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SNIPER: A novel assay for Human Papillomavirus testing among women in Guizhou, China

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INTRODUCTION

Human Papillomavirus (HPV) has been identified as the necessary cause of cervical cancer (1). There are approximately 500,000 new cases and 270,000 deaths annually world-wide, with 80% of those in developing countries(2). Cytology based screening programs require a complex healthcare infrastructure unattainable for most developing countries. This leaves the majority of women in the developing world without access to effective cervical cancer screening(3). The societal impact is large with cervical cancer primarily occurring in women in their 40's (the greatest cancer killer of women under the age of 50) when women are still raising and supporting families (4). Based on the nearly absolute etiologic link between high risk HPV DNA and cervical cancer new approaches for the prevention of cervical cancer have emerged, including primary HPV screening for secondary prevention of cervical cancer through the identification and treatment of cervical pre-cancer and early cancer. Randomized clinical trials have confirmed evidence from prior epidemiologic studies that high risk HPV DNA testing offers superior sensitivity compared to cervical cytology. Based on this evidence the International Agency for Research on Cancer concluded that HPV DNA testing for primary cervical cancer screening is an acceptable alternative to cervical cytology.

To make HPV DNA testing more accessible to developing countries, a low-cost, rapid, easy to use test, with exceptional test characteristics is needed. Genetel Pharmaceuticals Ltd., has developed the SNIPER series of HPV DNA testing kits, which have been approved for use in China since June 2006. Here, we report clinical validation of the SNIPER HPV DNA assay in a prospective cross-sectional screening study in Guizhou Province, China.

MATERIALS AND METHODS

Study Population

The institutional review boards of both the Cleveland Clinic Foundation and Peking University Shenzhen Hospital approved all written protocols. Between March and April of 2008, 1,000 women were recruited by the Renmin Hospital in the Buyi-Miao Autonomous District of Guizhou Province (BMAD), China in collaboration with the Peking University Shenzhen Hospital and Preventive Oncology International, Inc. affiliated with the Cleveland Clinic. In brief, women were excluded if they were pregnant, under the age of 30, did not have an intact

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uterus, or had a history of pelvic irradiation or cervical cancer. Women who provided written informed consent were enrolled. This manuscript reports on the 1,000 eligible women who submitted to HPV testing with the SNIPER assay, as an initial screening test, as well as to liquid based cytology (SurePath).

Clinical Protocol

Women who were HR-HPV positive (one of 13 high-risk types by the SNIPER assay) or positive on cytology ($\geq$ ASCUS) were requested to return for colposcopy and biopsy using the Preventive Oncology International (POI) micro-bopsy protocol. The Cervix was visually divided into quadrants for examination: Quadrant I (12:00 – 3:00), Quadrant II (3:00 – 6:00), Quadrant III (6:00–9:00) and Quadrant IV (9:00–12:00). Colposcopic diagnoses were made by quadrant. All patients were biopsied according to our standard protocol using a 2 mm POI biopsy forceps. All colposcopically detected abnormalities were biopsied by quadrant. In normal quadrants, biopsies were obtained at the squamo-columnar junction (2, 4, 8, and 10 o'clock depending on the quadrant). All patients had an endocervical curettage performed. Therefore all patients had a minimum of 5 biopsies [SCJ 2, 4, 8, and 10 o'clock and an endocervical curettage (ECC)].

Cervical histology represents the “criterion standard”. A team of pathologists read the pathology, and one gynecologic pathologist served as the final reference and quality control. All women found to have high-grade pre-cancer or true invasive cancer were cared for according to standard treatment protocols under the direction of the staff from the Peking University Shenzhen Hospital under the direction of Dr. Ruifang Wu.

