Clinical significance of signal pattern of high-risk human papillomavirus using a novel fluorescence *in situ* hybridization assay in cervical cytology

C.-M. Ho[1–3], B.-H. Lee[4], S.-F. Chang[5], T.-Y. Chien[1], S.-H. Huang[6], C.-C. Yan[4] and W.-F. Cheng[7,8]

1) *Department of Obstetrics and Gynecology, Gynecologic Cancer Center, Cathay General Hospital, Taipei,* 2) *School of Medicine, Fu Jen Catholic University, Hsinchuang, Taipei Hsien,* 3) *School of Medicine, Taipei Medical University, Taipei,* 4) *King Car Food Industrial Co, Ltd, Yuan Shan Research Institute, Elan,* 5) *Graduate Institute of Medical Sciences, Taipei Medical University,* 6) *Department of Pathology, Cathay General Hospital,* 7) *Graduate Institute of Oncology, National Taiwan University and* 8) *Department of Obstetrics and Gynecology, College of Medicine, National Taiwan University, Taipei, Taiwan*

Abstract

The present study aimed to evaluate a novel fluorescence *in situ* hybridization (FISH) assay for detecting the high-risk human papillomavirus (HR-HPV) DNA and signal pattern in cervical cytology specimens and for identifying cervical intraepithelial neoplasia (CIN) lesions. One hundred and ninety-six liquid-based cytology specimens with CIN were recruited. The signal pattern (punctate, mixed punctate and diffuse, and diffuse) detected by FISH was compared with E6 mRNA and correlated with histological classification. FISH and E6-type specific polymerase chain reaction (PCR) had fair to good agreement for detecting HPV DNA across all grades of CIN (kappa coefficient, 0.37–0.73). Among 44 samples of negative FISH and positive E6 type-specific PCR in HPV 16, 18, 31, 33, 52 and 58, 82% (36/44) of E6 mRNA were not detected, in contrast to 41% (48/118) of positive FISH and positive E6 type-specific PCR ($p < 0.0001$). Among HR-HPV DNA positive cases tested by the FISH assay, the specificity of predicting CIN3 using the punctuate pattern is higher than that using E6 mRNA (96.3% vs. 44.8%). The punctate pattern was 0% in patients with <CIN1 lesions, 8.7% for CIN1 lesions, 6.1% for CIN2 lesions, and 34.0% for CIN3 lesions (p 0.001). The odds ratios were 8.7-fold higher (2.7–27.8, $p < 0.0001$) for the punctate pattern versus the mixed punctate and diffuse pattern, and the diffuse pattern, for predicting CIN3 lesions. The novel FISH assay is comparable to PCR for detecting HPV DNA in cervical cytology with CIN lesions. The punctate signal pattern detected by the FISH assay can be more biologically and clinically relevant for clinically detecting CIN3 lesions.

Keywords: Cervical intraepithelial neoplasm, fluorescence *in situ* hybridization, high-risk human papillomavirus, signal pattern
Original Submission: 6 October 2009; **Revised Submission:** 2 December 2009; **Accepted:** 28 January 2010
Editor: E. Gould
Article published online: 9 February 2010
Clin Microbiol Infect 2011; **17:** 386–394
10.1111/j.1469-0691.2010.03186.x

Corresponding author: W.-F. Cheng, Graduate Institute of Oncology, College of Medicine, National Taiwan University, 7, Chung-Shan South Road, Taipei, Taiwan
E-mail: wenfangcheng@yahoo.com

Introduction

Cervical cancer is a major health burden in women. A total of 493 000 women were diagnosed with cervical cancer and 273 000 died around the world in the year 2002. Worldwide, cervical cancer is the second most common female cancer in the world, with a mean age standardized incidence rate of up to 18.8 per 100 000 women, and it is a major cause of mortality for women in developing countries [1]. Persistent infections with high-risk types of human papillomavirus (HR-HPV) represent a necessary cause of cervical cancer [1–4]. HR-HPV DNA can be detected in up to 99.7% of cervical squamous cell carcinomas [2,4] and in 94–100% of cervical adenocarcinomas and adenosquamous carcinomas [5].

HPV is a common virus infection among women, particularly in younger age groups, and most infections are transient and asymptomatic. Patients with persistent infection with these HPV types have a clearly enhanced risk of developing cervical carcinoma [6]. Large-scale screening studies have shown that HPV testing is more sensitive than a cytology method for the detection of high-grade cervical lesions [6]. However, the low specificity of current assays and commercial kits hampers the use of HPV testing in screening. Although the combination of HPV DNA detection and cytology is more suitable for risk assessment of the progression to cervical intraepithelial neoplasia (CIN) grade 3 and

carcinoma than cytology alone [7], the false positive rate of the HPV DNA test and the psychological burden for women with HPV DNA positive is still high, and needs to be improved. Hence, the detection of the signal pattern of HPV DNA of high-risk HPV types using the fluorescence *in situ* hybridization (FISH) method would be attractive and might serve as a better risk evaluation factor than the DNA test for the detection of the development of CIN3.

