Human Pathology (2015) **46**, 1376–1381

ELSEVIER

Human PATHOLOGY

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Original contribution

High-risk human papillomavirus infection involving multiple anatomic sites of the female lower genital tract: a multiplex real-time polymerase chain reaction–based study☆,☆☆,★

Yiang Hui MD[a], Pradip Manna PhD[b], Joyce J. Ou MD, PhD[a], Spencer Kerley MD[b], Cunxian Zhang MD, PhD[c], C. James Sung MD[a], W. Dwayne Lawrence MD, MSc[a], M. Ruhul Quddus MD, MPhil (Path)[a,*]

[a]*Department of Pathology and Laboratory Medicine, The Warren Alpert Medical School of Brown University and Women & Infants Hospital, Providence, RI 02905*
[b]*Physicians Reference Laboratory, Overland Park, KS 66210*
[c]*Kent Hospital, Warwick, RI 02886*

Received 18 March 2015; revised 16 May 2015; accepted 21 May 2015

Keywords:
hrHPV;
Multifocal infection;
Multisubtype infection;
CIN;
VIN;
VAIN

Summary High-risk human papillomavirus infection usually is seen at one anatomic site in an individual. Rarely, infection at multiple anatomic sites of the female lower genital tract in the same individual is encountered either simultaneously and/or at a later date. The current study identifies the various subtypes of high-risk human papillomavirus infection in these scenarios and analyzes the potential significance of these findings. High-risk human papillomavirus infection involving 22 anatomic sites from 7 individuals was identified after institutional review board approval. Residual paraffin-embedded tissue samples were retrieved, and all 15 high-risk human papillomavirus were identified and viral load quantified using multiplex real-time polymerase chain reaction–based method. Multiple high-risk human papillomavirus subtypes were identified in 32% of the samples and as many as 5 different subtypes of high-risk human papillomavirus infection in a single anatomic site. In general, each anatomic site has unique combination of viral subtypes, although one individual showed overlapping subtypes in the vagina, cervix, and vulvar samples. Higher viral load and rare subtypes are more frequent in younger patients and in dysplasia compared with carcinoma. Follow-up ranging from 3 to 84 months revealed persistent high-risk human papillomavirus infection in 60% of cases.

1. Introduction

High-risk human papillomavirus (hrHPV) is associated with malignant and premalignant lesions of the female genital tract including vulvar (VIN), vaginal (VAIN), and cervical (CIN) intraepithelial neoplasia and squamous cell carcinoma (SqCa). hrHPV DNA can be found in 96% of

☆ Competing interests: Pradip Manna and Spencer Kerley work for Physician Reference Laboratory. Other authors declare no conflict of interest.

☆☆ Funding/support: No funding was obtained for this article.

★ The finding of this study was presented, in part, at the 104th annual meeting of the USCAP; Boston, MA; March 21-28, 2015.

* Corresponding author. Department of Pathology and Laboratory Medicine, Women & Infants Hospital, Alpert Medical School of Brown University, 101 Dudley St, Providence, RI 02905.
E-mail address: mquddus@wihri.org (M. R. Quddus).

http://dx.doi.org/10.1016/j.humpath.2015.05.022
0046-8177/

patients with cervical cancer [1]. More than 170 types have been identified [2], with cancer cases most commonly attributed to HPV types 16, 18, 31, 33, 35, 45, 52, and 58 [3]. The International Agency for Research on Cancer also considers HPV types 39, 51, 56, and 59 to be hrHPV. Furthermore, carcinogenesis attributable to phylogenetically similar types including types 68, 73, and 82 has been demonstrated molecularly [1,4].

High-risk HPV–infected dysplasia or carcinoma is usually noted in a single anatomic site of the female lower genital tract. Only rarely are lesions associated with hrHPV infection seen involving multiple anatomic sites in a single individual. Sometimes these infections are noted simultaneously, or at times the second site involvement is noted subsequently at a later date.

Infections with some subtypes such as HPV-31 are reported to have better prognosis than others [5,6]. Also, HPV-16 and HPV-33 appear to have the highest risk of progression to invasive disease [7]. Multiple hrHPV subtypes have been reported to be found within 1 lesion [8]. It is notable that multiply infected cervical SqCas show the same HPV genotypes from primary to metastatic lesions [9]. Although previous reports have suggested that infection with multiple subtypes may be associated with progression of cervical dysplasia [10], it does not appear to be prognostically significant in the setting of single lesions [6].

