Full Length Research Paper

The study of association between bacterial vaginosis and cervical intraepithelial neoplasia at Xiangya Hospital (Changsha-Hunan, China)

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This study was to explore the relationship between bacterial vaginosis and cervical dysplasia. Vaginal discharge gram stain, liquid-based thinlayer cystoFast test (TCT), human papilloma virus (HPV) types 16, 18 test, colposcopy and directed biopsy were performed among 46 patients with cervical dysplasia served as investigate group and 100 patients with cervicitis served as control group. There was statistically significant increase in the incidence of bacterial vaginosis among cervical dysplasia group (52.18%) compared with control group (10.0%) (P<0.05). Their human papilloma virus (HPV) 16, 18 positive rates were 60.9 and 3% respectively (P<0.05). Human papilloma virus (HPV) types 16, 18 and bacterial vaginosis co-infection incidence were 41.3 and 0% respectively (P< 0.05). Both human papilloma virus (HPV 16, 18) and bacterial vaginosis (BV) negative results were 28.3 and 87.0% (P<0.05). Logistic regression test indicated that human papilloma virus (HPV) 16, 18 and bacterial vaginosis were independent risk factors for cervical dysplasia. Bacterial vaginosis facilitates the infection of HPV type 16, 18 and thus enhances development and progression of cervical dysplasia.

Key words: Bacterial vaginosis (BV), Human papilloma virus types 16, 18 (HPV 16, 18), cervical dysplasia (CIN).

INTRODUCTION

The hypothesis of a causal relationship between human papilloma virus (HPV) and cervical intraepithelial neo-plasia (CIN) was first proposed. Since then, a large body of experimental, clinical, and epidemiologic research has accumulated supporting an etiologic role for some types of human papilloma virus (HPV).

Although a strong and consistent association between human papilloma virus (HPV) and cervical neoplasia has been clearly established, the discrepancy between human papilloma virus prevalence and the incidence of cervical neoplasia suggests that infection with human papilloma virus alone is insufficient for the development of cervical intraepithelial neoplasia or cervical cancer and underscores the importance of other cofactors including cigarette smoking or immunosuppression and a history of sexually transmitted

diseases, such as bacterial vaginosis, Chlamydia Trachomatis and Trichomonas vaginalis (Holland and Frei, 2001; Aghajanian et al., 2007).

Bacterial vaginosis (BV) is the most common cause of vaginal discharge in women of childbearing age, accounting for 40 to 50 percent of cases. It is not due to single organism. Instead it represents a complex change in the vaginal flora characterized by a reduction in con-centration of the normally dominant hydrogen-peroxide producing Lactobacilli and an increase in concentration of other organisms, especially anaerobes. These include *Gardnerella vaginalis*, *Mycoplasma homonis*, *Prevotella* spp., *Porphyromonas* spp., *Bacteroides* spp., anaerobic *Peptostreptococcus* spp., *Fusobacterium* spp. and *Atopobium vaginae*. These anaerobes produce large amounts of proteolytic carboxylase enzymes, which break down vaginal peptides into a variety of amines that are volatile, malodorous, and associated with increased vagi-nal transudation and squamous epithelial cell exfoliation,

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Table 1. Nugent criteria of vaginal discharge Gram stain scoring system.

Score	Lactobacilli	*Gardnerella vaginalis* and *Prevotella buccae*	*Mobiluncus* spp
0	4+	0	0
1	3+	1+	1+ or 2+
2	2+	2+	3+ or 4+
3	1+	3+	
4	0	4+	

Total scores = Lactobacilli+*Gardnerella vaginalis* and *Prevotella buccae*+*Mobiluncus* spp; 0 denotes no bacteria was seen, 1+ less than 1 bacteria, 2+ 1 to 4 bacteria, 3+ 5 to 30 bacteria, and 4+ more than 30 bacteria. Score range: 1-10; >6 positive diagnoses of bacterial vaginosis, 4-6 intermediate, and < 4 indicates normal.

resulting in the typical clinical features observed in patients with bacterial vaginosis (Eschenbach et al., 1989; Hill 1993).

The rise in pH also facilitates adherence of *Gardnerella vaginalis* to the exfoliating epithelial cells, thereby creating the "clue cells" that are diagnostic of the disorder.

Some studies show that the presence of *Gardnerella vaginalis* on the cervix, as detected on Pap smear, is associated with high grade squamous intraepithelial neoplasia; however, a causal relationship has not been proven while other studies report no association (Klomp et al., 2008; Discacciati et al., 2006).