One limitation of using *in situ* hybridization for HPV detection is its low sensitivity, with a detection limit was approximately 50 viral copies per cell. We developed a novel method for detecting HPV DNA and signal pattern in one procedure by FISH. In the present study, we tested the performance of our novel FISH assay to determine the effectiveness of the FISH assay for detecting HR-HPV in thin-layer cervical specimens. In addition, the signal pattern (punctate, mixed punctate and diffuse, and diffuse) detected by FISH was compared with E6 mRNA and correlated with different grades of cervical lesions. Using this novel FISH assays, we address an important issue with respect to using the signal pattern in high-risk HPV type detection for determining the status of CIN3 in women and identifying a more biologically and clinically relevant testing method.

Materials and Methods

Study design and population

This was a cross-sectional study conducted in 2005. The study protocol was reviewed and approved by the Institutional Review Board of Cathay General Hospital. All of the participants provided their written informed consent before being enrolled in the study. A total of 196 liquid-based cervical swabs were collected from 58 women with cytological low-grade squamous intraepithelial lesions (LSIL) and 138 women with cytological high-grade squamous intraepithelial lesions (HSIL). All of these 196 patients were examined by colposcopy and the diagnosis was confirmed by biopsy or a loop electrosurgical excision procedure/cone. Each case was ascertained by histological diagnosis based on the most severe histology by biopsy or a loop electrosurgical excision procedure/cone. Thus, we obtained specimens from 196 women: 29 women with <CIN1 lesions (abnormal cytology, biopsy negative), 34 women with CIN1 lesions, 44 women with CIN2 lesions, and 89 women with CIN3 lesions. Specimens were tested for HPV DNA genotyping using a modified MY11/GP6+ polymerase chain reaction (PCR) for HPV DNA amplification, followed by HPV genotype-specific hybridization on a genechip. An E6 type-specific PCR was performed to validate multiple infections. A total of 196 specimens including 165 positive HPV DNA tests in types 16, 18, 52, 58, 31, 33, 39, 45, 51, 56, 59, 68, 6 and 11, and 31 with negative HPV DNA detected by E6 type-specific PCR, were examined by FISH. Among the 165 samples with positive E6 type-specific PCR, 162 samples were positive in HPV 16, 18, 52, 58, 31 and 33. Among the 162 samples, 118 samples had positive FISH with positive E6 type-specific PCR, and 44 samples of negative FISH, but with positive E6 type-specific PCR, in HPV 16, 18, 31, 33, 52 and 58, were examined by quantitative reverse-transcriptase PCR (QRT-PCR). The signal patterns detected by FISH were defined as punctate, mixed punctuate and diffuse, and diffuse patterns, and further correlated with different histopathologies of cervical lesions.

Collection of sample material

The cervical swabs were collected with a cytobrush by study physicians, during routine and abnormal Pap smear office visits, and put into 20 mL of thinprep collection media, and then stored at room temperature, no more than 2 weeks before the allotment of samples was made.

Allotment of samples. After sample collection, two 2 mL aliquots of the liquid based cytology (PreservCyt®; Cytyc Corporation, Malborough. MA, USA) sample were removed. Samples were spun for 10 min at 2800 ***g***. Subsequently, all traces of PreservCyt® medium were removed and the pellets were store at −70°C for DNA and RNA extraction. One 10-mL aliquot of the liquid-based cytology sample was removed for preparation of thin-layer slides within 2 weeks. Residues materials were stored as pellets at −70°C.

Control cell lines. CaSki cells (contains 60–600 HPV 16 DNA copies per cell), HeLa cells (contains 10–50 HPV 18 DNA copies per cell) and Jurkat cells (HPV-negative) were used simultaneously to assess the entire procedures inclusive of DNA extraction, RNA extraction, PCR HPV typing and FISH. Control cells were suspended for 48 h in 20 mL of the same preservation fluid used for collecting cervical samples and further processed as described for cervical smears.

Extraction of DNA. One aliquot of pellet material was for DNA extraction via the QIAamp DNA Blood Mini kit (Qiagen Inc.,Valencia, CA, USA) in accordance with the manufacturer's instructions. Extracted DNA was eluted in 100 μL AE buffer (10 mM Tris, pH 8.5). DNA was stored at −20°C until analysis. The concentration of DNA extract was quantitated by the Quant-iT™ PicoGreen® reagent (Invitrogen Corp., Carlsbad, CA, USA) in accordance with the manufacturer's instructions.