official title: The Mechanism of Vaginal Flora and Its Metabolites in the Pathogenesis of Cervical Cancer

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Study Protocol

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1. Brief Summary

The disorder of vaginal microflora and its metabolites is considered to be a facilitating factor to human papillomavirus-mediated cervical cancer. However, the mechanism is still unclear. This study intends to carry out a cross-sectional study and a cohort study. The cross-sectional study intends to recruit 300 premenopausal non-pregnant women, dividing them into five groups, with 60 in each group: HPV negative [Ctrl HPV (-)], HPV positive [Ctrl HPV (+)], low-grade squamous Intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL) and newly diagnosed invasive cervical cancer (ICC). Obtain basic information through the questionnaire, and collect vaginal secretion and blood samples. At the same time, patients who are diagnosed with cervical cancer for the first time will be included in the cohort study. Collect the same kind of information. The follow-up period is set to be 3 years, and samples will be collected every six months. If any condition changes within the 3 years, samples should be collected. If new treatments are taken, samples should be taken before and after treatment. And if the lesion turns negative after treatment within the 3 years, complete the follow-up. Using 16S rRNA gene sequencing, metabolomics, and immunological methods to determine the vaginal microbiota and its metabolites and inflammation condition, select biomarkers related to the onset of cervical cancer. construct a cervical cancer risk model and outcome prediction model, and reveal the mechanism of vaginal flora and its metabolites in the pathogenesis and development of cervical cancer. Therefore provides a new direction for the prevention and treatment of cervical cancer.

2. Research Background

Cervical cancer is the fourth most common gynecological malignancy in the world, caused by carcinogenic human papillomavirus[1]. According to the statistics of the World Health Organization in 2018, there are nearly 570, 000 new cases of cervical cancer and about 311, 000 deaths worldwide every year. There are 106, 000 new cases of cervical cancer and about 48, 000 deaths in China every year. In recent

years, a lot of data show that the incidence rate of cervical cancer in young women is increasing gradually. Cervical cancer is becoming younger and younger[2], which poses a great threat to the health of women.

At present, the etiology of cervical cancer is not clear. Studies at home and abroad shew that the occurrence of cervical cancer is related to the age of first sexual intercourse, the number of sexual partners, the age and frequency of first delivery, personal hygiene conditions, smoking, oral contraceptives and other factors[3-5]. It has been recognized that the persistent infection of high-risk human papillomavirus (HR-HPV) is the necessary conditions for cervical intraepithelial neoplasia (cervical intraepithelial neoplasia, CIN) and carcinogenesis. The progression from HPV infection to cervical cancer is slow and complex, which takes about 5-10 years, and only few patients eventually develop cervical precancerous or cancerous. Therefore, there may be other carcinogenic factors or cooperate with HPV in this process[6-7]. The factors increase the susceptibility of cervical epithelial cells to HPV, and increased expression of HPV virus. Therefore ensure the persistence of HPV infection, and finally lead to cancer. As an important factor to maintain the stability of vaginal microenvironment, whether vaginal flora plays a certain role in the process of HR-HPV continuous infection has always been concerned.

The vaginal microenvironment is mainly composed of endocrine regulation, anatomical structure and microbial flora. Every 6 - 8 kinds of microorganisms can be isolated from each woman of childbearing age, mainly including bacteria, fungi, protozoa and viruses. Under normal conditions, these microorganisms are mainly colonized in the vaginal mucosa. Among different microorganisms, microorganisms, host, and environment adapt, restrict and coordinate with each other, to achieve a dynamic micro-ecological balance. Under the action of some factors, such as long-term use of immunosuppressants or antibiotics, vaginal flushing or surgery, the vaginal micro-ecological balance is destroyed, which will lead to a variety of diseases [8]. In recent years, more and more scholars believe that vaginal microorganisms may play a key role in the occurrence of cervical lesions[9]. Exposure of the cervix to the unbalanced vaginal microenvironment may promote microbial invasion and then

