

Application of Chromogenic *In Situ* Hybridization (CISH) for Human Papillomavirus (HPV) Genotyping

Ekaterina Kldiashvili¹ and Sophio Bojgua²

- 1 Georgian Telemedicine Unio (Association), Tbilisi, Georgia
- 2 New Vision University, Tbilisi, Georgia

Abstract

Background: The research aimed evaluation of the effectiveness of CISH for HPV genotyping on cervical smears and compare the results of genotyping with cytopathology findings of routine screening.

Methods and findings: Gynecological cytology cases (n=1000) were taken from the clinical laboratory. Cases were diagnosed routinely by application of liquid based cytology method. Patients with diagnosed atypia were recalled for obtaining of material for HPV genotyping. This has been performed by usage of CISH method.

Conclusion: CISH is effective and easy for implementation method for HPV genotyping on cervical smears. There has been revealed 92.2% concordance in average between genotyping results and cytopathology findings of routine screening.

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Introduction

Obvious decreasing in cervical cancer mortality has been observed in countries with organized cervical cancer screening programs [1]. Liquid based cytology is a technique that enables cells to be suspended in liquid medium and spread in monolayer for better morphological assessment. It includes the preparation and evaluation of cells collected in liquid fixative. It is being introduced to improve the sensitivity of the Pap test [2]. Liquid based preparations are increasingly being used both for gynecologic and non-gynecologic cytology, including fine needle aspirations [3]. Two technologies, ThinPrep (Hologic, Marlborough, MA, USA) and BD Sure Path (BD Diagnostics – TriPath, Burlington, NC, USA) have been more widely used [2]. The advantages of liquid based cytology include improved sensitivity and specificity since fixation is better and nuclear details are well-preserved. Abnormal cells are not obscured or diluted by other epithelial or inflammatory cells. There is, therefore, a lower rate of unsatisfactory cervical cytology samples. The residual cell suspension can be used to make further cytological preparations or used for other tests like detection of human papillomavirus DNA. Other ancillary techniques like immunocytochemistry can also be performed on the residual sample [4]. The more widely used technologies for liquid based cytology require expensive equipment [2].

Nearly all sexually active men and women are exposed to human

papillomavirus (HPV) at some stage in their lives; it does not cause health problems. HPVs that infect the anogenital tract fall into two broad groups: those that cause warts (low-risk) and those associated with cancer (high-risk). Persistent infection with high-risk HPV types causes all cervical cancer, most vulvar, vaginal and anal cancers; approximately half of penile cancers, as well as an increasing subset of oropharyngeal cancers, and HPV is also implicated on cancer precursor conditions in the cervix, anus, vulva and vagina. In some instances, HPV status will determine the approach to cancer treatment. The rising number of HPV-related cancers is a major public health issue. The concept of a virus causing cancer is frightening. The association of cervical pre-cancer with HPV has clear psychosocial adverse effects. Health professionals must be prepared to discuss HPV status because affected patients may want to know the cause of their condition and may question the implications for their sexual partners. Although discussions about HPV between patients and health professionals are becoming more common in cervical disease, patients express concern about the stigma attached to sexual transmission. There is little rigorous research into how clinicians communicate with patients for the other cancers associated with HPV. Patients and the public know very little about HPV. Despite the introduction of HPV vaccination in schools, and HPV testing within cervical screening programmes in the UK

Corresponding author:

Ekaterina Kldiashvili

✉ ekldiashvili@gmail.com
ekldiashvili@yahoo.com

Executive Director, Georgian Telemedicine Union (Association) 75 Kostava str., 0171 Tbilisi, Georgia.

Tel: 995599957328

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and several other countries, systematic reviews demonstrate consistently poor knowledge and lack of awareness that HPV is a sexually transmitted causative factor for cervical cancer. Furthermore, women who are found to be HPV positive during cervical screening experience distress, anxiety and a notable lack of understanding. Health care professionals do not know enough about HPV-associated cancers, a developing area of research where there are still many uncertainties and good quality patient information is lacking [5].

More than 200 types of HPV have been recognized on the basis of DNA sequence data showing genomic differences. Based on their association with cervical cancer and precursor lesions, HPV is grouped to high-risk and low-risk HPV types. Low-risk HPV types include types 6, 11, 42, 43, and 44. High-risk HPV types include types 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 70 [6]. It is recognized that persistent infection of the HPV is required for the development of invasive cervical cancer [7]. While infection with HPV is common, especially in sexually active young women, most infections are transient and are characterized by self-recurrence without clinical consequences. However, some women develop persistent HPV infections and are at risk for cervical cancer and its precursors. Cervical cancer is heralded as being the third most common cancer in women followed by breast and colorectal cancer. World Health Organization (WHO) has reported that approximately 530,000 women worldwide are diagnosed with cervical cancer, and the mortality of incidence ratio changed from 52 to 88% in developing countries [8]. Although cervical cancer screening programs by cytology tests (e.g., conventional Pap-smears, LBS) has decreased the incidence and death rate in many countries in the past few decades, cervical cancer still remains a leading cause of death in women due to the high rate of false positive results is limited reproducibility of cytology tests [9,10]. Besides, the sensitivity and specificity of the test have been questioned. The low sensitivity of cytology tests would put women at risk of developing invasive cervical cancer. Thus, much concern has arisen recently to develop a better screening test and/or design for disease prevention, especially the role of HPV testing.

