Commentary

Detecting Every Genital Papilloma Virus Infection

What Does It Mean?

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Approximately 20 years have elapsed since the discovery that human papillomaviruses (HPV) are associated with genital squamous neoplasia. Nearly 10,000 articles, chapters, and books on the subject have been written. The focus of attention has varied to include studies of molecular mechanisms of papillomavirus-induced genital neoplasia, profiles of the pathology of this process, epidemiology, and clinical management. Conclusions reached in many of these studies have depended heavily on the methods by which papillomavirus nucleic acids were detected.

Before the current era of polymerase chain reaction (PCR) technology, the methods of choice for identifying HPV in crude or extracted DNA and tissue sections were Southern blot, dot blot, and *in situ* hybridization. Sensitivities of these assays varied but, with the exception of dot blot hybridization of unpurified genomic DNA, the information gained was usually reproducible. Refinements in HPV detection schemes progressed to PCR. Its establishment as a reliable assay required that this technique (and its proponents) endure the growing pains typical of any highly sensitive assay. The great potential advantages of PCR were not only its sensitivity but also its flexibility. As new HPV types were discovered, new primer sets or variations of existing ones could be designed to include these new targets in the detection arsenal. The spectrum of detectable HPV types was maximized because the target region was most frequently the L1 open reading frame encoding the major capsid protein wherein most HPVs share considerable sequence homology.^{1,2}

Although PCR enjoyed the center stage of HPV testing, certain issues remained unsettled before its use became widespread. One was specificity, ie, ability to minimize false positive results. This problem has been discussed at length and is not limited simply to laboratory contamination. It has been effectively addressed by practices or techniques designed either to separate PCR products

from PCR preparations or to render PCR products unusable as substrates. Two other, more subtle problems exist. The first is that irrespective of the technique used, HPV will be detected in the absence of clinical disease; the more sensitive the test, the higher the rate of detection in patients without morphological or cytological abnormalities. Occult or latent HPV is presumably not due to laboratory contamination because it has been demonstrated in conventional hybridization assays.³ Moreover, a proportion of morphologically normal HPV positives will eventually develop a cervical lesion.⁴ Presumably, many young women harbor various amounts of detectable HPV. The study in this issue by Kleter et al reveals a highly sensitive HPV detection technique that may maximize the detection of these viruses.⁵ A second and related problem is the interpretation of HPV in tissues remote to the genital tract squamous epithelium, such as esophagus, breast, colon, prostate, upper genital tract, placenta, bladder, and in certain skin disorders, including Kaposi's sarcoma.⁶⁻²⁰ The application of sensitive PCR-based assays to these sites has produced variable results depending on the study, leading to considerable confusion over whether HPV is really involved in the pathogenesis of tumors in other sites. The confusion is reminiscent of studies of herpes simplex virus (HSV) in the 1970s. Somewhat analogous to investigations of HPV in the PCR era, studies linking HSV to neoplasia were encouraged by technological achievements including the then novel production of antibodies or probes to HSV, which produced conflicting results and debate over the role of this virus in cervical cancer.²¹ (Predictably, with the advent of PCR this relationship is being examined again.) Although the role of HPV in genital neoplasia is undisputed, the precise relationship of this virus to tumors in other sites has been unresolved, primarily because advances in PCR detection techniques may have temporarily exceeded our ability to understand their significance.

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One standard by which HPV detection methods are measured is detection rates in cervical squamous neoplasia, specifically squamous cell carcinomas. Southern blot hybridization has scored positive for HPV in less than 80% of carcinomas.²² The possibility that HPV-negative cervical cancers existed was suggested by these findings and encouraged by studies identifying p53 mutations in HPV-negative cervical tumor cell lines.²³ Because PCR failed to detect HPV in all cancers, the issue could not be resolved, although some studies produced high enough detection rates to refute the argument for another pathway.²⁴ The study by Kleter et al is most impressive, revealing that 100% of cervical cancers contain HPV.⁵ To achieve this result, the authors developed an elegant assay which maximized sensitivity. It should be emphasized that the development of this assay drew heavily on the prior experiences of others, who are cited in their report.^{1,2,25}

