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Original Contributions

A comparison of the detection of biomarkers in infections due to low risk versus high-risk human papillomavirus types

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ABSTRACT

Adjunctive immunohistochemistry tests for human papillomavirus (HPV) infection include p16 and Ki67 as well as the more recently discovered biomarkers importin-β, exportin-5, Mcl1, and PDL1. The purpose of this study was to compare the expression of these biomarkers in HPV infection due to the high-risk types such as HPVs 16, 18, 31, 33, 35, and 51 versus lesions that contain the low risk types HPV 2, 6 or 11. We studied 35 lesions with low risk HPV types (verruca vulgaris = 10 cases, condyloma acuminatum = 15 cases, CIN 1 with HPV 6/11 = 10 cases) and 25 CIN 1 or 2 lesions with a high-risk HPV type. The 25 high-risk positive CIN 1–2 cases had strong expression of the panel p16, Ki67, importin-β, exportin-5, Mcl1, and PDL1 where each protein localized to the cells in the parabasal aspect of the lesion. In comparison, neither p16, importin-β, exportin-5, Mcl1, nor PDL1 were increased in the epithelia of the lesions with the low risk HPV types; Ki67 showed variable expression. HPV viral capsid L1 protein and viral DNA were excellent markers of infection in the lesions with low risk types. Thus, p16, importin-β, exportin-5, Mcl1, and PDL1 are not only biomarkers of high-risk HPV infection but can also differentiate such lesions from those that contain low risk HPV types. Low risk HPV infections can be best differentiated from their mimics by viral L1 capsid detection and/or HPV DNA by in situ hybridization.

1. Introduction

Human papillomavirus (HPV) is one of the most common sexually transmitted diseases [1-5]. It is estimated that over 6 million new cases of HPV infection of the cervix, vulva, penis, and oral cavity occur in the United States each year. Non-sexually spread HPV infection, most commonly manifested as verruca vulgaris, is frequent with over 3 million new cases each year in the United States. Over 150 HPV types have been identified, based on having < 50% homology to each of the known HPV types, with about 40 types able to cause disease in humans [1-5].

HPV infection is an obligatory prerequisite for cervical cancer. Importantly, although many HPV types have been shown to be able to induce squamous cell cancer, other types completely lack this ability [1-5]. The latter “low risk” HPV types includes HPV 2, the most common type in verruca vulgaris, and HPVs 6 and 11, which are the cause of vulvar/penile condyloma acuminatum, and also are responsible for about 20% of CIN 1 (LGSIL) lesions. In comparison, the high-risk for cancer types include HPVs 16, 18, 31, 35, 51 as well as HPVs 5 and 8. The latter two types are the most common in squamous cell cancer that evolves from epidermodysplasia verruciformis [6-10]. Much work has focused on elucidating the molecular differences between the low risk and high-risk HPV types. Although much of this attention has focused on the HPV open reading frames E6 and E7, which can transform squamous cells only from high-risk types, it is clear that there are other molecular events that are important in allowing high-risk types to play a key role in oncogenesis [2,4].

Treatment of HPV lesions on the skin usually involves either topical salicylic acid and/or cryotherapy. In each case the proposed mechanism is death of viral containing cells that, in turn, induces a viral clearing immune response. Although success rates vary considerably, in at least 50% of cases the lesion will recur [11]. For cervical HPV disease, laser ablation has long ago surpassed cryotherapy as the standard treatment with success rates typically at least 80%. Interestingly, recurrent CIN lesions in immunecompetent women invariably contain a different HPV type from the primary lesion, suggesting that a given woman will develop a type specific immunity after treatment [1-5]. This is in part the basis of the highly successful HPV vaccine that has shown protective rates of over 95% for the specific HPV types found in the given formulation.