Our research aimed evaluation of the effectiveness of CISH for high-risk HPV DNA (16/18/31/33/35; 16/18; and 31/33) detection and genotyping on cervical smears and compare the results of genotyping with cytopathology findings of routine screening.

Methods

One thousand patients attending gynecologist at outpatient clinic for routine visit during 2015 (from February 2015 until November 2015) were selected for study. These were 18-65 years old nonvaccinated for HPV gynecologically asymptomatic females. The median age of screened group was 37 years. Specific inclusion criteria as not been used for patients recruitment. Informed consent from all cases was taken.

All cases were taken by usage of the ThinPrep reagents (Hologic). The cervical smear was obtained by rover cervical brushes and washed in the sampling solution ThinPrep (Hologic). One package of sampling materials (cervical brush and vial with sampling solution ThinPrep) has been used per patient. After obtaining

and before laboratory processing the samples were stored at room temperature. The delay time between obtaining of samples and their laboratory processing did not exceed 2 hours. The smears have been prepared on glass slides by the application of the ThinPrep 2000 Processor (Hologic) accordingly with the provided for gynecology sample instructions, the program #4 of the processor has been used. One glass slide has been prepared for each screened patient. Prepared wet smears have been fixed in absolute alcohol during 30 min and stained accordingly with Papanicolaou staining protocol (<http://www.nottingham.ac.uk/pathology/protocols/papcytol.html>). The Bethesda 2001 System terminology (<http://nih.techriver.net/bethesdaTable.php>) has been used for reporting of cervical smears. The average time required for processing and reporting of the sample was 4 hours (92.7% of cases). The Papanicolaou stained smears were evaluated by light microscopy (Konus, 5601-Biorex-2) under x4, x10, x40 and x100 objective lens. The stained smears have been archived accordingly with Georgian requirements to medical data storage and documentation.

The re-usage of the rest of samples containing ThinPrep solution containing samples for additional smear preparation was impossible because of insufficient for smear preparation amount of liquid. Therefore, patients with diagnosed atypia were recalled within 5 working days (to avoid self-recurrence and/or new infection cases) for obtaining of material for HPV genotyping. The short time period has been elaborated to exclude and avoid situation of viral clearance and/or possible new infections. The HPV genotyping has been performed by CISH method on conventional smears. The smears have been fixed in absolute alcohol during 30 min. HPV specific DNA detection in the smear were performed by ZytoFast HPV type 16/18/31/33/35 Probe Kit (ZytoVision) and HPV genotyping by ZytoFast HPV type 16/18 and type 31/33 Probe Kits (ZytoVision) according to the manufacturer's instructions. Positive and negative controls were used as reference for the color appearance and reaction quality assurance.

Briefly, for enzyme digestion the smears were treated by pepsin solution on 37°C during 5 minutes and then washed by distilled water at room temperature. For fixation smears were incubated in 1% formaldehyde solution (5 min, room temperature). After heat pretreatment step (incubation in EDTA for 15 min, 98°C; wash distilled water for 1 min, room temperature) air dried smears were hybridized with dig-labeled probes (5 µL, 5 min at 75°C and 60 min at 37°C). The detection of HPV DNA in smears was performed by rabbit-anti-DIG (30 min at 37°C) and anti-rabbit-AP-polymer (30 min, 37°C). For counterstain Mayer's hematoxylin was used (1 min, room temperature). The stained smears were evaluated by light microscopy (Konus, 5601-Biorex-2) under x40 and x100 objective lens. Smears with stained red cells (AP-Permanent Red) on the blue background were considered positive for HPV DNA (16/18/31/33/35) and HPV concrete genotype (16/18, 31/33).

Results

Sampling quality

In our study one thousand cases were studied. The cellularity was low in liquid based cytology as compared with conventional

smears. The nuclei overlap to the lesser extent, so better cell morphology can be studied in the liquid based cytology technique.

Screening results

894 cases (89.40%) were interpreted as the negative for intraepithelial lesion (NILM), epithelial cell abnormalities were diagnosed in 103 (10.3%) cases, and glandular cell abnormalities were diagnosed in 3 (0.3%) cases. Cases with abnormal cervical cytology included 79 cases (76.7%) with atypical squamous cells of undetermined significance (ASC-US); 16 cases (15.53%) with a diagnosis of atypical squamous cells of undetermined significance, suspicious but not diagnostic for high grade squamous intraepithelial neoplasia (ASC-H); 6 cases (5.83%) with a diagnosis of low grade squamous intraepithelial lesion (LSIL); 2 case (1.94%) with a diagnosis of high grade squamous intraepithelial lesion (HSIL). The results of screening are summarized in **Table 1**.

