Tech Sample

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During a routine gynecologic examination of an asymptomatic 26-year-old woman, a small, flat, cervical lesion was noted. Colposcopy of the cervix was performed, and a biopsy specimen was sent for histologic examination. Routine H&E staining showed numerous clear cells in the cervical epithelium but no dysplasia.

Questions to be considered:

1. What is the significance of the clear cells in the cervical epithelium?
2. What other techniques may be used to confirm the presence of the human papillomavirus (HPV)?
3. What is the significance of the type of HPV found in lesions of the cervix?

DETECTION, IDENTIFICATION, AND TYPING OF THE HUMAN PAPILLOMAVIRUS IN INFECTIONS OF THE CERVIX

Learning Outcomes

Upon completion of this exercise, the participant should be able to

- *identify the first indicators of human papillomavirus infection in cervical tissue.*
- *perform further techniques to confirm the identity of the virus.*
- *discuss the clinical significance of the virus type in the management of the patient's condition.*

Identification and typing of human papillomavirus (HPV) infection in genital lesions is of paramount importance for effective patient management. HPV types may be low risk (HPV 6 and 11) or high risk (HPV 16 and 18) for progression to malignancy, and the virus can be demonstrated in the laboratory by various means. Cervical tissue may be fixed in formaldehyde and processed to paraffin using in-house methods. Sections of these lesions stained with hematoxylin and eosin usually show the presence of koilocytes (clear cells) in the epithelium. Koilocytes are pathognomic for HPV and are generally regarded as the result of preceding events in virus replication. The keratinization process of the epithelium is a prerequisite for the formation of viral particles, and the synthesis of viral protein is dependent upon the replication of the viral DNA. By using an antiserum to the major capsid protein, immunostaining can be carried out on conventional paraffin sections to detect the presence of the virus. In benign lesions, the viral DNA remains episomal and the antigen can be easily detected using this method. In malignant lesions however, the viral DNA usually integrates into the host DNA, thereby disrupting the genes that are responsible for protein production. Consequently, the protein will not be synthesized in sufficient quantities and immunostaining will not be able to detect it. Viral DNA is present in infected cells irrespective of the severity of the lesion. Molecular in situ hybridization methods, in conjunction with polymerase chain reaction (PCR) amplification methods, can be used to demonstrate HPV DNA in paraffin sections using type specific DNA probes. In this way, the identification of those women at risk of developing cervical cancer can be ascertained and effective patient management carried out.

Detection of HPV

The infection of the squamous epithelium of the genital tract by different types of HPV produces a wide spectrum of morphological phenomena, resulting in clinical, subclinical, or latent infection.[1] *Clinical HPV infection* is defined as any lesion that causes symptoms or is visible to the naked eye. *Subclinical infection* does not cause symptoms and can be diagnosed only with technical aids, such as a colposcope. *Latent HPV infection* is associated with no morphologic abnormalities and can be detected only by using virologic methods. All three manifestations may occur and recur during a given infection. Colposcopically, the criterion for abnormal epithelium is leukoplakia, a detectable white area on the cervix after the application of acetic acid. Cytologically, the presence of koilocytes (koilocytosis) is pathognomonic for HPV infection. Koilocytes are clear cells that are found in the squamous epithelium and are readily identified in paraffin sections of cervix by staining with H&E (**Slide 1**). These viral changes also can be recognized in cervical smears.

Squamous epithelium is the body's first line of defense, and has evolved to be a hostile environment for invading pathogens. It is here that HPV succeeds in establishing and maintaining infections by first entering a basal cell, presumably through a breach in the superficial layer. The infected cell must not be one that is already committed to maturation or the infecting virus will simply be shed from the surface. Instead, the cell must be actively dividing, allowing the virus to spread and persist. The presence of the virus causes abnormal cellular maturation in the form of genital warts (condylomata acuminata), that may be visible on the external genitalia (low-risk HPV types) or as small flat plaques on the cervix (high-risk HPV types).[2]