In addition, it is theoretically possible that nitrosamines may be an important agent in the development of premalignant disease of the cervix (Barrigton et al., 1997).

Therefore, the aim of this study was to explore the relationship between bacterial vaginosis and cervical intraepithelial neoplasia.

PATIENTS AND METHODS

A cohort of 46 subsequent patients diagnosed with cervical cell lesion form April 2009 to July 2009 at the gynecology out patient department of Xiangya hospital (Central South University, Changsha, Hunan, China) was assigned as the investigate group (Age: 22 to 64 years old, mean age: 39.22±10.55, number of pregnancy: 0 to 7, average number of pregnancy: 2.91± 1.55).

At the same time, among 230 patients diagnosed with chronic cervicitis inflammation, 100 patients were randomly selected and they served as the control group (Age: 19 to 66 years old, mean age: 38.56± 10.32, number of pregnancy: 0 to 8, average number of pregnancy: 2.78± 1.73). Their age and number of pregnancies in the two groups showed no significant difference (P>0.05). Exclusion criteria included antibiotic use within one week or vaginal medica-tion. Informed consent was obtained from each woman, the protocol was reviewed and approved by the ethic committee of Xiangya hospital.

All patients underwent vaginal smear gram stain, thinlayer cystoFast test (TCT) combined with Bethesda system, colposcopy and directed biopsy.

Staining reagents were prepared in the laboratory of Xiangya hospital. Human papilloma virus (HPV) type 16, 18 nucleotide am-plification fluorescent assay test was performed by using reagent kit (QIAGEN Co. Ltd-China).

Method

Vaginal smear gram stain

The vaginal swab specimens were taken from the lateral fornix. They were smeared directly on glass slides and air dried for a standard gram stain.

Gram- stained slides were evaluated according to the Nugent et al. (1999), each slide was examined under an oil immersion objective (x 20 magnifications) using the Nugent criteria of vaginal discharge Gram stain scoring system (Table 1).

In this study, there were twenty four (24) patients with Nugent score >6 (52.18%), fourteen (14) patients with Nugent score 4 to 6 (30.43%) and eight (8) patients with Nugent score <4 (17.39%) in the investigate group, while the control had ten (10) patients with Nugent >6 (10%), thirty one (31) patients with Nugent 4 to 6 (31%) and fifty nine patients (59) with Nugent <4 (59%).

Thinlayer cystoFast test (TCT)

Liquid-based thinlayer cell (thinlayer-cystoFast test: TCT) is a new method of cervical cell sample preparation certified by United States Food and Drug Administration (FDA). It includes the preparation and evaluation of cells collected in liquid fixative. The advantages of liquid-based cytology include improved sensitivity and specificity as fixation is better and nuclear details are well preserved. The residual cell suspension can be used to make further cytological preparations or used for other tests like detection of human papilloma virus (HPV) DNA (Kavatkar et al., 2008).

In our study, samples were collected in the usual way by using a brush-like device. The brush head was then detached into a vial containing the fixative solution prepared in the laboratory of Xiangya hospital, where they were mixed to disperse the cells. Cellular debris such as blood or mucus was removed and thinlayer cervical cells were deposited on the microscope slide which was then stained. The Bethesda system (2001) for reporting cervical cytology was used: A typical squamous cell (ASC) of undetermined significance (ASC-US) cannot exclude HSIL (ASC-H), low-grade squamous intraepithelial lesion (LSIL) encompassing: human papilloma virus/ mild dysplasia/ cervical intraepithelial neoplasia (CIN) 1, high-grade squamous intraepithelial lesion (HSIL) encompassing: moderate and severe dysplasia, carcinoma in situ, cervical intraepithelial neoplasia (CIN) 2 and cervical intraepithelial neoplasia (CIN) 3.

Colposcopy and directed biopsy

The result of cervical biopsies was reported according to the cervical intraepithelial neoplasia (CIN) classification system as mild

(CIN 1), moderate (CIN 2), or severe (CIN 3) dysplasia or carcinoma in situ (CIS) (Solomon et al., 2002).

Identification of human papilloma virus (HPV) type 16, 18

Reagents from QIAGEN Co. Ltd-China isolation kit were used, and DNA extraction was carried out by using DNA extraction solution 2 according to the instruction supplied by the manufacturer. Purified DNA was transferred to the clean tubes and stored at -20°C prior to analysis. DNA negative controls were also extracted in the same manner.