Sample Preparation from Cervical Swabs

Samples for HPV DNA testing were collected using a conical shaped cervical brush (after obtaining a cytology sample) and placed in 2 ml of saline solution (pH 7.4). Before being processed for HPV DNA using the SNIPER assay, samples were maintained between 2°C and 30°C for up to 1 week. Then, 1 ml of this suspension was used as described in the following protocol. The solution was spun at 3,000g for 10 min at room temperature (RT). The supernatant was discarded, and the pellet was washed with 50 μ l of lysis buffer in 10mM Tris-HCl (pH 7.4) and then spun at 3,000g for 10 min at RT. The supernatant was discarded, and 50 μ l of a lysis buffer in 10 mM Tris-HCl (pH 7.4) was added to suspend the pellet. The pellet was then vortexed to distribute the cells evenly. The pellet was boiled in a heating block at 100°C for 10 min and then cooled down to room temperature. The sample was then centrifuged at 13,000 ~ 15,000g for 10 min, and the supernatant was preserved for HPV detection. A 2 μ l portion of it was used for the SNIPER™ HR-HPV assay.

High-risk HPV testing with SNIPER™ HR-HPV assay

Oligonucleotide primers and probes were designed by using Primer Express software (version 2.0; Applied Biosystems, Foster City, Calif.). For PCR amplification, a pool of HPV primers was designed to amplify HPV DNAs from 13 HR-HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68). DNA polymerase extends the annealed primers along the target templates to produce an approximately 200 bps double-stranded HPV target DNA molecule. The fluorescent probe sequences are specific for polymorphic regions of L1 bound by these primers. The 5' of probes were labeled with fluorophore FAM and the 3' of probes were labeled with non-fluorescent quencher (Applied Biosystems, Foster City, Calif.). The probes were designed to ensure a higher melting temperature than for the primers. The PCR amplification was performed in a 40 μ l volume containing 37.5 μ l of PCR master mix (SNIPER™ HR-HPV assay, Genetel Pharmaceuticals Ltd. Shenzhen, China); 0.4 μ l of Taq polymerase; 0.1 μ l of uracil-*N*-glycosylase (UNG, Fermentas) and 2 μ l of DNA sample. Amplification and detection was performed by using an ABI Prism 7000 sequence detection

system (Applied Biosystems). The amplification included an initial hold step of 2 min at 50 °C and 10 min at 95 °C, followed by a three-step cycle consisting of 15 s at 95 °C, 30s at 52 °C and 1 min at 62 °C. In the analysis of clinical samples 40 cycles were used due to the high efficiency of the PCR. PCR tubes, including all PCR components but without template DNA, were used to ensure that the reagents mix were free of contamination.

Statistical Analysis

The performance characteristics of the SNIPER assay were evaluated by calculating the sensitivity, specificity, positive and negative predictive values according to the standard definitions. All women in this study either received the reference standard (cervical biopsies) or were negative on HPV and cytology, which has been shown in previous studies to predict a women being histologically negative(5). Due to this the above estimates were calculated directly in this study. Since women in this study had multiple biopsies, biopsy data were collapsed across women to generate variables using the highest-grade biopsy in any quadrant as the final histological diagnosis. All confidence intervals are exact binomial confidence intervals. Performance of the SNIPER assay was determined both for $\geq$ CIN II and $\geq$ CIN III. Receiver Operating Characteristic (ROC) curves were drawn using the current cut-off for positivity, for both $\geq$ CIN II and $\geq$ CIN III. All data analysis was performed using STATA 10.0 (StataCorp LP, College Station, Texas).

RESULTS

One thousand women were screened with the SNIPER assay. Of those 175 (17.5%) tested positive for HPV with the assay and another 36 women tested negative for HPV but had positive Pap smears. These women were asked to return for follow-up. Of the 211 women asked to return all but 21 or 90 percent of the women returned for colposcopy and biopsy. Demographic characteristics for the 979 women who completed screening are presented in Table 1. Median age and proportions of women having $\geq$ CIN II and $\geq$ CIN III differed between women with and without HR-HPV. Regardless of HR-HPV status women reported a median number of 3 pregnancies and 1 live birth. The majority of women screened were pre-menopausal, married, non-smokers with approximately 35% reporting vaginal discharge regardless of HR-HPV test status. Twenty-five women had $\geq$ CIN II on histology and 16 were diagnosed as $\geq$ CIN III.