cause cervical lesions [10]. Clinical studies have found that the vaginal flora diversity of HPV positive people is higher than that of negative people, among which the proportion of Lactobacillus is decreased, and a variety of anaerobic bacteria such as Gardnerella are over proliferated[11-14]. Lactobacillus protects vaginal mucosal epithelial cells from pathogens, secretes corresponding substances, promotes the formation of biofilm, and can inhibit the adhesion and invasion of pathogens. With the decline of Lactobacillus, the vaginal micro-ecological balance is destroyed, inflammatory factors increased, and the susceptibility of cervical epithelium to HPV is improved[15]. Lactobacillus can also interfere with the material metabolism of cancer cells, inhibit the formation of carcinogens, effectively eliminate carcinogenic factors to induce tumor cell apoptosis, and inhibit the proliferation of cancer cells by secreting polysaccharides. Gardnerella can act on CD59 regulatory molecules, activate inflammatory signaling pathways, release a large amount of cytolysin, causing apoptosis of vaginal epithelial cells, reduce Lactobacillus colonization, and increase the risk of imbalance of vaginal microenvironment[16].

Although a large number of research results shew that vaginal flora imbalance is closely related to HPV infection, the causation relationship between vaginal flora and its metabolites and cervical lesions is not clear. Prospective cohort studies and laboratory studies are needed. In addition, in view of the complexity of vaginal microenvironment and the diversity of influencing factors of cervical lesions, the relationship and interaction between HPV infection and vaginal flora imbalance in cervical lesions need to be further explored.

As mentioned in the review, this study intends to carry out cross-sectional research and cohort research, obtain the basic information of the research objects through questionnaire, and collect vaginal secretion and blood samples every time the patients review the clincal department as scheduled. Therefore construct a cervical cancer risk model and outcome prediction model, and reveal the mechanism of vaginal flora and its metabolites in the pathogenesis and development of cervical cancer. Therefore provides a new direction for the prevention and treatment of cervical cancer.

3. Research Purpose (with clear objectives)

3.1 To explore the correlation between vaginal flora and its metabolites and cervical cancer, and to build a risk assessment model of cervical cancer pathogenesis;

3.2 Explore the mechanism of the development and conversion of vaginal flora and its metabolites in cervical cancer, and construct a prediction model of cervical cancer outcome;

3.3 Screen the biomarkers related to the incidence of cervical cancer and therefore provide new ideas for the screening and treatment of cervical cancer.

4. Research Design

This study intends to carry out a cross-sectional study and a cohort study.

The cross-sectional study intends to recruit 300 premenopausal non-pregnant women, dividing them into five groups, with 60 in each group: HPV negative [Ctrl HPV (-)], HPV positive [Ctrl HPV (+)], low-grade squamous Intraepithelial lesion (LSIL group), high-grade squamous intraepithelial lesion (HSIL group) and newly diagnosed invasive cervical cancer (ICC group).

Obtain basic information through the questionnaire, and collect vaginal secretion and blood samples every time the patients review the clincal department as scheduled. At the same time, patients who are diagnosed with cervical cancer for the first time will be included in the cohort study. Collect the same kind of information. The follow-up period is set to be 3 years, and samples will be collected every six months. If any condition changes within the 3 years, samples should be collected. If new treatments are taken, samples should be taken before and after treatment. And if the lesion turns negative after treatment within the 3 years, complete the follow-up.

5. Research Scheme and Technical Route

5.1 Selection of the Study Population

5.1.1 Cross-Sectional Study

5.1.1.1 Calculation of Sample Size

In the study of medical microbiome, microbiome is regarded as exposure and disease is regarded as outcome in most cases. Therefore, traditional sample size calculation methods are rarely used in microbiome research because previous exposure (microbiome) information is difficult to obtain. The most commonly used sample size and efficiency calculation methods in microbiome research include t-test, analysis of variance χ 2 test and Dirichlet polynomial model [17]. Taking the t-test as an example, the sample size and efficiency calculation are determined in three steps. Firstly, a small amount of amplicon data are obtained through preliminary experiments. Secondly, the R-package "vegan" was used to calculate the α Diversity parameter Shannon index of each sample[18]. Finally, the power.t.test() function is used to calculate the sample size and efficiency. In the process of studying Crohn's disease, Gonzalez A[19] calculated the sample size through the diversity parameter Faith's PD of the microbial community: the PD of 100 B1 Crohn's disease was normally distributed. The mean and standard error of PD were 13.5 and 3.45, respectively (see Figure 1). In general, the statistical test level used in most studies is 5% and the degree of assurance is 80%. Therefore, 110 patients were enrolled (55 in each group).