PCR amplification and detection

Samples were subjected to PCR-Fluorescent Probing with PE Gene Amp 5700 (QIAGEN Co. Ltd-China). PCR conditions were as follows: 40 µl of reaction system II, 37.8 µl of PCR reaction solution, 0.2 µl of Taq Enzyme and 0.06 µl of Uracil-N-Glycosylase (UNG). PCR amplifications that target a portion of HPV gene L_1 (approximately 450 bp [base pair]) were performed on each sample preparation and HPV L_1 consensus primer MY09/MY11 was used. To control internally the quality of the isolated DNA, the 110 bp sequence of β-globin gene was co-amplified using PC03 and PC04 primers in the multiplex PCR with the MY primers.

PCR conditions were 1 cycle of 94°C for 1 min follow ed by 40 cycles of 95°C for 5 s, 60°C for 40 s, followed by a final extension at 40°C for 3 min. Both probe and primers hydrolysis stim ulated fluorescence. The increase in fluorescence was measured, and was a direct consequence of target amplification during PCR (Kalvatchev et al., 2003; Kurtycz et al., 1996).

The results were interpreted with the software of PE Gene Amp 5700 through the presence of crossing of fluorescence curve with the threshold line. C_T value undetermined (the fluorescence curve did not cross the threshold line) or C_T value 40 (0) in the channel FAM interpreted as DNA negative for human papilloma virus (HPV) type 16, 18.

Human papilloma virus (HPV) type 16 reaction solution detection C_T value ≤ 37 in the channel FAM was interpreted as DNA positive for HPV16. Human papilloma virus (HPV) type 18 reaction solution detection C_T value ≤ 37 in the Rox channel was interpreted as DNA positive for HPV18. C_T value range of 38 to 40 (42) was doubtful and required that sample be re-analyzed on PCR stage. If the same or positive result was achieved the DNA HPV identification was considered to be positive. If not the DNA HPV identification was considered to be negative.

Experimental data was statistically processed with SPSS 17.0 software. Rate comparison using χ^2 test: variables showed statistical significance in signal factor analysis, which led to logistic regression analysis with a = 0.05, P<0.05 defined as statistically significant.

RESULTS

Comparison of the vaginal secretion Gram stain scores

There were twenty four (24) patients (52.2%) with Nugent score >6 and positive bacterial vaginosis (BV) among the investigate group. The control group had ten (10) patients (10%) with positive bacterial vaginosis (BV).

The investigate group had significantly increased risk of bacterial vaginosis (BV) compared to the control group.

The difference was statistically significant (P<0.05) (Table 2).

Comparison of HPV type 16 and 18 assay results

Twenty-eight cases (60.9%) in the cervical dysplasia group had positive result as compared to three cases (3%) in the control group (P<0.05) (Table 3).

Result of logistic regression analysis

Those with Nugent score >6 and positive HPV type 16, 18 positive cases were assessed with logistic regression analysis and the results revealed significant risk for cervical dysplasia (Table 4).

Interrelationship between bacterial vaginosis and HPV type 16, 18 co-infections

Nineteen (19) patients (41.3%) in the cervical dysplasia group had combined bacterial vaginosis and HPV type 16.18 co-infection, while none was found in the control group (p<0.05). Furthermore, thirteen (13) patients (28.3%) had negative result for both bacterial vaginosis and HPV 16, 18 infections, the control group had 87 cases (87%) (p<0.05) (Table 5).

Results of pathological examination

The results were as follows: Three (3) cases of chronic inflammation, nine (9) cases of cervical intraepithelial neoplasia (CINI), eleven (11) cases of cervical intraepi-thelial neoplasia (CINII), and fourteen (14) cases of cervical intraepithelial neoplasia (CINIII) and nine (9) cases of squamous carcinoma. These results illustrated the high positivity rate of thinlayer cystoFast test (TCT) in cervical cancer screening.

DISCUSSION

The epidemiologic risk factors for cervical intraepithelial (CIN) are similar to those for cervical cancer and include multiple sexual partners, early onset of sexual activity, a high risk sexual partner (history of multiple sexual part-ners, human papilloma virus (HPV) infection, and lower genital tract neoplasia). A history of sexually transmitted diseases (STDs), as well as cigarette smoking, human immunodeficiency virus (HIV), acquired immune deficiency syndrome (AIDs) (Aghajanian et al., 2007).

During the past decade, both animal data and molecular biologic evidence confirmed the malignant transformation potential of human papilloma virus-induced lesions (http://

Table 2. Comparison of incidence of Bacterial vaginosis.

