 (11.4%)     | 0.481|
| Mycoplasma genitalium                                | 1 (0.8%)        | 1 (1.0%)      | 0             |      |
| Ureaplasma urealyticum                               | 3 (2.3%)        | 3 (3.1%)      | 0             |      |
| Herpes simplex virus type 2                         | 0               | 0             | 0             |      |
| Common pathogens of reproductive tract infections (n)|                 |               |               |      |
| No infection                                         | 72 (54.5%)      | 52 (53.6%)    | 20 (57.1%)    | 0.719|

Quantitative data with a non-normal distribution are presented as median (IQR). Categorical data are presented as n (%). *Mann-Whitney U-test. **Fisher’s exact test, otherwise, chi-square test. IQR: interquartile range; ASCUS: atypical squamous cells of undetermined significance; LSIL: low-grade squamous intraepithelial lesion; ASC-H: atypical squamous cells-cannot exclude high-grade; HSIL: high-grade squamous intraepithelial lesion; SCC: squamous cell carcinoma; HPV: human papillomavirus.
**HPV typing test**

For 3 days before the sampling, the patient had to avoid irrigating the vagina with vaginal medication, avoid using acetic acid and iodine solution, and avoid sexual intercourse for 24 hours.

The cervical cytology samples were obtained using a special cervical secretion sampler and stored at 2-8 °C, and sent for testing as soon as possible. The DNA was extracted using a fully automated nucleic acid extractor (HBNP-4801A, Kaipu Biology, Shanghai, China) according to the instructions of the DNA Extraction Kit (Chaozhou Kaipu Biochemistry Co., Ltd., Chaozhou, China). The extracted DNA was amplified on a PCR system (ABI7500, Applied Biosystems, Foster City, CA, USA) according to the kit’s instructions. The positivity and subtypes of HPV were determined according to the chip results.

**Vaginal flora examination and analysis**

According to the experimental procedures of the STD Ten Joint Inspection Kit (Chaozhou Kaipu Biochemistry Co., Ltd., Chaozhou, China), the DNA samples of cervical cells was extracted as templates for PCR amplification. The reaction system included the PCR mixture, DNA polymerase, and DNA template. The amplification procedures were 1) 95 °C for 9 minutes, 2) 40 cycles of 95 °C for 30 seconds, 58 °C for 30 seconds, and 72 °C for 40 seconds, 3) 72 °C for 5 minutes, and 4) held at 16 °C. The amplified products were conducted flow-through hybridization in an HB-2012a flow-through hybridization instrument, and whether a certain pathogenic microorganism was positive or not was determined according to the results of hybridization.

**Statistical analysis**

All data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY, USA). The Shapiro-Wilk method was used to test the normality of the continuous data. Continuous data with a normal distribution are presented as means ± standard deviations and were analyzed using Student’s t-test. The continuous data with a non-normal distribution are presented as median (IQR) and were tested using the Mann-Whitney U-test. Categorical data are presented as n (%) and were analyzed using the chi-square test or Fisher’s exact test. Logistic regression analysis was used to analyze the risk factors of cervical lesions. Factors with a significant difference in univariable logistic regression analyses (p < 0.05) were included in multivariable logistic regression analysis and p < 0.05 was considered statistically significant.

**Table 2. Univariable and multivariable logistic regression analysis for cervical precancerous lesions.**

| Variables                      | Univariable analysis | Multivariable analysis |
|--------------------------------|----------------------|------------------------|
|                                | OR (95%CI)           | p                      | OR (95%CI)           | p                      |
| **Age group**                  |                      |                        |                      |                        |
| < 45 years                     | Reference            |                        | Reference            | 0.132                  |
| ≥ 45 years                     | 0.602 (0.246-1.473)  | 0.266                  | 0.465 (0.171-1.260)  |                        |
| **HPV type**                   |                      |                        |                      |                        |
| High-risk type                 | 1.475 (0.653-3.329)  | 0.350                  |                        |                        |
| 16 type                        | 3.261 (1.438-7.394)  | 0.005                  |                        |                        |
| 18 type                        | 0.320 (0.069-1.489)  | 0.146                  |                        |                        |
| Medium-risk type               | 0.597 (0.157-2.268)  | 0.449                  | 0.452 (0.092-2.221)  | 0.328                  |
| Low-risk type                  | 0.724 (0.313-1.677)  | 0.451                  | 0.605 (0.222-1.645)  | 0.325                  |
| **High-risk group**            |                      |                        |                      |                        |
| Non-type 16 or 18              | Reference            |                        | Reference            |                        |
| Only type 16                   | 3.103 (1.320-7.296)  | 0.009                  | 2.825 (1.121-7.120)  | 0.028                  |
| Only type 18                   | 0.264 (0.032-2.168)  | 0.215                  | 0.148 (0.015-1.422)  | 0.098                  |
| Both type 16 and 18            | 1.455 (0.126-16.836) | 0.764                  | 2.632 (0.168-41.241) | 0.491                  |
| **Infection flora of genital tract** |                  |                        |                      |                        |
| No infections                  | Reference            |                        | Reference            |                        |
| Infections                     | 1.300 (0.618-2.737)  | 0.490                  |                        |                        |
| *Chlamydia trachomatis*        | 0.351 (0.075-1.646)  | 0.184                  | 0.142 (0.024-0.855)  | 0.033                  |
| *Ureaplasma parvum*            | 1.250 (0.582-2.684)  | 0.567                  | 1.068 (0.455-2.505)  | 0.881                  |
| *Mycoplasma*                   | 3.040 (0.951-9.717)  | 0.061                  | 7.750 (1.603-37.474) | 0.011                  |

HPV: human papillomavirus.
Results

General characteristics of the patients

A total of 132 patients were included; 97 patients were younger than 45 years of age, with a median age of 35 (8.0), and 35 patients ≥ 45 years of age, with a median age of 55 (11.0). There were no significant differences in cytology, type of cervical lesion, HPV type, and common pathogens of the reproductive tract (all p > 0.05). The proportion of patients with HPV subtypes other than HPV16 and HPV18 was higher in the older patients than in the younger ones (84.6% vs. 51.4%, p = 0.037) (Table 1).