The SNIPER assay was 93.3% [(77.9, 99.2) 95% CI] sensitive and 86% [(84, 88) 95% CI] specific for the detection of $\geq$ CIN II (Table 2) versus 63.3% [(43.9, 80.1) 95%CI] sensitive and 93.4% [(91.6, 94.9) 95% CI] specific for ASCUS abnormal cervical cytology. For the detection of $\geq$ CIN III the SNIPER assay was 94.1% [(71, 100) 95% CI] sensitive and 85% [(83, 87) 95% CI] specific versus 76.5% [(50.1, 93.2) 95% CI] sensitive and 92.8% [(91.0, 94.4) 95% CI] specific for ASCUS positive cervical cytology (Table 3). Positive and negative predictive values were also calculated. The probability that a person who tested HR-HPV positive actually had $\geq$ CIN II was 17.4 % [(11.9, 24) 95% CI] for SNIPER assay versus 23.2 [(14.6, 33.8) 95 % CI] for ASCUS positive cytology. For the detection of $\geq$ CIN III the PPV was 9.94 [(5.79, 15.6) 95% CI] for SNIPER and 15.9% [(8.72, 25.6) 95% CI] for ASCUS positive cytology. The predictive value of a negative test approached 100% for $\geq$ CIN II and $\geq$ CIN III for both cytology and the SNIPER assay. Areas under the curve for the sniper assay for the detection of $\geq$ CIN II and $\geq$ CIN III 0.8966 and 0.8952 respectively are almost identical.

DISCUSSION

Testing for HPV has been shown to have superior sensitivities compared to conventional cytology and can improve patient management when used in conjunction with cytology (6–8). Testing for HR-HPV is also under investigation for use as a primary screening tool (9–13).

The high negative predictive value for HR-HPV may allow for increases in the recommended screening interval especially for women found to be negative by both cytology and HR-HPV testing (14). Hybrid Capture 2(HC2), a commercially available HPV assay detects the 13 most common HR-HPV types (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), and has been widely used. In China as well as many other parts of the world many “home brew” assays have emerged that are used by local laboratories and occasionally on a wider scale. Many of these assays are developed to be considerably less expensive than HC2. Genetel Pharmaceuticals Ltd. is a sophisticated laboratory in China that has developed an HPV test similar in spectrum to HC2. The assay has demonstrated analytical sensitivity comparable to HC2 and has received approval by the SFDA (Chinese FDA). In this trial we chose to test the SNIPER Assay and perform diagnostic verification with our well-established POI micro-biopsy protocol.

In this study, we showed that testing for HR-HPV with the SNIPER assay was far more sensitive than cervical cytology (93.3 vs. 63.3) for the detection of $\geq$ CIN II in a population of women in Guizhou, China. The same was true for the detection of $\geq$ CIN III (94.1 vs. 76.5). In this population with a rate of HR-HPV of 17.5 percent, positive predictive values were higher for cytology for both detection of $\geq$ CIN II and $\geq$ CIN III (23.2 vs. 17.4 and 15.9 vs. 9.9) respectively, while negative predictive values approached 100% for both SNIPER and cytology. Median age (39 vs. 41) was significantly different between those without and with HR-HPV.

Comparing the performance of the SNIPER assay to the historical performance of HC2 in the Shanxi Province Cervical Cancer Screening Study (SPOCCS) I and II, the SNIPER assay was slightly less sensitive and more specific. In SPOCCS I the sensitivity for $\geq$ CIN II from a direct cervical sample was 95% while the specificity was 85%, for detection of $\geq$ CIN III sensitivity was 98% and specificity was 100% (5). In SPOCCS II HC2 was 96.8% sensitive and 79.7% specific for the detection of $\geq$ CIN II (15).