Group	BV positive	BV negative	Total	BV positive/ total
Investigate gp	24	22	46	52.18%
Control gp	10	90	100	10%
P value				P < 0.01

Positive bacterial vaginosis (BV positive), Negative bacterial vaginosis (BV negative)

Table 3. Comparison of HPV positive cases.

Group	HPV16,18 positive	HPV16,18 negative	Total	HPV16,18 positive/ total
Investigate gp	28	18	46	60.87%
Control gp	3	97	100	3%
P value				P < 0.01

Human papilloma virus types 16, 18 (HPV16, 18) Group (gp)

Table 4. Logistic regression results.

Item	Regression coefficient	Wald χ^2	P value	OR	95% CI
HPV16/18 (+)	3.49	26.75	<0.01	32.77	8.74 ～122.96
Nugent score< 6	1.49	8.53	0.003	4.43	1.63 ～12.04

Table 5. Comparison of HPV infection with or without BV.

Group	BV(+) HPV16,18(+)	BV(-) HPV16,18(-)	Total	BV(+) HPV16,18(+)	BV(-) HPV16,18(-)
Investigate	19	13	46	41.30%	28.26%
Control	0	87	100	0	87%
P Value				P＜0.01	P＜0.01

Bacterial vaginosis and human papilloma virus co-infection (BV (+) HPV (+)).
Both bacterial vaginosis and human papilloma negative results (BV (-) HPV (-)).

emedicine.medscape.com/article/253513-overview).

This study not only confirmed that the cervical cell lesions group had higher human papilloma virus (HPV) type 16, 18 infection incidence (P<0.05) (Table 3) but also showed the high rate of human papilloma virus type 16, 18 infection in positive bacterial vaginosis patients. There were significant differences (P<0.05) (Table 5).

Ka Hyun et al. (2009) demonstrated the significant correlation between bacterial vaginosis (BV) and the presence of cervical intraepithelial neoplasia (CIN). However, no statistically significant relationship between bacterial vaginosis (BV) and cervical intraepithelial neoplasia (CIN) was demonstrated by multivariate analysis. In their study, bacterial vaginosis (BV) was diagnosed if three of the following four findings were present: Pre-

sence of thin, grey vaginal secretions coating the vaginal wall, vaginal pH>4.5 and presence of clue cells of vaginal smears.

Michelle et al. (2006) investigated the association between bacterial vaginosis (BV) and squamous intrae-pithelial lesion (SIL) concluded that bacterial vaginosis (BV) was not associated with the development of squa-mous intraepithelial lesion (SIL). But when women with high squamous intraepithelial lesion (HSIL) were considered, an increased frequency of bacterial vaginosis (33%) was found compared with that in women with no cytological abnormalities (12%). The presence of clue cells in Pap smear was used as bacterial vaginosis (BV) diagnosis.

In our study, we used thinlayer-cystoFast test (TCT)

combined with Bethesda system to diagnose bacterial vaginosis (BV) and the results were highly consistent with biopsy diagnosis.

Schiff et al. (2000) assessed the risk factors for cervical intraepithelial neoplasia (CIN) among southwestern American Indian women by using case-control methods showed that bacterial vaginosis (BV), as assessed by the presence of clue cells upon light microscopic examina-tion, was a risk factor for cervical intraepithelial neoplasia (CIN) in their study.

However they did not find history of bacterial vaginosis, or bacterial vaginosis score related to CIN in their study subjects.

Tavares-Murta et al. (2008) evaluated the local immune response in patients with bacterial vaginosis (BV) and cervical intraepithelial neoplasia (CIN), as assessed by cytokine and Nitric oxide (NO) concentrations yielded that in patients with bacterial vaginosis or cervical intraepithe-lial neoplasia, the cytokines interleukin (IL-6), interleukin (IL-8), and interleukin (IL-10) and nitric oxide (NO) were at higher concentration in endocervix than in vaginal secretions. They concluded that similar local immune profiles were observed in the bacterial vaginosis and cervical intraepithelial neoplasia groups, suggesting a possible common denominator in these conditions.

This study further confirmed that when Nugent score>6, the risk of cell lesion increased, suggesting that bacterial vaginosis might enhance the development and progression of cervical cell lesion.

Despite the small sample size and very short duration of this study, the results suggest that bacterial vaginosis enhances cervical cell lesion and also increases the risk for human papilloma virus (HPV) infection. However, con-firmation of this association requires further investigation involving a larger number of women.

We therefore recommend that examining human papilloma virus co-infection and cervical cell lesions are important measures to consider when performing aggressive therapy in recurrent bacterial vaginosis, which will subsequently help in prevention of cervical carcinogenesis.

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