Factors associated with cervical precancerous lesions

The factors associated with cervical precancerous lesions were analyzed (Table 2). The univariable analysis showed that HPV-16 and only HPV-16 infections were associated with cervical precancerous lesions. The multivariable analysis showed that only HPV-16 infection lesions (OR: 2.825, 95% CI: 1.121-7.120, p = 0.028, non-type 16 or 18 HPV as reference), Chlamydia trachomatis infection (OR: 0.142, 95% CI: 0.024-0.855, p = 0.033, non-infection as reference), and Mycoplasma infection (OR: 7.750, 95% CI: 1.603-37.474, p = 0.011, non-infection as reference) were independent risk factors for cervical precancerous lesions.

Discussion

There is a causal association between HPV infection and CIN [4]. Vaginal microflora imbalance can favor HPV infection [19,20]. Therefore, this study aimed to analyze the correlation between abnormal vaginal microflora and different types of HPV infection, and cervical precancerous lesions during the perimenopausal period. The results suggest that menopause was not associated with cervical precancerous lesions. The prevention of HPV-16 and regulation of Vaginal microflora, especially Chlamydia trachomatis and Mycoplasma, could be significant to prevent the occurrence of cervical precancerous lesions.

CIN and CC are caused by a persistent infection with oncogenic types of HPV [1,24,25]. HPV-16 and HPV-18 are the genotypes with the highest oncogenic potential, but about 15 other genotypes are also considered oncogenic [1,24-26]. HPV-16 is the major subtype associated with CC [1,24,25,27]. In the present study, HPV-16 was independently associated with cervical lesions, as supported by the literature.

Bacterial vaginosis is due to the imbalance of the vaginal microflora and is common in women of childbearing age. A previous systematic review reported a correlation between bacterial vaginosis and HPV infection, suggesting that the presence of bacterial vaginosis increases the risk of HPV infection [28]. Indeed, the vaginal microflora plays an essential role in preventing HPV infection and accelerating HPV virus clearance [19,20]. The mechanism might involve an increase in mucin-degrading enzymes in the vagina of patients with bacterial vaginosis. These enzymes might promote HPV virulence by disrupting the protective mucosal barrier [28]. Another explanation might be that the anaerobic bacteria often involved in bacterial vaginosis produce ammonia and carcinogenic ammonia nitrite, causing cervical epithelial cell transformation and exfoliation. A recent meta-analysis showed that bacterial vaginosis is associated with HPV infection and CIN [29]. A study showed that precancerous lesions were more frequently detected in smears of bacterial vaginosis than non-vaginosis smears [30]. Zhang et al. [31] reported that the cervical microbial diversity was reduced in patients with CIN II/III.

There is a relationship among Mycoplasma, Chlamydia, and HPV infections, and cervical lesions [32]. Nevertheless, whether Mycoplasma and Chlamydia infections participate in the development of CIN and CC actively or indirectly through a higher susceptibility to HPV infection is still controversial [29]. Still, a meta-analysis revealed that the rates of Mycoplasma and Chlamydia infections were higher in HPV-positive patients [29]. Ureaplasma urealyticum has been associated with initiating abnormalities and HPV persistence of viral cells and is considered a cofactor for HPV to promote CIN leading to CC [33,34], but this association was not observed in the present study. Valadan et al. [35] reported an association between Chlamydia trachomatis infection and CIN. The possible mechanism for this association is that Chlamydia adsorbs to the genital mucosa after infection, causing inflammation of the genital mucosal epithelial cells, reducing cervical and vaginal immune barriers, and facilitating HPV infection [36].

The changes in the vaginal environment (pH, hormones, and nutrients) during menopause are associated with changes in the composition of the vaginal microflora [37,38]. These changes often result in a decreased abundance of Lactobacillus species and lactic acid production, increasing vaginal pH and infections’ susceptibility. The involvement of hormones has been highlighted by a study that showed that women who receive hormonal replacement therapy have vaginal microflora similar to that of premenopausal women, with high proportions of Lactobacillus, but the mechanisms are unknown [39].
In the present study, there were no differences between younger and older women regarding the proportion of HPV and other vaginal infections. Furthermore, age was not independently associated with cervical lesions. This study has limitations. The patients were from a single-center, and the sample size was small. Only the presence of pathogenic microorganisms was examined, and the normal microflora species were not measured. Because of inconsistent reporting in the patient charts, age had to be used as a surrogate of the menopausal status, which introduced bias. Of note, this study included women who had indications for LBC, HPV test, leucorrhea routine test, or routine urine test. Therefore, they do not represent the general population of women.

In conclusion, HPV-16, Chlamydia trachomatis infection, and Mycoplasma infection were independently associated with cervical lesions. Menopause was not associated with cervical precancerous lesions. The results suggest that treatment of HPV-16 and regulation of vaginal flora (such as Chlamydia trachomatis and Mycoplasma) could be significant to prevent the occurrence of cervical precancerous lesions.

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Authors’ Contribution

Yu Huan and Ma Li, Li Qian carried out the studies, participated in collecting data, and drafted the manuscript. Yu Huan, Bian Meilu and Liang Haiyan performed the statistical analysis and participated in its design. Yu Huan and Liang Haiyan participated in the acquisition, analysis, or interpretation of data and draft the manuscript. All authors read and approved the final manuscript.

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