ORIGINAL ARTICLE INFECTIOUS DISEASES

The association of uterine cervical microbiota with an increased risk for cervical intraepithelial neoplasia in Korea

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Abstract

Recent studies have suggested potential roles of the microbiome in cervicovaginal diseases. However, there has been no report on the cervical microbiome in cervical intraepithelial neoplasia (CIN). We aimed to identify the cervical microbiota of Korean women and assess the association between the cervical microbiota and CIN, and to determine the combined effect of the microbiota and human papillomavirus (HPV) on the risk of CIN. The cervical microbiota of 70 women with CIN and 50 control women was analysed using pyrosequencing based on the 16S rRNA gene. The associations between specific microbial patterns or abundance of specific microbiota and CIN risk were assessed using multivariate logistic regression, and the relative excess risk due to interaction (RERI) and the synergy index (S) were calculated. The phyla Firmicutes, Actinobacteria, Bacteroidetes, Proteobacteria, Tenericutes, Fusobacteria and TM7 were predominant in the microbiota and four distinct community types were observed in all women. A high score of the pattern characterized by predominance of *Atopobium vaginae*, *Gardnerella vaginalis* and *Lactobacillus iners* with a minority of *Lactobacillus crispatus* had a higher CIN risk (OR 5.80, 95% CI 1.73–19.4) and abundance of *A. vaginae* had a higher CIN risk (OR 6.63, 95% CI 1.61–27.2). The synergistic effect of a high score of this microbial pattern and oncogenic HPV was observed (OR 34.1, 95% CI 4.95–284.5; RERI/S, 15.9/1.93). A predominance of *A. vaginae*, *G. vaginalis* and *L. iners* with a concomitant paucity of *L. crispatus* in the cervical microbiota was associated with CIN risk, suggesting that bacterial dysbiosis and its combination with oncogenic HPV may be a risk factor for cervical neoplasia.

Keywords: *Atopobium vaginae*, cervical intraepithelial neoplasia, human papillomavirus, *Lactobacillus crispatus*, *Lactobacillus iners*, microbiome, pyrosequencing
Original Submission: 22 September 2014; **Revised Submission:** 4 January 2015; **Accepted:** 26 February 2015
Editor: D. Raoult
Article published online: XXX

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Introduction

The importance of the microbiota in health maintenance or disease susceptibility is well known; the gut microbiota is known to control the absorption of nutrients [1]. Imbalance in the gut is associated with Crohn's disease, type 2 diabetes and chronic allergies [2], for which dysbiosis, not a single pathogen change but shifts in the relative abundance of microbes, has long been suggested. Furthermore, the association of microbial dysbiosis with several cancer types has been suggested in areas surrounded by mucosal membranes where

http://dx.doi.org/10.1016/j.cmi.2015.02.026

bacteria live densely [3]. Although only modest effects of dysbiosis on cancer have been presented so far, the long duration of dysbiosis and possible combined effects with other risk factors suggest greater clinical implications [4]. Although human papillomavirus (HPV) has been the major risk for cervical precancerous lesions or cancer [5]; recently, the potential role of the cervicovaginal microbiome in cervical cancer through the elevation of pH has been reported [6]. In addition, the role of the cervicovaginal microbiome in HPV infection has also been reported [7], suggesting a possible role in cervical cancer through potentiation of HPV infection. Although there have been reports on the microbiome in premenopausal or postmenopausal women [8], and in bacterial vaginosis (BV) [9], no study on the microbiome related to cervical precancerous lesions or cancer has been presented. The purpose of this study was to assess the association between cervical intraepithelial neoplasia (CIN) the and cervical microbiota identified using pyrosequencing.

Materials and methods

Study participants and design

The Korean HPV cohort study, which was established to identify epidemic, genetic, viral and ecological factors associated with the development of cervical neoplasia in Korean women, recruited 1096 women, aged 18–65 years, from March 2006 to the present. The study participants were randomly selected from the gynaecological oncology clinics of six tertiary medical centres in Korea. Detailed information regarding the inclusion/exclusion criteria and the design of the baseline measurements for the HPV cohort were presented in a previous study [10]. All study participants provided written informed consent in accordance with good clinical practices. Cervical swabs were collected for a Papanicoaou smear test. Immediately after sampling, each cervix brush (Rovers Medical Devices, Oss, the Netherlands) was rinsed in a vial of PreservCyt solution (Cytyc Co., Marlborough, MA, USA), and the vial was placed in a Thin Prep Processor (Cytyc Co.). A second swab was collected for high-risk (HR)-HPV DNA detection and microbiota analysis using a Cervical Sampler Brush (Digene Co. Gaithersburg, MD, USA). Half of the second swab was used immediately for HR-HPV DNA detection and the other half was stored at −80°C for subsequent DNA extraction, for 2 months up to a maximum of 5 years. A total of 125 women were randomly selected from the 1096 enrolled women, and baseline samples obtained from 120 women (50 controls, 70 with CIN) were analysed; for five of the samples, metagenomic DNA extraction failed. This study was approved by the Institutional Review Board of the Korean National Cancer Centre (NCCNCS-06-062) and by the ethics committees of the Korean National Cancer Centre and Korea University Guro Hospital.

Cytological screening and HR-HPV DNA detection

The cervical cytological findings were classified according to the Bethesda system [11]. HPV DNA detection was performed using the commercially available Digene Hybrid capture II DNA Test (Qiagen, Gaithersburg, MD, USA). The results of a chemiluminescent HPV DNA test were measured in relative light units (RLU) with a probe designed to detect 13 types of HR-HPV. The test results were read as positive at concentrations of 1 pg/mL or greater than the RLU/cut-off ratio (RLU of specimen/mean RLU of two positive controls).

DNA extraction and pyrosequencing

Metagenomic DNA samples were extracted using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA). Target fragments of the 16S rRNA gene corresponding to the V1–V3 regions were amplified using bar-coded primers. Amplifications were performed in a final volume of 50 μL containing 10 × *Taq* buffer, a dNTP mixture (Takara, Shiga, Japan), 10 μM of the bar-coded fusion primers, and 2 U of *Taq* polymerase (ExTaq, Takara). The amplification conditions and pyrosequencing procedure have been previously described [12]. The beads recovered following emulsion PCR were deposited on a 454 Pico Titer Plate, and sequencing was performed using a Roche/454 GS Junior system (Roche, Branford, CT, USA).

Pyrosequencing data analysis

The raw sequences were sorted using their unique barcodes, and low-quality reads (with an average quality score <25 or a read length <300 bp) were removed [12]. The primer sequences were trimmed using a pairwise sequence alignment, and sequences were clustered to correct for sequencing errors. Representative sequences in each cluster were identified using EzTaxon-e, a public database that contains sequences of the 16S rRNA genes of species type strains, and the taxonomic positions of relevant uncultured sequences were identified [13] using the highest pairwise similarity among the BLASTN results. Chimeric sequences were detected and removed by the UCHIME algorithm, which detects chimeric sequences by alignment of a query sequence with two parent sequences in a reference database [14], and the diversity indices were calculated using the MOTHUR program. The heat-map analysis was performed using the MULTI-EXPERIMENT VIEWER. Pyrosequencing reads are available in the EMBL SRA database (http://www.ebi.ac.uk/ena/data/view/PRJEB5760).

Please cite this article in press as: Oh HY, et al., The association of uterine cervical microbiota with an increased risk for cervical intraepithelial neoplasia in Korea, Clinical Microbiology and Infection (2015), http://dx.doi.org/10.1016/j.cmi.2015.02.026