Two of the most critical variables in PCR detection are probe selection and detection technique. The two most commonly used probe sets, MY9/MY11 and GP5⁺/GP6⁺, target the L1 region of HPVs and are designed to identify a wide range of HPV types.^{1,2} Both work extremely well, identifying HPV sequences in up to 90% of squamous precursors in either archival tissue or Papanicolaou smear preparations.⁵ Their respective PCR product sizes are about 450 and 150 base pairs. The two techniques are similar to the slightly more sensitive GP primers and the more efficient MY primers at detecting mixed HPV infections.^{1,2,26} The issue of sensitivity reflects in part primer size; assays targeting smaller products typically are more sensitive, particularly in formalin-fixed tissues where DNA preservation may influence the capacity of the assay to consistently amplify HPV.^{5,27} This author experienced one instance in which the index of HPV-positive squamous cancers was doubled by reducing the product size from 450 to 200 base pairs when analyzing archival tissue samples.²⁸ Kleter et al approach the theoretical limit by reducing the target product size to 65 base pairs, resulting in an amplified segment of only 25 base pairs between the primers. Their strategy is nicely illustrated in Figure 1 of their paper. With only 6 different primers (see Table 2 in Kleter et al), they succeed in detecting HPV in 100% of cervical cancers.⁵

The detection technique is also critical, inasmuch as its design will determine not only sensitivity but also how easily contaminants are detected. PCR analyses are extremely prone to contamination; the problem is a complex one. Studies relying on primer-mediated amplification followed by fragment length polymorphism analysis of the product provide a landscape of products in which lane-to-lane contamination can be identified immediately.²⁵ It is the preferred strategy of this author because samples can be concurrently typed and compared to one another on the same autoradiograph.³⁰ It has several drawbacks, including the need for polyacrylamide gels, isotopes for maximum sensitivity, and a large enough product (450 bp) to analyze after restriction digest. A clever variant of this approach digests the product with multiple enzymes and exposes the filter containing the transferred DNA to a probe targeting certain digested fragments, the size of

which will reveal the HPV type.³¹ Still another uses single-strand conformational polymorphism analysis.³² Direct probing of products on dot blots or the equivalent with oligonucleotide probes (which naturally target the interval sequence) is highly sensitive, but requires both that the probe be specific and that there be no contamination. Theoretically, trace laboratory contamination by a reaction product could wreak havoc in this system, producing a variable number of additional positives which might be much more conspicuous in a lane-to-lane comparison of restriction length polymorphisms.³³

Kleter et al have opted for the probe strategy, using an enzyme-linked detection assay in a microtiter-based hybridization system.⁵ As described in their Methods section, the PCR reaction incorporates a biotin label that fixes the product to streptavidin-coated microtiter wells. After rinsing away the unbound material, HPV sequences within the PCR products bound to the streptavidin were detected by digoxigenin-labeled oligonucleotide probes corresponding to the 25-base pair interval flanked by the primers (see Figure 1 and Table 1 of Kleter et al). An alkaline phosphatase-linked antibody to digoxigenin completes the assembly, which, after addition of substrate, will produce a quantifiable result. There remains the issue of setting the threshold for sensitivity, which the authors arbitrarily placed at an OD of 2.5 times background.⁵ This system is identical to that in a prior publication^{25,29} and analogous to the Hybrid Capture Technique (HCT) of Digene Corporation (Beltville, MD), which uses an RNA probe to identify the HPV products, an antibody to the RNA-DNA hybrid, and a tagged enzymatic reaction (chemiluminescence) read by a luminometer.³⁴ The clear advantage of these systems is the avoidance of isotopes; the trade-offs are the arbitrary selection of a cutoff for distinguishing positives from negatives and no provision (other than a negative control) for identifying potential contaminants. However, Kleter et al show convincingly that the latter two concerns, at least in their hands, are not factors that affect their system. Moreover, they support their assay with sequence data from the PCR products.⁵