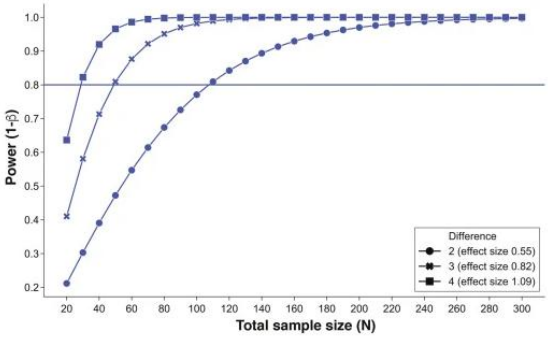

Figure 1: Standard sample size calculation. It was determined significant differences in gut flora diversity (Faith PD) between groups B1 and B2 / B3, and the degree of assurance is 80% at three effect values (PD difference 2,3,4, equivalent to Cohen D 0.55,0.82, and 1.09).

The pre-experiment could not be carried out, so the α diversity distribution of the flora of patients with cervical cancer could not be obtained. In addition, no

published articles were found to provide the data required for calculation. Therefore, it is planned to design this study with reference to the sample size of the above study, details as follows:

Recruit 300 premenopausal non-pregnant women, age 18-60, dividing them into five groups, with 60 in each group (> 55, ensuring an effect value of 0.8, the difference equivalent to 3 PD units): HPV negative [Ctrl HPV (-)], HPV positive [Ctrl HPV (+)], low-grade squamous Intraepithelial lesion (LSIL group), high-grade squamous intraepithelial lesion (HSIL group) and newly diagnosed invasive cervical cancer (ICC group).

5.1.1.2 Inclusion Criteria: (1) Age 18 to 60 years women; (2) have a history of sexual life for 3 years or more; (3) women not in the menstrual period, pregnancy, or puerperium.

5.1.1.3 Exclusion Criteria: (1) Women who received antibiotics and antifungal treatment within one month before the sample collection (records); (2) Women suffering from the following diseases: other cancer, vaginal infections, bacterial vaginosis, vulvar infections, urinary tract infections or sexually transmitted infections including chlamydia, gonorrhea, trichomoniasis and genital herpes, type I or type II diabetes, AIDS Virus positive; (3) Women with abnormal vaginal secretions or dirt in the vagina, and women who used flushing substances within three weeks before the sample collection; (4) Have sexual intercourse or use vaginal lubricant within 48 hours before sample collection.

5.1.2 Cohort Study

The study subjects are patients first diagnosed with cervical cancer. A total of 100 patients (sample size estimated from the above cross-sectional study). The follow-up period is set to be 3 years, and samples will be collected every six months. If any condition changes within the 3 years, samples should be collected. If new

treatments are taken, samples should be taken before and after treatment. And if the lesion turns negative after treatment within the 3 years, complete the follow-up.

5.1.3.1 Inclusion Criteria: (1) Women first diagnosed with cervical cancer; (2) have a history of sexual life for 3 years or more; (3) women not in the menstrual period, pregnancy, or puerperium.

5.1.3.2 Exclusion Criteria: (1) Women who received antibiotics and antifungal treatment within one month before the sample collection (records); (2) Women suffering from the following diseases: other cancer, vaginal infections, bacterial vaginosis, vulvar infections, urinary tract infections or sexually transmitted infections including chlamydia, gonorrhea, trichomoniasis and genital herpes, type I or type II diabetes, AIDS Virus positive; (3) Women with abnormal vaginal secretions or dirt in the vagina, and women who used flushing substances within three weeks before the sample collection; (4) Have sexual intercourse or use vaginal lubricant within 48 hours before sample collection.

5.1.4 Withdrawal / Early Stop of the study

(1) If the patient quits the study before completing the evaluation specified in the study protocol, it is seemed to withdraw and stop the study early. (2) Patients can withdraw / stop the study due to pregnancy. (3) If the patients requests to withdraw from the project for their own reasons, they will withdraw / stop the study unconditionally.

5.2 Research Technique

5.2.1 Informed Consent

Before starting the study, the researcher clearly and verbally explains the details of the study and its potential risks and benefits to the patient or her authorized person. The patient or her authorized person and the researcher need to sign and date the informed consent form. Only after

signing the informed consent can the patients enter the screening and then participate in the study.