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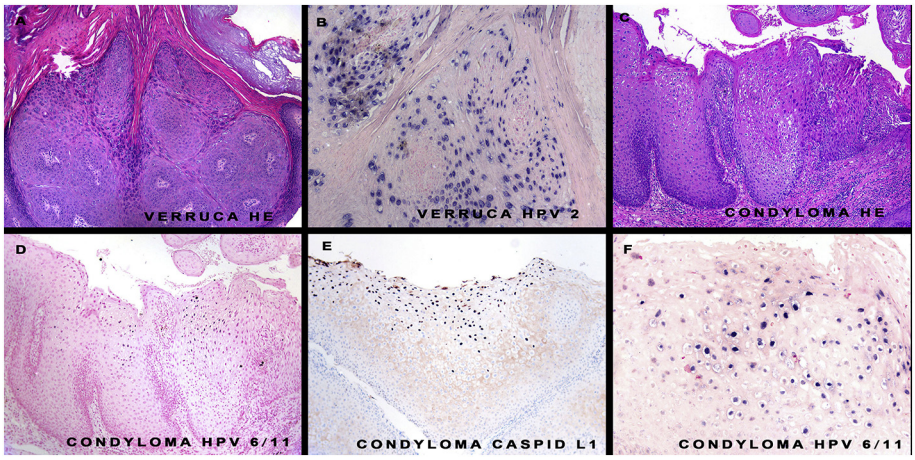

Fig. 1. Histology and molecular detection of condyloma and verruca vulgaris. Panel A shows the acanthosis, rare perinuclear halos, and marked hyper/parakeratosis typical of a verruca vulgaris. The infectious virus, which are most abundant towards the surface, number in the hundreds/infected cell and, thus, are easily detected by in situ hybridization for HPV 2 (panel B). Similarly, a vulvar condyloma (panel C) on H&E exam shows marked acanthosis and many varying sized perinuclear halos that contain HPV 6/11 DNA (panel D). Verruca/condyloma contain large amounts of infectious virions based on the strong signal seen in many cells for the L1 capsid protein (panel E) and, in the serial section, HPV DNA (panel F).

The histologic diagnosis of HPV infection, whether verruca vulgaris, condyloma acuminatum, or CIN, has a relatively high false positive and false negative rate [1-5]. All acute HPV infections demonstrate cell crowding, perinuclear halos, and varying degrees of nuclear atypia that can be mimicked by many non-viral conditions. Thus, there has been much effort to develop biomarkers of HPV infection to improve diagnostic accuracy.

The purpose of this manuscript was to compare the host response to low risk versus high-risk HPV types by studying the classic biomarkers, p16 and Ki67, as well as newer biomarkers that include importin-β, exportin-5, Mcl1, and PDL1.

2. Materials and methods

2.1. Formalin fixed, paraffin embedded tissues

Formalin fixed, paraffin embedded tissues were available from the files of the Ohio State University Medical Center and Phylogeny Medical Laboratory. Thirty five skin biopsies with active HPV disease diagnosed as either verruca vulgaris (10 cases, each from the face or digits), condyloma acuminate (15 cases from the vulva, penis, or perianal area) or CIN 1 (LGSIL) (10 cases with documented HPV 6/11 infection) were available for study. Twenty-five cases of CIN 1 or 2 (LGSIL or HGSIL) in which high risk HPV types had been identified by in situ hybridization were studied for comparison. The age range/mean age for the cases with low risk HPV infection was 25 to 73 with a mean of 42 years old as compared to high risk HPV type infection where the age range was 21 to 45 with a mean of 37 years old. The negative controls included: omission of the primary antibody, use of a rabbit IgG clone, and histologically normal skin/cervical mucosa adjacent to the lesion. We also studied 15 cases from the vulva that were deemed “equivocal for condyloma” based on the histologic findings that were suggestive but not diagnostic of the disease.

2.2. In situ hybridization

HPV DNA in situ hybridization was done with a variety of individual probes that can detect HPV 1,2,5,7,8, 13, and 57 as previously reported [5,10,12] that are types associated with skin and not genital tract lesions. Also used was the HPV “genital tract high risk consensus probe” from Enzo Life Sciences (Farmingdale, NY) that can detect over 20 different HPV types that includes HPVs 16, 18, 30, 31, 33, 35, 45, 51, 58, and 70 as well as the HPV “low risk” 6/11 probe from the same company. In brief, after protease digestion, the genomic HPV probes labeled with biotin were co-denatured with the tissue DNA, hybridized for 15 h, washed at intermediate stringency, and then detected with using the HPV in situ hybridization kit from Enzo. The chromogens nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate yields a blue signal with nuclear fast red as the counterstain. The ultrasensitive hyper-biotinylated HPV 6/11 probe from Enzo was also used.