At least three features of a robust HPV detection assay establish its value: its adaptation to fixed tissues, percentage of squamous precursors or invasive cancers tested that score positive, and relationship of the HPV types detected to the biology of the lesions under study. A 65-base pair product maximizes the chance that HPV will be detectable, even in tissues with considerable DNA degradation. But the proportion of lesions that score positive and whether they contain the predicted HPV type(s) are still matters of concern, and the latter concern is not trivial. The literature describes instances in which low-risk HPVs have been identified in genital or nongenital malignant tumors, only a minority of which could be explained. Therefore, a highly sensitive test that did not consistently identify the high-risk HPV types in cancers would be of questionable value. However, Kleter et al show not only that their system can detect HPV in a very high proportion of abnormal smears, but also that the index of high-risk types increases as a function of the severity of the morphology.⁵ In contrast to other systems, including those

using the MY or GP primers and yielding HPV detection rates of 70–90%, these authors detected HPV in a compelling 98% of dysplastic smears. Moreover, the ratio of high- to low-risk HPV types detected increased as a function of the cytologic abnormality, starting at 3 to 1 for normal smears, progressing to nearly 30 to 1 for dysplastic smears, and ending at nearly 200 to 1 for invasive carcinomas.⁵ This is impressive evidence that the strategy outlined in their report will not only detect HPV in nearly all squamous precursors, but also identify the type of HPV characteristically associated with cervical neoplasia.

Are there real advantages to such a sensitive HPV DNA detection assay? The answer is yes, depending on the situation. This technology lays to rest the question of whether a significant minority of cervical cancers evolve via an HPV-negative pathway. This assay would also be valuable in attempts to resolve the relationship between HPV and unusual morphological phenotypes in the cervix or elsewhere, particularly in work with small numbers of cases where assay sensitivity is particularly critical. High sensitivity might resolve the controversy of whether HPV DNA in pelvic lymph nodes is a marker for occult metastases in cervical carcinomas.^{35,36} In vulvar cancer, where both HPV-positive and -negative cancers exist, a closer look at this dichotomy with a highly sensitive HPV detection technique might reveal new insights into the relationship of HPV to certain tumor phenotypes.³⁷ Epidemiologists, who have periodically struggled with suboptimal HPV DNA data, would delight in an even more sensitive assay. Reanalysis of extragenital (and variably HPV-positive) tumors with a highly sensitive and specific method could either reveal important and novel associations with this virus or dismiss the question of cryptic infection.

The most pressing question, and one which tempers this author's enthusiasm for a more sensitive HPV test, concerns its clinical utility. To their credit, the authors avoid offering this assay as a panacea for HPV-mediated clinical management. A central problem with using HPV testing, specifically PCR-based HPV testing, in the clinical setting is the relationship of a positive result to clinical disease. The problem can be subdivided into three parts. The first is the utility of HPV testing in smears which are clearly dysplastic. Most studies show that at least 50% of abnormal smears contain high-risk HPV types and the study by Kleter et al places the index at nearly 100%. Hence, the triage of patients with dysplastic smears by HPV testing yields little new information other than to validate significance of cytologic atypia. A second issue is the significance of HPV in a population. Studies have shown that women who score positive for high-risk HPVs have a greater chance of developing a high-grade precursor lesion.⁴ However, this risk varies and likely will decrease as a function of increasing rates of viral detection. One study using dot blot and Southern blot hybridization put the risk at 28%.⁴ Recent studies using PCR have estimated the risk of developing a high-grade squamous intraepithelial lesion (SIL) following detection of HPV16 to be less than 10%, although the risk is 100 times higher than for HPV-negative women.³⁸ The index of HPV positives achieved by the techniques of Kleter et al in a

random population of young women cannot be determined because the authors targeted only women with a history of smear abnormality. However, 23% of women with normal smears and a history of treated SIL scored positive for HPV.⁵ If similar rates exist for randomly selected women of reproductive age, the findings, although correct and free of laboratory contamination, are of limited value unless they can be correlated with a substantial risk of subsequent cervical neoplasia. Considering the marked differences observed in HPV detection between smears of younger (15–30 years) and older (≥ 35 years) women and the transient nature of many HPV "infections" detected by molecular assays, a highly sensitive HPV detection assay is more likely to worsen than resolve the dilemma of managing HPV-positive but cytologically negative women.^{39–41} Nevertheless, it all but eliminates the risk of high-grade dysplasia or cancer (at least in the short term) for the HPV negatives.³⁸