The current study did not compare SNIPER to HC2. Data from a study of 1,777 samples showed the overall concordance of the SNIPER assay with HC2 for the detection of 13 HR-HPV types was 96.2% (16). Based on these data the SNIPER assay is being used as the primary method for HR-HPV testing in many labs within China. A recent comparison of HC2 and SNIPER was conducted in Shenzhen city among 1,000 Chinese women aged 30–65 years (17). For the detection of high-risk HPV a sensitivity and specificity of 93.7% and 81.1% for SNIPER and 95.2 % and 84.4% for HC2 was found (18). Personal communications with individuals experienced with the procedures of both HC2 and the SNIPER assay found the two assays similar in labor intensity (non-automated) with the SNIPER requiring about hour less time (18). Of interest, although the SNIPER Assay is sold to hospitals at a considerably lower cost than HC2, the patient charges are similar in most instances. The final important point is the issue of clinical relevance. HC2 has demonstrated over many years of clinical testing and many years of clinical application that its sensitivity and specificity at the levels chosen to be the positive and negative cut-points are clinically relevant and the assay has worked extremely well in the care of patients. This balance is critical and only well verified studies and follow-up of negative testing can fully define clinical relevance.

In conclusion it seems that on balance the SNIPER assay is functionally competitive and in terms of cost holds an advantage over HC2 in a Chinese healthcare market and potentially others around the world.

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Table 1

Demographic characteristics of 979 Chinese women screened with the SNIPER assay.

Characteristic	HPV Negative N=818	HPV Positive N=161	P-Value *
Median age (years)	39.0 (30–54)	41.0 (30–50)	.015
Median number of pregnancies (range)	3 (1–9)	3 (1–10)	.113
Median number live births (range)	1 (0–6)	1 (1–4)	.211
Menopausal N (%)			
Yes	42 (5 %)	11 (7%)	0.444
No	776 (95%)	150 (93%)	
Married N (%)			
Yes	803 (98%)	154 (96%)	0.073
No	15 (2%)	7 (4%)	
Smokers N (%)			
Yes	12 (1%)	4 (2%)	0.317
No	806 (99%)	157 (98%)	
Reporting Vaginal Discharge N (%)			
Yes	288 (35%)	58 (36%)	0.857
No	530 (65%)	103 (64%)	
CIN 2 or greater			
Yes	2 (0.3%)	28 (17%)	0.000
No	816 (99.7%)	133 (83%)	
CIN 3 or greater			
Yes	1 (0.02%)	16 (10%)	0.000
No	817 (99.8%)	145 (90%)	

* P-value from Fishers exact test

Table 2

Sensitivity, specificity, positive-predictive value (PPV), negative-predictive value (NPV), for SNIPER assay, and Pap smear among 979 women examined from a colposcopy clinic in Guizhou, China based on the detection of CIN2+ on histology (n= 30) and a HPV prevalence of 17.5%.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
SNIPER assay	93.3 (77.9, 99.2)	86.0 (83.6, 88.1)	17.4 (11.9, 24.1)	99.8 (99.1, 100)
Pap smear	63.3 (43.9, 80.1)	93.4 (91.6, 94.9)	23.2 (14.6, 33.8)	98.8 (97.8, 99.4)

Table 3

Sensitivity, specificity, positive-predictive value (PPV), negative-predictive value (NPV), for SNIPER assay, and Pap smear among 979 women examined from a colposcopy clinic in Guizhou, China based on the detection of CIN3+ on histology (n= 17) and an HPV prevalence of 17.5%.

	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
SNIPER assay	94.1 (71.3, 99.9)	84.9 (82.5, 87.1)	9.9 (5.79, 15.6)	99.9 (99.3, 100)
Pap smear	76.5(50.1, 93.2)	92.8 (91.0, 94.4)	15.9 (8.72, 25.6)	99.6 (98.9, 99.9)