2.3. Immunohistochemistry

Our immunohistochemistry protocol has been previously published [5,10,12-14]. The biopsies were tested for the following antigens: Ki67, p16, importin-β, exportin-5, PDL1 (ABCAM, Cambridge MA), Mcl1, (Enzo Life Sciences), and HPV consensus capsid 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 was used instead of the Leica HRP polymer (catalogue # ADI-950-113-0100) as this reduced background [15].

3. Results

3.1. Documentation of the HPV-related diagnosis by H&E stains that were serial sections to those used for biomarker analysis

The 60 biopsies (verruca vulgaris = 10, condylomata = 15, CIN 1 = 25, and CIN 2 = 10) were examined by hematoxylin and eosin (H&E) stain to confirm the original pathology diagnosis; these were serial sections to those used for the immunohistochemistry and in situ hybridization. H&E analysis did confirm that 10 lesions from non-genital sites were verruca vulgaris based on marked hyperplasia with hyper and parakeratosis associated with perinuclear halos in the granular cell region (Fig. 1). Similarly, the 15 condylomata from the vulva, anus, or penis each showed marked acanthosis associated with prominent perinuclear halos that varied in size and shape (Fig. 1). The 35 CIN lesions each showed the cellular crowding, nuclear atypia, and variable sized perinuclear halos diagnostic of the disease (Fig. 2).

3.2. HPV in situ hybridization confirmation of low risk versus high-risk viral types

The 60 biopsies were then tested by in situ hybridization to document the specific HPV type present in a given lesion. Probes used included biotin labeled genomic probes specific for HPV 6/11, HPV 2, and a “consensus” high- risk probe cocktail. For the lesions positive with the latter probe cocktail, additional in situ hybridization was done using the individual probes for HPVs 16, 18, 31, 33, 35, and 51.

Each of the 10 verruca vulgaris lesions was positive for HPV 2 whereas each of the 15 condylomata was positive for HPV 6 or 11. Representative images are shown in Fig. 1. Note that the viral signal was typically intense, indicative of many hundreds of viruses per

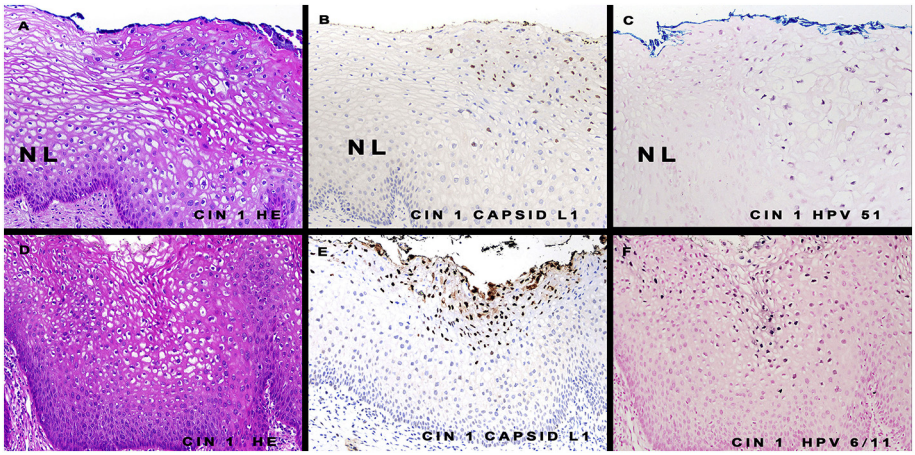

Fig. 2. Histologic and viral features of CIN 1 associated with low risk versus high-risk HPV types. Panels A and D show the H&E findings of two different CIN 1 lesions where each show equivalent acanthosis, cell crowding, variably sized perinuclear halos and nuclei that vary in size, shape, and chromaticity. The lesion in panel A shows the strong demarcation between the normal cervix (NL) and CIN. Note that the lesion is A contains the high-risk HPV 51 (panel C) whereas the other lesion contains HPV 6/11 (panel F). Since both lesions represent the acute phase of HPV infection with high copy viral DNA, they also contain many infectious virions as seen with the immunohistochemistry test for the L1 capsid protein (panels B and E, respectively).

infected cell [2,12]. These infections produce infectious virions and, indeed, the immunohistochemistry test for viral capsid protein L1 was strongly positive (Fig. 1). Note the similar distribution between the viral DNA and capsid protein that is an indicator of the specificity of each test. Finally, note that viral infected cells in the verruca and condylomata were typically not detectable towards the basal part of the lesion.