The strongest argument for HPV testing has been its application to the management of women whose Papanicolaou smear abnormalities are not diagnostic for squamous precursor lesions but fall out of the "comfort zone" of benign cellular changes.⁴² These nondiagnostic squamous atypias or "atypical squamous cells of undetermined significance" (ASCUS) are emblematic of how introducing a particular diagnostic term or language into routine cytopathology practice alters patient management.⁴³ The term ASCUS vexes the practitioner who desires a clear-cut diagnosis and choice of management, while raising the remote, but usually needless, specter of cancer to both doctors and patients. By definition, a diagnosis of ASCUS implies diagnostic uncertainty and has inherently poor reproducibility between cytopathologists. For this reason, ASCUS, now ensconced as a villain of modern cytopathology practice, has been proposed as a suitable target for HPV-directed triage.⁴² The commitment to this concept is so strong that a large National Cancer Institute-sponsored study has been initiated to explore the role of HPV testing in the triage of ASCUS.

For HPV-mediated clinical management of ASCUS to be feasible, the presence of HPV nucleic acids in a smear must be predictive of biopsy-proven disease. Biopsy-proven disease permits decisive (ablative) management. Accordingly, if the HPV-positive ASCUS smear correlates strongly with a morphologically demonstrable lesion, colposcopy is justified. Conversely, if an HPV-negative ASCUS carries an extremely low risk of a lesion, the patient can be followed. Whether this strategy is cost-effective is unclear, but some studies show clearly that about 45% of HPV-positive and 5% of HPV-negative ASCUS smears will be followed by a diagnosis of high-grade SIL.⁴² Although other studies have reached similar results, the clinical value of this information remains a matter of debate.⁴⁴

The technique used for most commercial HPV testing has been the HCT, a sensitive and specific marker for HPV. HCT employs a high-risk probe set and most results are interpreted without discriminating among the particular high-risk HPV types that may be present.³⁴ HCT is not as sensitive as standard PCR techniques and by inference would not be as sensitive as the assay de-

scribed by Kleter et al.⁴⁵ However, potential gains or losses from using a more sensitive assay are worth discussing. Because the assay of Kleter et al is more sensitive, it is likely that an incremental increase in HPV-positive ASCUS cases would be achieved, reducing the false negative (HPV negatives followed by positive biopsies) rate. If this reduction were significant, a nagging concern with HPV testing would be resolved. It probably would, based on the extremely low risk for subsequent disease in HPV-negative women with normal smears.³⁸ However, the advantage obtained by the increase in assay sensitivity will likely be purchased with the loss of specificity.⁴⁶ The increase in total numbers of HPV positives would increase the number of women referred to colposcopic triage, including many whose biopsies would not prove to be abnormal. This is due to two problems in the correlation of histological, cytologic, and HPV data. One has become obvious over the past few years and has introduced a measure of reality into clinical and laboratory practice. The other is less conspicuous, but could be exacerbated by the clinical use of highly sensitive HPV assays to manage ASCUS.

The first problem is that a significant proportion of dysplastic Papanicolaou smears will not be corroborated on biopsy. This pertains primarily to low-grade dysplasias, where the rate is as high as 50%. For high-grade lesions, approximately 15% will not be confirmed on followup biopsy. This was initially attributed to diagnostic overcalls by cytopathologists. However, discrepancies in smear and biopsy occur just as frequently when cytologic criteria are rigidly applied and despite the presence of HPV.^{42,47,48} Explanations for disparities in cytology and biopsy findings include small cervical lesions that are missed clinically, overlooked or occult vaginal or vulvar lesions producing abnormal cells, and, in some cases, lesion regression before colposcopic exam. Thus, even high rates of HPV positivity will not ensure colposcopic and/or histological confirmation. The assay by Kleter et al showed a significant increase in their HPV detection rate over standard PCR techniques and, by inference over HCT, particularly in normal smears.⁵ Given that the histological confirmation rate in ASCUS is approximately 25%, highly sensitive HPV assays threaten to yield an even smaller fraction of women referred to colposcopy in which a lesion is eventually diagnosed by biopsy.