HPV carcinogenesis appears to be multifactorial and continues to be investigated. The virus infects basal cells of stratified squamous epithelium. Methylation of host tumor suppressor genes such as for p16 (*CDKN2A*) and suppression of p53 and Rb by HPV oncoproteins E6 and E7 contribute to genomic instability that facilitates tumorigenesis. HPV oncoprotein E5 augments the action of E6 and E7 by facilitating viral replication, cellular proliferation, and subversion of host defense mechanisms [11,12]. Although HPV integrates into the host genome, there are more than 190 reported integration loci indicating that this process is random [13]. Most infected individuals do not develop lesions. However, the risk of developing premalignant and malignant lesions is increased when infections persist for more than 1 year [14]. Furthermore, repeated detection of the same type of hrHPV is also associated with greater risk of developing higher-grade lesions [15].

Polymerase chain reaction (PCR) assays are well established in the detection of HPV and typically target the L1, E6, and E7 open reading frames, which are retained even after integration into the host genome. Multiplex real-time PCR assays were used to establish efficacy of the Gardasil vaccine and have been found to be comparable to the widely available linear array assay [16–18]. Type-specific detection of hrHPV using multiplex real-time PCR allows for detection of coinfection, monitoring recurrent infection, and determination of viral load [8]. Viral load of HPV-16 has been shown to correlate with the development of CIN-3 [19].

It is not well studied whether mutifocal HPV infection is caused by multiple subtypes of hrHPV infection or any particular subtype of hrHPV. Similarly, the role of high viral load in causing multifocal site of involvement is not known.

This study aimed to use this method to identify hrHPV subtypes involved in patients with multifocal lesions, quantitate viral loads in these patients, and provide clues to which subtypes may predominate in future with the advent of vaccination programs.

2. Materials and methods

Twenty-two samples, including high-grade squamous intraepithelial lesion (HSIL) of the uterine cervix (CIN), vagina (VAIN), vulva (VIN), and vulvar SqCa, from different anatomic locations from 7 patients were selected for this study. The patients' ages ranged from 22 to 81 years (mean, 43 years; median, 41 years), with HSIL or SqCa from patients with lesions of multiple anatomic sites encountered in the Department of Pathology from 2007 to 2013 retrieved after the institutional review board approval. Cases were examined based on morphologic criteria according to the World Health Organization classification of tumors [20]. Low-grade dysplasia of the cervix (CIN-1), vagina (VAIN-1), and vulva (VIN-1) were excluded from the study. Hematoxylin and eosin–stained sections were reviewed from each case to confirm the presence of the lesion of interest. Representative lesional tissue was obtained from the paraffin-embedded tissue blocks. Five to 10 tissue sections were obtained to extract DNA using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Valencia, CA; Cat No. 56404).

HPV testing was performed on the isolated DNA using the proprietary COMPLeTe Care HPV test (Physicians Reference Laboratory, Overland Park, KS), a multiplex real-time PCR assay. Four multiplex reactions targeting 4 hrHPV types with 8 μL of extracted DNA were performed using a LightCycler 480 instrument. This enabled detection of all 15 hrHPV types. Standard curves were generated using controls for each of the 15 hrHPV subtypes as well as an internal *β-globin* control for each run.

The hrHPV subtypes tested consisted of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82. The COMPLeTe Care HPV test (PRL, Overland Park, KS) uses the region of the HPV genome encoding for *E7*, which is required for oncogenesis and is stable upon integration into host chromosomes. The *β-globin* gene is simultaneously amplified and quantified by the assay as an internal control.

Absolute viral load and cell counts were calculated using the quantitative standards. Viral load and *β-globin* were determined based on the crossing point above the corresponding baselines in relation to the quantitative standards [8]. Viral loads were normalized to a cell count of 1000 cells. The quantification range of the assay is 10^{-1} to 10^{-6} copies/reaction, and the qualitative lower limit of detection is less than 10 copies/reaction. Copies less than 10 are qualified as "positive." The number of viral copies was then correlated with sites of lesions. The clinical follow-up of these cases when available was noted.