Thirty-five cervical lesions with the diagnosis of CIN 1 (25 cases) or CIN 2 (10 cases) were tested for HPV DNA using the HPV 6/11 probe and the high risk viral probe cocktail. In situ hybridization showed that 10 of the CIN 1 lesions contained HPV 6/11 whereas each of the other 25 CIN cases was positive with the high-risk HPV probe cocktail. Of the CIN lesions with high risk types, eight contained HPV 16, three HPV 18, five HPV 31, one HPV 33, one HPV 35, four HPV 51, and three lesions contained other high risk HPV types as each lesion was negative for the HPV 6/11 probe. As evident in Fig. 2, one could not differentiate the CIN 1 lesions that contained either HPV 6/11 from those which contained a high risk HPV type based on the histologic findings. Fig. 2 also demonstrates that these lesions contained many infectious virions given that abundant L1 capsid protein was detected in the same distribution as the viral DNA.

3.3. Correlation of low risk versus high-risk HPV types with immunohistochemistry detection of host proteins that served as potential biomarkers

We had previously shown that the host response to the high risk HPV infection in CIN lesions included a marked over-expression of Ki67 and p16 as well as other novel biomarkers that included Mcl1, PDL1, importin-β, and exportin-5 [5]. To further corroborate this finding, we studied 25 CIN lesions with high-risk HPV types for each of these biomarkers. The epithelia of the histologically normal areas and the normal cervical controls were negative for p16, importin-β, Mcl1, and PDL1 and showed weak expression of Ki67 and exportin-5 that in each case localized to the basal cells. Importin-β was evident in the smooth muscle of the larger blood vessels and Mcl1 was present in scattered mononuclear cells in the dermis/submucosa that served as internal controls. Each of the 25 CIN lesions that contained high-risk types was indeed strongly positive for p16, Ki67, importin-β, exportin-5, and Mcl1 (Fig. 3); PDL1 was evident in 21/25 lesions. Note that each biomarker was present in the cells towards the base of the CIN lesion and not in the cells towards the apical aspect which, as noted in Fig. 2, typically contained the proliferating viral DNA, as previously reported [5].

We next compared the data from the high-risk CIN HPV lesions to the 10 CIN lesions with low risk HPV types. Neither p16, importin-β, Mcl1, nor PDL1 was evident in the epithelia of the CIN 1 lesions that contained HPV 6/11 (Fig. 3). Exportin-5 was detected in these CIN 1 lesions but only in the basal cells, as in the baseline adjacent normal tissue. Ki67 showed a variable distribution, at times only present in the basal cells though more commonly it was also evident in cells in the middle aspect of the lesion (Fig. 3).

Next, the distribution of these biomarkers was analyzed in the verruca vulgaris and condylomata. Neither p16 (Fig. 4), importin-β, Mcl1, nor PDL1 was evident in the epithelia of the verruca or condylomata. Exportin-5 was at times seen in scattered basal cells of these lesions but in a distribution equivalent to baseline. Ki67 showed a very variable distribution at times located in basal cells of the lesion, usually in lesions with few cells with detectable viral DNA, and in most cases in cells towards the middle part of the lesion, commonly when many viral infected cells were evident in the lesion.