The second problem is the impact of ASCUS interpretation on ASCUS triage by HPV testing. Because rates and usage of ASCUS vary as a function of observer experience and skill, the role and significance of HPV testing will vary. In some practices, ASCUS smears interpreted as "favor reactive" have a significantly lower rate of biopsy-proven high-grade SIL than those scored as "favor dysplasia".^{49,50} This has important implications for patient management. In this author's experience, a significant minority of smears interpreted as favor reactive (8%) are positive for high-risk HPVs. While the triaging of this minority to colposcopy may seem appropriate, it should be stressed that a similar proportion of benign reactive smears in young women will also score for high-risk HPV.⁵¹ Thus, PCR-based HPV testing may not clearly distinguish those women with minor Pap smear atypias

from those with reactive epithelial changes. If so, the rationale for referring these women for colposcopy is questionable. The combination of a highly sensitive HPV detection assay, a young sexually active population, and an aggressive cytopathologist could lead to the needless referral of numerous women for colposcopy.

The authors have wisely avoided the thorny clinical issues in presenting an elegant alternative to the molecular detection of genital papillomaviruses. Irrespective of its clinical utility, an assay that can combine high sensitivity, specificity, and a non-radioactive detection system is useful in the study of papillomavirus-related diseases. Because the initial reports of a new technique are the most optimistic, this primer combination will likely be scrutinized by others who will, in turn, recommend additional modifications. Such studies will determine whether Kleter et al, with the assistance of their predecessors, have approached the theoretical maximum of human papillomavirus detection.

References

1. Manos MM, Ting Y, Wright DK, Lewis AJ, Broker TR, Wolinsky SM: Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. *Cancer Cells* 1989, 7:209-214
2. de Roda Husman AM, Walboomers JM, van den Brule AJ, Meijer CJ, Snijders PJ: The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* 1995, 76:1057-1062
3. Wagner D, Ikenberg H, Boehm N, Gissman L: Identification of human papillomavirus in cervical swabs of deoxyribonucleic acid in situ hybridization. *Obstet Gynecol* 1984, 64:767-772
4. Koutsky LA, Holmes KK, Critchlow CW, Stevens CE, Paavonen J, Beckmann AM, DeRouen TA, Galloway BA, Vernon D, Kiviat NB: A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection. *N Engl J Med* 1992, 327:1272-1278
5. Kleter B, van Doorn L-J, ter Schegget J, Schrauwen L, van Krimpen K, Burger M, ter Harmsel B, Quint W: Novel short-fragment PCR assay for highly sensitive broad-spectrum detection of anogenital human papillomaviruses. *Am J Pathol* 1998, 153:1731-1739
6. Chan KW, Wong KY, Srivastava G: Prevalence of six types of human papillomavirus in inverted papilloma and papillary transitional cell carcinoma of the bladder: an evaluation by polymerase chain reaction. *J Clin Pathol* 1997, 50:1018-1021
7. Lu QL, Lalani el-N, Abel P: Human papillomavirus 16 and 18 infection is absent in urinary bladder carcinomas. *Eur Urol* 1997, 31:428-432
8. Di Lonardo A, Venuti A, Marcante ML: Human papillomavirus in breast cancer. *Breast Cancer Res Treat* 1992, 21:95-100
9. Gopalkrishna V, Singh UR, Sodhani P, Sharma JK, Hedau ST, Mandal AK, Das BC: Absence of human papillomavirus DNA in breast cancer as revealed by polymerase chain reaction. *Breast Cancer Res Treat* 1996, 39:197-202
10. Fidalgo PO, Cravo ML, Chaves PP, Leitao CN, Mira FC: High prevalence of human papillomavirus in squamous cell carcinoma and matched normal esophageal mucosa: assessment by polymerase chain reaction. *Cancer* 1995, 76:1522-1528
11. Turner JR, Shen LH, Crum CP, Dean PJ, Odze RD: Low prevalence of human papillomavirus infection in esophageal squamous cell carcinomas from North America: analysis by a highly sensitive and specific polymerase chain reaction-based approach. *Hum Pathol*, 1997, 28:174-178
12. Lu S, Syrjanen K, Havu VK, Syrjanen S: Failure to demonstrate human papillomavirus (HPV) involvement in Bowen's disease of the skin. *Arch Dermatol Res* 1996, 289:40-45
13. Cheng JY, Sheu LF, Lin JC, Meng CL: Detection of human papillomavirus DNA in colorectal adenomas. *Arch Surg* 1995, 130:73-76