The earliest indication that HPV was related to cervical infection was the description of superficial cells showing perinuclear clear zones with small hyperchromatic nuclei and peripheral cytoplasmic condensation in patients with cervical dysplasia and carcinoma.[3] These clear cells are degenerating cells destined to die or be shed from the epithelium as a result of HPV infection. These cells were first described in 1933 by Papanicolaou[4] and showed the same characteristics as the clear cells (termed koilocytes by Koss and Durfee[5]) that are now known to be specific for HPV infection. Although the largest number of viral particles are found in the nuclei of koilocytes in the superficial layers of the epithelium, not all koilocytes contain virus. This suggests that the koilocyte is not an indicator of the maturation of the virus particle but is more likely to be the result of preceding events in virus replication. HPV may be present in cells other than koilocytes, indicating that

the absence of koilocytosis does not rule out infection by the virus.[6]

Identification of HPV

Papillomaviruses of human, bovine, and canine origin share at least one common antigenic determinant that is a genus-specific antigen. The genomes are designated E (early) and L (late), depending on their time of synthesis.[7] The antiserum reacts with the major capsid protein of the virus and, because the antigens are stable during formalin fixation and routine tissue processing, the application of immunostaining is made possible in conventional paraffin wax sections (**Table**). A brown precipitate located in the nuclei of epithelial cells indicates the presence of the antigen (**Slide 2**).

Progression toward the formation of viral particles requires expression of late-region products that are linked to the keratinization process of the epithelium. In invasive carcinomas, the viral DNA is integrated in the host genome, resulting in the disruption of the genes responsible for protein production. However, some of the viral DNA can remain episomal, and the detection of antigen is possible because the expression of the late genes is not interrupted. Large numbers of mature virions are needed to detect the capsid antigen. Although HPV DNA replication precedes capsid antigen production, replication also may occur without the production of detectable antigen.[8] If the protein is not being synthesized by the virus in sufficient amounts, immunostaining cannot be expected to detect it. Consequently, although positive immunostaining for the antigen confirms HPV infection, a negative reaction does not exclude the virus.

Typing the HPV Virus

Morphologic changes associated with HPV infection are characteristic, yet immunostaining provides little information about the type of HPV present. There are currently more than 70 types of HPV, and the ability to distinguish between different viral types is crucial for predicting the likelihood that benign lesions will become malignant. Although antibodies to detect HPV in a type-specific manner have been developed,[8] the detection of HPV DNA or RNA is the most reliable way of diagnosing subclinical disease; for latent infection, it is the only way. Various molecular techniques, such as the Southern blot technique, in situ hybridization (ISH), and the polymerase chain reaction, are currently in use. The only method that does not destroy the morphologic features of the specimen is ISH, although its sensitivity is rather low. However, the unprecedented sensitivity of polymerase chain reaction–DNA amplification methods provides numerous advantages for the detection of HPV DNA, and its use with ISH methods provides a useful tool for the detection of HPV in the nuclei of squamous epithelial cells in paraffin sections.[9] Many ISH techniques are available in commercial kit form, and the use of type-specific HPV DNA probes is compatible with routine in-house hybridization methods.

The HPV types commonly associated with genital tract infection are types 6, 11, 16, and 18. The HPV

Table. Immunoperoxidase Staining of Papillomavirus Structural Antigen.

Fixative: 10% formalin
Sections: 4-μ thick
Reagents

- Avidin-biotin peroxidase kit
  rabbit IgG Vectastain ABC kit (Vector Laboratories, Burlingame, California)
- Tromethamine-buffered saline (TBS), pH 7.6
  Sodium chloride 8.0 g
  Tromethamine [tromethamine (hydroxymethyl) methylamine] 0.6 g
  1N hydrochloric acid 3.8 mL
  Distilled water to 1.0 L
  Check pH and adjust if necessary

Procedure

1. Deparaffinize and hydrate sections to distilled water.
2. Rinse slides in TBS, pH 7.6.
3. Incubate slides in 0.5% hydrogen peroxide in methanol for 10 minutes.
4. Rinse briefly in TBS.
5. Incubate slides in dilute normal rabbit serum (DAKO, Carpinteria, California) for 20 minutes.
6. Pour off serum and immerse the sections in rabbit antibovine papillomavirus (DAKO) diluted 1:80 with TBS for 20 minutes.
7. Wash in TBS for 5 minutes, continue incubation in biotinylated antirabbit immunoglobulin (Vector Laboratories) for 30 minutes.
8. Wash in TBS for 5 minutes, then incubate the sections in Vectastain ABC reagent for 60 minutes.
9. Wash in TBS for 5 minutes, then develop the peroxidase for 8 minutes in 0.05% diaminobenzidine in TBS with 0.1 mL of 1% hydrogen peroxide added before use to activate the solution.
10. Dehydrate the sections in alcohol, clear in xylene, and place on coverglasses without counterstaining and coverslip.