3.4. Differentiation of verruca vulgaris and condylomata from their mimics

Thus, the data strongly suggested that detection of p16, importin-β, exportin-5, Mcl1, or PDL1 could not be used to diagnose lesions with low risk HPV infections or differentiate them from their mimics. Fig. 1 did demonstrate how these lesions typically contain high numbers of virions per infected cell which would allow in situ hybridization for HPV 2 (verruca) or 6/11 (condyloma) or immunohistochemistry for L1 capsid protein (either lesion since a “consensus” capsid protein was used) to detect such lesions. In this regard, we studied 15 vulvar lesions deemed equivocal for a diagnosis on condyloma based on the histologic findings. Immunohistochemistry for the L1 capsid protein did demonstrate that 3/15 of these lesions were positive in cells towards the surface. In situ hybridization for HPV 6/11 DNA demonstrated that the same 3 lesions were viral DNA positive (Fig. 4). Thus, it was concluded that the latter three lesions were indeed condylomata and the other twelve were not induced by HPV infection. The ultra-sensitive hyperbiotinylated HPV 6/11 probe from Enzo Life Sciences gave similar data to the standard probe from the same company, indicating that the viral copy number in these cases tended to be high (data not shown).

Fig. 4 also demonstrates that the biomarkers can assist in differentiating low grade from high-grade vulvar/penile/perianal lesions. On histologic examination of several vulvar lesions, both condyloma and VIN 1–2 were considered. The biomarkers p16 (Fig. 4), importin-β, exportin-5, Mcl1, and PDL1 were each negative in these cases. HPV capsid L1 protein and HPV 6/11 DNA in situ hybridization were positive in the same distribution (Fig. 4). Thus, the lesions were condylomata and not VIN.

4. Discussion

The main finding of this study is that HPV types that can induce malignant change, such as HPVs 16 and 18, elicit a wide-ranging host response that includes the biomarkers p16, Ki67, importin-β, exportin-5, Mcl1 and, to a lesser extent, PDL1. However, the HPV types that

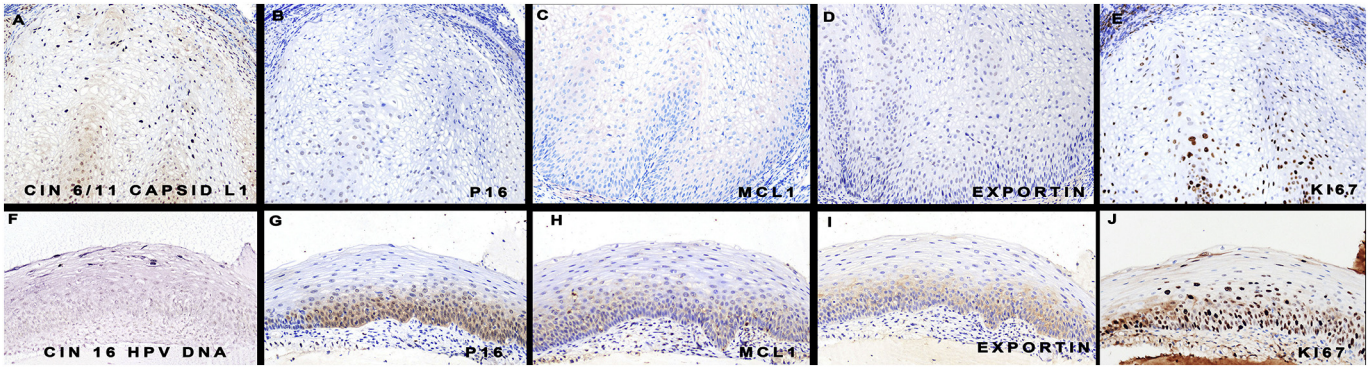

Fig. 3. Variable expression of a panel of biomarkers in low risk versus high-risk HPV CIN infections. Panel A shows the strong expression of the L1 capsid protein in a CIN 1 lesion that contains HPV 6/11. Note that the lesion does not express p16 (panel B), Mcl1 (panel C), or exportin-5 (panel D); importin-β and PDL1 were likewise not expressed (not shown). In comparison, the serial sections of the CIN 1 lesion that contains HPV 16 DNA (panel F) show strong expression towards the parabasal layer of p16 (panel G), Mcl1 (panel H), exportin-5 (panel I) as well as importin-β and PDL1 (not shown). Each lesion showed Ki67 expression in cells above the basal layer (panels E and J, respectively).