HPV

HPV DNA has been revealed in 95 cases (92.2%) from 103 cases with diagnosed epithelial cell abnormalities. These cases were positive for high-risk HPV of 16/18/31/33/35 type. 16/18 types of HPV were revealed in 78 cases (82.11%), 31/33 types of HPV were revealed in 17 cases (17.89%). 8 cases (7.8%) were negative for HPV DNA. All these cases were diagnosed as ASCUS during LBC screening. The correlation between HPV DNA detection and genotyping by application of 16/18 and 31/33 HPV types probes with LBC screening diagnosis are summarized in **Table 2**.

Discussion

The article presents a small pilot study of 1000 women in Georgia. As a result of the review and analysis of the data it can be concluded, that the liquid based cytology technique gives results comparable to the conventional technique with better morphology [3]. Furthermore, among the specific objectives of a pilot study was standardization of the classification system for cervical lesions to improve communication and clinical care. For this purpose the trainings for healthcare professionals have been performed. These trainings present the data on the comprehensive organization of screening, standards of cervical smear obtaining, sample processing tasks and etc.

Table 1 The screening results of intraepithelial lesion, epithelial cell abnormalities, glandular cell abnormalities.

Category Number of cases	NILM	ASCUS	ASC-H	LSIL	HSIL	AGC
Totally – 1000 cases (%)	894 (89.4%)	79 (76.7%)	16 (15.53%)	6 (5.83%)	2 (1.94%)	3 (0.3%)

Table 2 The correlation between HPV DNA detection and genotyping.

CISH HPV LBC screening diagnosis	HPV DNA (16/18/31/33/35)	HPV DNA (16/18)	HPV DNA (31/33)
ASCUS	71 (74.73%)	51 (53.68%)	16 (16.84%)
ASC-H	16 (16.85%)	8 (8.42%)	6 (6.31%)
LSIL	6 (6.32%)	5 (5.26%)	1 (1.05%)
HSIL	2 (2.10%)	2 (2.10%)	--

Cancer is an emerging public health issue in Georgia [11]. While the data available are known to be underestimates of true cancer incidence and cancer-related mortality, the statistics portend a troubling outlook for women in particular. The leading female cancers in Georgia are breast, cervical and ovarian – all in the reproductive system. Unfortunately, about half of all cancers are diagnosed in the late stages, obviating the opportunity for effective treatment.

Screening for cervical cancer is now being widely discussed as part of the larger context of a general effort to improve women’s health through education, disease prevention and early intervention [12]. These discussions have established the following: 1) most women have never had a Pap test, 2) different classification (e.g., the Bethesda System, Papanicolaou, CIN) are used to communicate results of cytology tests, 3) the primary risk factor for cervical cancer, human papillomavirus, is having a partner with previous relationships.

The Pap test is the most successful screening tests utilized in medicine. However, for this test to be effective, three things must occur. First, an adequate specimen must be obtained from the patient and submitted to the laboratory. Second, the specimen must be properly prepared, screened, and interpreted. Third, it is imperative that when communicating the diagnosis, the laboratory uses terminology that is clearly understood by the clinician. To this end, several classification schemes and sets of terminology to provide more meaningful diagnostic information have been developed. The most informative and adequate is the Bethesda 2001 System (TBS) [8]. It is not only a simple listing of diagnostic terms, but is a comprehensive way to report cervical cytology that incorporates a descriptive diagnosis and evaluation of specimen adequacy with a heavy emphasis on clinical relevance [9,10].

The benefits of screening and early intervention are clear. Early intervention is available and can be performed in Georgia at minimal cost. There is no standardized approach for obtaining smears or interpreting results. The resulting ambiguity makes it difficult for clinicians to compare results of Pap-test, negatively affecting patient care. By the implementation of the present pilot the introduction of standardized and LBC approaches for cervical cancer screening has been performed.

It has been revealed, that LBC improved sensitivity and specificity of cervical cancer screening since fixation is better and nuclear details are well-preserved, the amount of unsatisfactory samples is decreased. It has been also revealed, that CISH is effective and easy for implementation method for HPV DNA detection and genotyping on cervical smears. The efficacy of the high-risk HPV type (16/18/31/33/35) probe for detection of HPV infection has been confirmed [12]. Furthermore, the prevalence of 16/18 type of HPV in atypical cases has been confirmed by the present pilot study. The 92.2% concordance in average between genotyping results and cytopathology findings of routine screening has been revealed. 7.8% cases of atypical cases were HPV negative; all these cases were diagnosed as ASCUS during LBC screening. It has been concluded, that severe inflammation as well as specific

inflammation (i.e., induced by *Candida* spp.) can be misdiagnosed. Taking into account the atypical cases follow up guidelines, it has been concluded, that shift towards HPV DNA based cervical cancer screening will be effective and appropriate. The increased costs of cervical cancer screening will be compensated by the higher specificity and prolonged screening intervals.

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