5.2.2 Questionnaire Survey

A self-designed questionnaire survey is adopted. After the questionnaire design is completed, a pre-survey is carried out to estimate the rationality and feasibility of the questionnaire. The patient's informed consent shall be obtained and sufficient time shall be given before the investigation. The questionnaire shall be filled in face to face by the trained investigator and the research object. During the filling process, no situation of inducing or interfering are allowed. The quality of the questionnaire shall be checked by another quality control person. If any problem is found, it shall be immediately checked, supplemented or corrected.

The content of the questionnaire includes:

(1) General information: name, place of origin, age, nationality, occupation, education level, family income, etc.

(2) Marriage and childbirth history: menstruation, marital status, pregnancy and parity, history of sexual life, contraceptive methods, etc.

(3) Lifestyle: smoking history, drinking history, sleep time, moderate to high-intensity physical activity, etc.

(4) Disease history and family history: diabetes, hypertension, venereal history, history of gynecological tumors.

(5) Testing and laboratory examinations: height, weight, gynecological examinations, HPV testing, TCT or cervical pathological diagnosis, etc.

5.2.3 Collection of Specimens

After emptying the urine, the subjects are asked to separate their legs, take the lithotomy position and lie on the gynecological examination bed to fully expose the

genitals and perineum. Open the vagina with a sterile vaginal speculum, use two sterile vaginal cotton swabs to collect secretions at the vaginal dome or the side wall of the middle part of the vagina, rotate for 10-15s, and put them into a dry sterile test tube after the swab fully absorbs the secretion to avoid contacting the vaginal mouth and vulva and prevent specimen pollution. Put the cotton swab into a dry sterile test tube and store it in a refrigerator at - 80° C for the extraction of total bacterial DNA. The vaginal pH value was measured with precision pH test paper.

Vaginal swabs were collected in quadruplicate, two for the metabolomics of vaginal secretions, one for vaginal microorganisms and the other for reproductive tract inflammatory factors.

Statistical Analysis Plan

5.2.4 Evaluation indicators and methods

1.Genital tract inflammation score: ELISA kit is used to detect the expression levels of 7 cytokines (IL-1 α , IL-1 β , IL-8, MIP-1 β , CCL20, RANTES and TNF- α , etc.) in the vaginal secretions, and determine a cumulative score according to the level of each cytokine. If 3 or more than 3 of the 7 cytokines ranks in the upper quartile of all participants, it`s considered high-level reproductive tract inflammation. A score of 5 to 7 is considered high-grade genital tract inflammation, 1 to 5 is low-grade genital tract inflammation, and a score of 0 is no inflammation.

2.Blood inflammatory factors: Use ELISA kit to detect 7 kinds of inflammatory factors (IL-1 α , IL-1 β , IL-8, MIP-1 β , CCL20, RANTES and TNF- α .) in the blood sample.

3.16sDNA sequencing and biological information analysis: Extract DNA with a total bacterial DNA extraction kit, using bacterial DNA as a template, bacterial 16S rDNA V3~V4 variable regions as targets, and barcode-equipped universal primers for PCR amplification. The PCR products will be sequenced using Illumina NovaSeq

sequencing technology. After quality control, trimming, denoising, splicing, and chimera removal of the obtained raw data and reads, the high-throughput original base sequence is obtained, and the data will be analyzed using Qiime2 software. Data analysis includes operational unit (OTU) clustering, genetic enrichment analysis, principal component analysis (PCoA), community structure diversity (α and β diversity), and analysis of bacterial genus differences between groups (using linear discriminant effect analysis of LefSe), correlation analysis, intestinal flora prediction model (random forest model).

4.The metabolite composition and content in vaginal secretions: The non-targeted metabolomics method is used to detect the metabolite composition and content in vaginal secretions. Quantitative analysis of metabolomics in each group, principal component analysis (PCOA, group analysis), differential metabolite spectrum analysis (increased/decreased metabolites in each group), correlation analysis (correlation analysis of inflammatory factors and metabolites). Correlation analysis between microbiology and metabolomics (including correlation analysis between different species and different metabolites, Scatter plot analysis, etc).

5.2.5 Statistical Method

R software will be used for statistical analysis. The data results are expressed as mean ± standard deviation ($x \pm s$). The mean between the two groups is compared by two independent sample t-test. Kruskal Wallis and Wilcoxon rank sum test are used for statistical analysis of vaginal flora data; Spearman rank correlation is used for correlation analysis. $P < 0.05$ is considered to have statistically significant difference.

6.Reference Documentation

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