cannot induce malignant change, such as HPV 2 (verruca vulgaris), and HPVs 6/11 (condylomata) do not induce these biomarkers, although Ki67 expression can be suprabasal in these lesions, especially when viral DNA synthesis is robust. However, infections due to HPVs 2, 6, and 11 usually have higher viral copy numbers and more virions when compared to the high-risk HPV types. The diagnostic implications of this work for the surgical pathologist are two-fold: 1) HPV DNA in situ hybridization and/or L1 capsid protein detection by immunohistochemistry are the preferred methods to differentiate low risk HPV infections from their mimics; 2) detection of the biomarker panel p16, Ki67, importin-β, exportin-5, and/or Mcl1 by immunohistochemistry is an excellent way to differentiate high risk HPV infections from their mimics or from low risk infections.

The study of the molecular differences between high and low risk HPV types viz-a-viz their ability to induce oncogenesis has focused mostly on open reading frames E6 and E7 for HPVs 16 and 18 [1-4]. These oncoproteins can inactivate the tumor suppressor proteins p53 and retinoblastoma protein, respectively. The viral ORF E2 down-regulates E6 and E7 expression. Thus, the central event in regard to ORF E6/E7 overexpression as a prelude to oncogenesis is viral integration into the host genome that disrupts viral ORF E2. Low risk HPV types are not able to integrate into the host genome.

HPVs 5 and 8, which cause epidermodysplasia verruciformis and have much more malignant potential than HPVs 16 and 18, have E6 and E7 ORFs that have functions not evident with HPVs 16 and 18 [6-10]. For example, HPVs 5/8 E6/E7 ORFs can inhibit the tumor suppressor NOTCH [9], hyperphosphorylate EGFR in conjunction with UV light [8], and inhibit caspase-14 mediated apoptosis [16]. Thus, the mechanisms by which the high-risk HPV types can induce oncogenesis are complex and vary to a degree among the different subtypes. Although more needs to be discovered to fully understand why only high risk and not low risk HPV types can induce oncogenesis, the data from this study shows another fundamental difference between these two key subgroups of HPV. High-risk HPV types induce a strong host response in the cells towards the basal aspect of the lesion that includes the proteins p16, Ki67, importin-β, exportin-5, Mcl1, and PDL1. Interestingly, these proteins are made in cells with either no detectable viral DNA/RNA/proteins or low copy viral DNA which are beneath the cells with the relatively high copy viral DNA and several viral RNAs and proteins [5,10]. Low risk HPV types lack the ability to induce these proteins. The reasons for this marked disparity await further research.

Few previous studies have investigated the use of biomarkers in differentiating high from low risk HPV driven lesions [17-19]. Most have relied on PCR typing which can easily differentiate low risk from high risk HPV types, although technical challenges and resource limitations render such testing impractical for use in most clinical

