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New biomarkers of human papillomavirus infection in acute cervical intraepithelial neoplasia

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ABSTRACT

Acute human papillomavirus (HPV) infection of the cervix (cervical intraepithelial neoplasia, CIN) is marked by high copy episomal viral DNA and L1/L2 capsid protein expression (productive infection) in the cells towards the surface that facilitate sexual viral transmission. Viral DNA is low copy and not associated with viral capsid protein expression in the less differentiated lower part of the CIN (nonproductive infection). The purpose of this study was to examine the host response in these two areas. Serial section and co-localization analyses demonstrated that in 29/33 (88%) of cases the NF-κB pathway was activated and localized to the suprabasal nonproductively infected cells in the CIN lesions. There was a concomitant increased expression of importin-β, exportin-5, Mcl1, p16, Ki67 and cFLIP in 32/33 (96%) of CIN lesions that likewise localized primarily to the nonproductively infected cells. Only Ki67 and exportin-5 were expressed, though much less so, in the adjacent, normal squamous epithelia. The viral proteins E1^E4 and L1 were localized to productively infected cells whereas E6/E7 protein/RNA was rarely present in early CIN. It is concluded that the host viral response to acute cervical HPV infection includes strong increased expression of proteins besides p16 and Ki67. These include importin-β, exportin-5, Mcl1, and cFLIP in cells with low copy and relatively quiescent viral DNA that, in turn, may serve as new biomarkers of this disease.

1. Introduction

Human papillomavirus (HPV) is the cause of cervical intraepithelial neoplasia (CIN) that is among the most common of the sexually transmitted diseases [1-5]. Both screening (Pap smear) and definitive diagnosis (cervical biopsy) of CIN rely on cytologic/histologic changes that include perinuclear halos, a disorganized cellular growth pattern, and variability in nuclear size/shape/chromaticity that show overlap with reactive, non-sexually transmitted conditions (1–5). Since CIN is a venereal disease and an obligatory prelude to cervical cancer, much effort has been directed to improve the diagnostic accuracy of CIN lesions in both Pap smears and cervical histopathology [1-5].

Over 90% of CIN lesions arise in the transformation zone since the virus has a strong predilection to infect the basal cells in areas of squamous metaplasia [1-5]. These infected basal cells typically contain 1–20 copies of episomal viral DNA that can be detected by PCR in situ or ultrasensitive in situ hybridization assays [3]. In acute HPV infection, viral DNA copy number increases dramatically as the cells mature with concomitant expression of ORFs E1, E2, and E4 that are involved in viral DNA replication and transfer of the viral genome to progeny cells. Towards the mucosal surface ORFs L1 and L2 are highly expressed which is essential for production of infectious virions [1-5]. ORF E2 also attenuates expression of the HPV oncoproteins ORF E6 and E7, which in turn, degrade the tumor suppressor p53 and retinoblastoma protein family, respectively, and thus are essential for the evolution to cervical cancer [1-5].

Cells often use nuclear trafficking of RNAs and proteins to repress or activate constitutively expressed proteins such as p53 and NF-κB [6-9]. Key proteins in nuclear transport include importin-β and exportin-5 [10,11]. Many viruses use importins and exportins to facilitate their infectivity [12-15]. The HIV-1 protein Rev. is essential for infectivity as its binding to importins and exportins is required for transport of late

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structural mRNAs of HIV-1 from the nucleus to the cytoplasm [12,13]. The protein ICP27 encoded by herpes simplex virus-1/2 is required for nuclear export of several viral mRNAs that is a prerequisite for infectivity [14]. Nuclear trafficking is important in HPV infection since the viral E6 protein cannot degrade p53 if the nuclear export of the p53 via exportin-5 is blocked [16]. Viruses, including HPV, have also developed various molecular mechanisms to limit host cell immune surveillance and caspase-mediated apoptosis that often involve viral modulation of the NF-κB pathway [7-9,17-19].

The purpose of this manuscript was to analyze the cellular response in the cervix to acute HPV infection. By using an ultrasensitive in situ hybridization assay, the less differentiated, low viral DNA copy number positive (nonproductive) cells in the suprabasal zone of the lesion could be differentiated from the terminally differentiated, high viral DNA copy number and capsid protein L1 positive (productive) cells in the subapical zone.

2. Materials and methods

2.1. Cervical tissues

Formalin fixed, paraffin embedded tissues were available from the files of Folio and Enzo Clinical Laboratories. Thirty-three cervical biopsies diagnosed as acute CIN were studied, together with seventeen aged matched controls that were biopsies of transformation zones with no histologic evidence of CIN taken because of an ASCUS Pap smear. The CIN diagnoses were confirmed on hematoxylin and eosin stains by a gynecologic pathologist (GJN) from serial sections used for the immunohistochemistry and in situ hybridization analyses. The diagnosis of acute CIN infection was based on: 1) well defined and frequent koilocytes towards the surface of the lesion; 2) high viral DNA copy number in these cells; 3) strong expression of L1 and E1^E4 protein in cells towards the surface of the lesion. Most of these lesions would be defined as CIN 1 or CIN 1 with areas consistent with CIN 2. Likewise, the seventeen histologically normal cervical biopsies were all reviewed by a gynecologic pathologist prior to use, and each was documented to be HPV DNA negative by in situ hybridization.