14. Terris MK, Peehl DM: Human papillomavirus detection by polymerase chain reaction in benign and malignant prostate tissue is dependent on the primer set utilized. *Urology* 1997, 50:150-156
15. Zimna K, Poreba E, Kedzia W, Gozdzicka-Jozefiak A, Kedzia H: Human papillomavirus (HPV) in upper genital tract carcinomas of women. *Eur J Gynaecol Oncol* 1997, 18:415-417
16. Noel JC, Andre J, Hermans P, Fayt I, Peny MO, De Thier F, Haot J, Burny A: Kaposi's sarcoma: evaluation of the role of human papillomaviruses. A study using the high sensitivity hot-start polymerase chain reaction detection method and a review of the literature. *Arch Dermatol Res* 1996, 288:786-788
17. Boyd AS, Annarella M, Rapini RP, Adler-Storthz K, Duvic M: False-positive polymerase chain reaction results for human papillomavirus in lichen planus: potential laboratory pitfalls of this procedure. *J Am Acad Dermatol* 1996, 35:42-46
18. Hermonat PL, Kechelava S, Lowery CL, Korourian S: Trophoblasts are the preferential target for human papilloma virus infection in spontaneously aborted products of conception. *Hum Pathol* 1998, 29:170-174
19. O'Leary JJ, Landers RJ, Crowley M, Healy I, O'Donovan M, Healy V, Kealy WF, Hogan J, Doyle CT: Human papillomavirus and mixed epithelial tumors of the endometrium. *Hum Pathol* 1998, 29:383-389
20. Proby CM, Shamanin V, Rausch C, Glover MT, de Villiers EM, Leigh IM: Novel human papillomaviruses identified in skin cancers and keratinocyte cell lines from renal transplant recipients (Meeting abstract). *Br J Dermatol* 1995, 132:644
21. Nahmias AJ, Sawanabari S: The genital herpes-cervical cancer hypothesis: 10 years later. *Prog Exp Tumor Res* 1978, 21:117-139
22. Chang DY, Hsieh CY, Chen RJ, Lee SC, Huang SC: Comparison of detection of human papillomavirus 16 DNA in cervical carcinoma tissues by Southern blot hybridisation and nested polymerase chain reaction. *J Med Microbiol* 1995, 43:430-435
23. Scheffner M, Munger K, Byrne JC, Howley PM: The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci USA* 1991, 88:5523-5527
24. Resnick RM, Cornelissen MT, Wright DK, Eichinger GH, Fox HS, ter Schegget J, Manos MM: Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *J Natl Cancer Inst* 1990, 82:1477-1484
25. Jacobs MV, van den Brule AJ, Snijders PJ, Helmerhorst TJ, Meijer CJ, Walboomers JM: A non-radioactive PCR enzyme-immunoassay enables a rapid identification of HPV 16 and 18 in cervical scrapes after GP5+/6+ PCR. *J Med Virol* 1996, 49:223-229
26. Qu W, Jiang G, Cruz Y, Chang CJ, Ho GY, Klein RS, Burk RD: PCR detection of human papillomavirus: comparison between MY09/MY11 and GP5+/GP6+ primer systems. *J Clin Microbiol* 1997, 35:1304-1310
27. Baay MF, Quint WG, Koudstaal J, Hollema H, Duk JM, Burger MP, Stolz E, Herbrink P: Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. *J Clin Microbiol* 1996 34:745-747
28. Yang Y-C, Shen J, Tate JE, Crum CP: Carcinoma of the cervix in young women in Taiwan: prognosis is independent of papillomavirus or tumor cell type. *Gynecol Oncol* 1997, 64:59-63
29. Lungu O, Wright T.C., Silverstein S: Typing of human papillomaviruses by polymerase chain reaction amplification with L1 consensus primers and RFLP analysis. *Mol Cell Probes* 1992, 6:145-152
30. Genest D, Stein L, Cibas E, Sheets E, Zitz J, Crum CP: A binary (Bethesda) system for classifying cervical cancer precursors: criteria, reproducibility and viral correlates. *Hum Pathol* 1993, 24:730-736