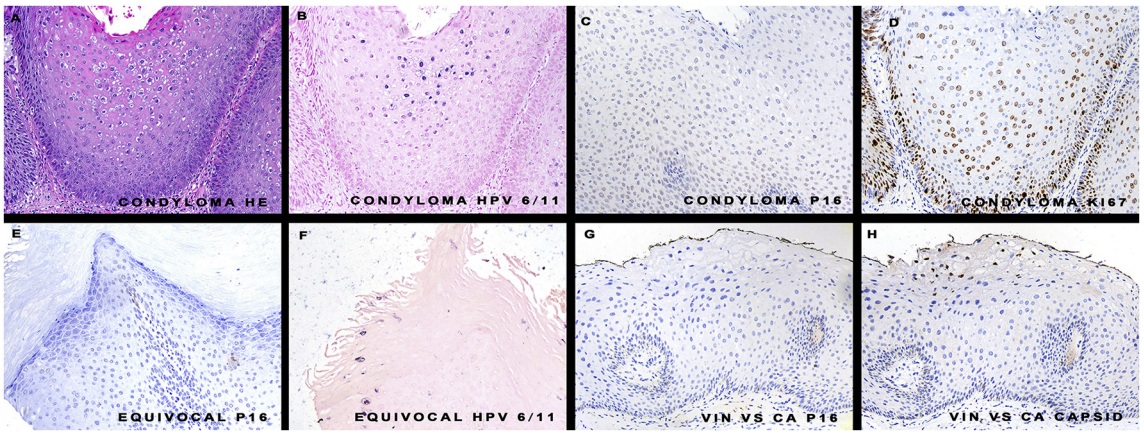

Fig. 4. Utility of immunohistochemistry and in situ hybridization for equivocal vulvar lesions. Panel A show an unequivocal vulvar condyloma that contains abundant HPV 6/11 DNA towards the apical part of the lesion (panel B). Note that the serial section lacks p16 expression (panel C) and shows increased Ki67 typical of such lesions that contain many virions (panel D). Panel E shows a vulvar lesion equivocal for a condyloma; the negative p16 test (panel E) and cells positive for HPV 6/11 DNA (panel F) allow the diagnosis of condyloma. Panel G shows a lesion in which the differential diagnosis included condyloma and VIN. The negative p16 test (panel G) and the strongly positive capsid antigen assay (panel H) confirmed the diagnosis of condyloma; HPV 6/11 was detected by in situ hybridization (not shown).

laboratories. Immunohistochemistry testing can discriminate normal tissue from low grade and high grade lesions, yet studies have not investigated their use in differentiating between low and high risk HPV infected tissues. For example, Ki67 is invaluable in identifying cell proliferation among condylomatata and CINs/SILs, but it is a poor marker for distinguishing low versus high risk HPV as seen in this study [17]. p53 has been highly variable across studies in terms of differentiating high and low risk HPV infections [18,20].

Cases to rule out HPV infection are seen many times each day in any busy dermatopathologist or gynecologic pathologist setting. The diagnostic surgical pathology laboratory typically offers a few adjunctive tests to assist in this regard: HPV DNA in situ hybridization, p16 and Ki67 immunohistochemistry. Although useful, a main focus of this current manuscript is that these tests have limited applications in the diagnostic setting. If the diagnostic issue is verruca vulgaris or genital tract condylomata versus a viral negative mimic, then p16 and Ki67 will not be of use. However, in situ hybridization for HPV 2 (verruca) or HPVs 6/11 (condylomata) should be definitive since each virus produces hundreds of copies/infected cell and these types cause > 95% of the respective lesion [2,4,5]. Immunohistochemistry for the viral capsid L1 protein is also an excellent way to separate the HPV positive from viral negative mimics which is clinically more important for condylomata given that this is a sexually transmitted disease [1-5].

Similarly, if the issue is verruca/condylomata versus a high grade HPV lesion, then the biomarkers p16 as well as importin-β, exportin-5, and Mcl1 can make this distinction. More commonly, the diagnostic issue is between high risk HPV infection (e.g. VIN, Bowen's disease, CIN 2–3, AIN) and a viral negative mimic. In these circumstances, it can be argued that immunohistochemistry for p16, Ki67, importin-β, exportin-5, and Mcl1 much improves the specificity of the testing, especially given the well-documented background issues with p16 and Ki67 [15]. If each test gives a positive result in the parabasal region of the lesion and the adjacent, histologically unremarkable tissue is negative, then the lesion is a high grade and not a mimic. Clearly, the converse is true if the biomarkers are negative.

The situation is slightly more complicated with CIN 1 lesions because about 15% will contain HPVs 6/11 [2,4]. Thus, about 85% of CIN 1 lesions will be positive for p16, Ki67, importin-β, exportin-5, and Mcl1. Since CIN 1 lesions usually do produce high copy infectious virions, in situ hybridization for HPV DNA using a consensus probe that can detect most of the 20 types associated with these lesions is a logical way to differentiate CIN 1 from its mimics, as is immunohistochemistry using a consensus capsid L1 antibody. In the United States the clinical treatment for CIN 1 is the same regardless of HPV type and, thus, it is unusual for the surgical pathologist to be asked to differentiate CIN 1 lesions with low risk types from those with high risk types. Such a distinction could be made with either in situ hybridization for the low risk and high-risk types and/or the panel of biomarkers including p16, importin-β, exportin-5, Mcl1, and PDL1.

Declaration of Competing Interest

There is no duality of interests to declare.

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