2.2. Organotypic raft cultures

Existing tissue from the following HPV16 positive and negative organotypic epithelial rafts was utilized. These were generated as described in [22,23]. Near diploid immortalized keratinocytes that form skin (NIKS) and HPV16 + NIKS were carried as previously described, with irradiated J2-3T3 mouse fibroblasts used as feeder cells [20,21]. Subsequently, a total of 1×10^6 human keratinocytes was plated on a collagen matrix that harbored embedded fibroblasts. Exposure to the liquid-air interface resulted in the generation of stratified epithelium with differentiation properties that reflect human epidermis. Rafts were harvested 14 days after lifting, fixed in 4% paraformaldehyde, and embedded in paraffin. Four micrometer sections were stained with hematoxylin/eosin and also tested for the relevant proteins by immunohistochemistry.

2.3. Cell lines

C33a (HPV DNA negative) and SiHa (1 integrated copy HPV 16/cell) cells were grown as previously described, and fixed in 10% neutral buffered formalin for 8–15 h before processing for immunohistochemistry or in situ hybridization [24].

2.4. In situ hybridization

HPV DNA in situ hybridization was done using a previously published protocol that can detect HPVs 6, 11, 16, 18, 30, 31, 33, 35, 42, 43, 44, 51, 52, 56, 68, 70, and other novel types [3,24-27]. In brief, after protease digestion, genomic HPV probes labeled with biotin were used as per the manufacturer's recommendations (PATHO-GENE AP HPV in situ screening and typing assays, Enzo Life Sciences HPV in situ kit, Farmingdale, NY, Enz-32884). The chromogens nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate yields a blue signal with nuclear fast red as the counterstain. These results were then compared to an ultrasensitive HPV in situ hybridization kit from Enzo Life Sciences, in which the probes are “hyperbiotinylated”; these are the PATHO-GENE PLUS HPV probes (Enz-32889).

HPV 16/18 E6/E7 RNA was detected by in situ hybridization using the ACD kit (ACD, Newark CA) as per the manufacturer's protocol.

2.5. Immunohistochemistry

Our immunohistochemistry protocol has been previously published [24-27]. The cervical biopsies were tested for the following antigens: activated caspase-3, bcl2, BAD, BIM, cFLIP, HPV 16 E1^4 protein, HPV 16/18 E6 protein, importin-β, exportin-5, NF-κB p65, bclX (Abcam, Cambridge MA), BAX, BAK, Mcl1 (Enzo Life Sciences, Farmingdale, NY), and HPV consensus protein L1 (Biocare, Pacheco, CA). The analyses were done on the automated Leica Bond platform with the modification that the Enzo Life Sciences peroxidase anti-mouse/rabbit conjugate (catalogue # ADI-950-113-0100) was used in place of the equivalent Leica conjugate as this reduced background.

2.6. Co-expression analysis

Co-expression analyses were done using the Nuance system (CRI) as previously published [24-26]. In brief, a given tissue was tested for two different antigens using fast red as the chromogen for one target followed by immunohistochemistry using DAB (brown) as the second chromogen with hematoxylin as the counterstain. The results were then analyzed by the Nuance and InForm systems where each chromogenic signal is separated, converted to a fluorescence based signal, then mixed to determine what percentage of cells were expressing the two proteins of interest.

3. Results

3.1. H&E results with HPV DNA by in situ hybridization

The 50 biopsies (33 acute CIN lesions and 17 cervical tissues negative for CIN) were first examined by hematoxylin and eosin stain using sections that were adjacent to those used for in situ hybridization and immunohistochemistry. Blinded pathology analysis confirmed twenty CIN1 (Fig. 1A) and thirteen CIN 1 lesions with foci of CIN 2, and 17 cervical biopsies that lacked the histologic features of CIN. HPV in situ hybridization was done using the Enzo PATHO-GENE assay on all 50 biopsies. Across samples, 30/33 (90%) of the early CIN lesions were positive for HPV DNA using the HPV DNA in situ assay (Fig. 1B). Note that high copy numbers of HPV DNA localized preferentially to the differentiated cells in the subapical zone of early CIN (Fig. 1B), and HPV DNA was not detected in the suprabasal layers in less differentiated squamous cells (Fig. 1B). Each of the 17 control tissues was negative for HPV DNA as was the histologically unremarkable squamous lining adjacent to the CIN (Fig. 1D). Immunohistochemistry analysis for viral ORF expression showed strong signals in each CIN for the HPV consensus capsid protein L1 that showed the same distribution as the high copy HPV DNA (Fig. 1E) and in the HPV 16+ cases for HPV E1^4 protein (Fig. 1F). HPV16/18 E6 protein was not detected in any of the CIN lesions that contained HPVs 16 or 18, though the HPV 16/18 E6 protein was found in each of five HPV 16+ cervical squamous cell cancers that served as positive controls (data not shown).

HPV typing using individual genomic probes [5,24] demonstrated that most of the lesions contained high risk HPV DNA. Specifically, only 3/30 of the early CIN lesions (10%) contained HPV 6/11 which is