Results

Positive staining of epithelial cells (koilocytes) containing the viral antigen show dark brown nuclear staining.

types 6 and 11 are commonly associated with benign genital warts but seldom are found in invasive carcinomas of the cervix. Conversely, HPV types 16 and 18 are almost exclusively found in cervical intraepithelial neoplasia and invasive cancers. There is a significant relationship between tumor grade and HPV type in cancer of the cervix. Determination of the physical state and expression of HPV DNA sequences at different stages of tumor development has shown that the HPV genome becomes integrated into the host genome very early in cervical cancer development.[1]

The most practical use for HPV testing is identifying women at risk for progression to invasive cancer, thereby allowing infections to be subdivided into high and low risk. Although the low-risk types (HPV 6 and 11) cause the genital warts that can be distressing and troublesome, they can be managed effectively. The exposure of squamous epithelium to HPV in combination with an altered immune state leads to subclinical or clinical infection. Subclinical, sometimes latent, and rarely clinical infection may progress to intraepithelial or invasive neoplasia if certain preconditions are met by the virus and by the infected cell. These include infection with high-risk types (HPV 16 and 18) and integration of the viral genome.

It was determined that the patient in the present exercise had a subclinical genital human papillomavirus (HPV) infection. Genital infections are sexually transmitted and are generally prevalent in populations of high promiscuity. Among healthy females, the infections are almost twice as frequent as they are among healthy males. In both sexes, the highest rate of positive cases occurs in the 16- to 35-year-old age group, the group of highest sexual activity.

Molecular technology in the histology laboratory is commonplace, and its use improves diagnostic accuracy and efficiency, especially in the identification of women who, despite normal cervical cytology, are likely to have or develop cancer of the genital tract. Only by the identification and typing of HPV of those at risk of invasive disease can effective management of the disease be accomplished.

Key to 35-mm Transparencies

Slide 1. Paraffin section of cervix obtained from a patient with subclinical genital infection. Note the numerous clear cells (koilocytes) in the squamous epithelium showing perinuclear clear zones and peripheral cytoplasmic condensation. (H&E, ×200)

Slide 2. Koilocytes in the cervical epithelium from the same patient showing positive nuclear staining for papillomavirus structural antigen using the method described in the Table. (Avidin-Biotin Peroxidase, ×200)

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Suggested Reading

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CMLE Documentation Questions

1. Infection by the human papillomavirus (HPV)
   A) is established by entering the superficial cells of squamous epithelium.
   B) produces lesions that will progress to invasive carcinoma.
   C) arises in combination with an altered immune state.
   D) always results in the integration of viral DNA into the host cell DNA.
   E) is demonstrated histologically by molecular methods only.

2. Immunostaining, using a genus specific papillomavirus structural antigen to demonstrate the virus
   A) is the only method by which the HPV type may be identified.
   B) will demonstrate the HPV, irrespective of the severity of the lesion.
   C) can be performed only on frozen sections.
   D) usually detects the viral capsid protein in the nuclei of koilocytes.
   E) indicates latent infection only.

3. Infection with high-risk HPV types 16 and 18
   A) is usually found in benign genital warts.
   B) generates lesions that always become malignant.
   C) produces only latent infection.
   D) does not persist in cervical lesions.
   E) can be demonstrated by using immunostaining and hybridization methods.

Note: If you wish to receive CMLE credit, enter the answers to these test questions on your 1999 Tech Sample Program Answer Sheet. Retain your answer sheet until the final exercise for this series has been distributed (there are 6 exercises in the Histotechnology series). DO NOT SEND THE ANSWERS TO THE ASCP AT THIS TIME.

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