31. Zehbe I, Sallstrom JF, Evander M, Edlund K, Rylander E, Wadell G, Wilander E: Nonradioisotopic detection and typing of human papillomaviruses by use of polymerase chain reaction and single-strand conformation polymorphism. *Diagn Mol Pathol* 1996, 5:206-213
32. Meyer T, Arndt R, Stockfleth E, Flammann HT, Wolf H, Reischl U: Strategy for typing human papillomaviruses by RFLP analysis of PCR products and subsequent hybridization with a generic probe. *Biotechniques* 1995, 19:632-639
33. Zitz J, McLachlin CM, Tate J, Mutter G, Crum CP: Analysis of isotope-labeled PCR-amplified HPV DNA by RFLP provides optimal sensitivity and contamination detection. *Mod Pathol* 1994, 7:407-411
34. Lorincz AT: Hybrid capture method for detection of human papillomavirus DNA in clinical specimens: a tool for clinical management of equivocal Pap smears and for population screening. *J Obstet Gynaecol Res* 1996, 22:629-636
35. Baay MF, Koudstaal J, Hollema H, Duk JM, Burger MP, Quint WG, Stolz E, Herbrink P: Detection of HPV-16 DNA by PCR in histologically cancer free lymph nodes from patients with cervical cancer. *J Clin Pathol* 1997, 50:960-961
36. Kobayashi Y, Yoshinouchi M, Tianqi G, Nakamura K, Hongo A, Kamimura S, Mizutani Y, Kodama J, Miyagi Y, Kudo T: Presence of human papilloma virus DNA in pelvic lymph nodes can predict unexpected recurrence of cervical cancer in patients with histologically negative lymph nodes. *Clin Cancer Res* 1998, 4:979-983
37. Andersen W, Franquemont D, Taylor PT, Crum CP: Vulvar squamous cell carcinoma and papillomaviruses: two separate entities? *Am J Ob Gyn* 1991, 165:329-336
38. Rozendaal L, Walboomers JM, van der Linden JC, Voorhorst FJ, Kenemans P, Helmerhorst TJ, van Ballegooijen M, Meijer CJ: PCR-based high-risk HPV test in cervical cancer screening gives objective risk assessment of women with cytomorphologically normal cervical smears. *Int J Cancer* 1996 68:766-769
39. Melkert PWJ, Hopman E, van Den Brule AJC, Risse EKJ, van Diest PJ, Bleker OP, Helmerhorst T, Schlipper MEJ, Meijer CJ, Walboomers JMM: Prevalence of HPV in cytomorphologically normal cervical smears, as determined by polymerase chain reaction, is age-dependent. *Int J Cancer* 1993, 53:919
40. Rosenfeld WD, Rose E, Vermund SH, Schreiber K, Burk RD: Follow-up evaluation of cervicovaginal human papillomavirus infection in adolescents. *J Pediatr* 1992, 121:307
41. Evander M, Edlund K, Gustafsson A, Jonsson M, Karlsson R, Rylander E, Wadell G: Human papillomavirus infection is transient in young women: a population-based cohort study. *J Infect Dis* 1995, 171:1026-1030
42. Cox JT, Lorincz AT, Schiffman MH, Sherman ME, Cullen A, Kurman RJ: Human papillomavirus testing by hybrid capture appears to be useful in triaging women with a cytologic diagnosis of atypical squamous cells of undetermined significance. *Am J Obstet Gynecol* 1995, 172:946
43. Crum CP: The Bethesda system: a perspective (Editorial). *Pathology Patterns, Am J Clin Pathol* 1991, 96:S2-S5
44. Adam E, Kaufman RH, Berkova Z, Icenogle J, Reeves WC: Is human papillomavirus testing an effective triage method for detection of high-grade (grade 2 or 3) cervical intraepithelial neoplasia? *Am J Obstet Gynecol* 1998, 178:1235-1244
45. Nindl I, Greinke C, Zahm DM, Stockfleth E, Hoyer H, Schneider A: Human papillomavirus distribution in cervical tissues of different morphology as determined by hybrid capture assay and PCR. *Int J Gynecol Pathol* 1997, 6:197-204
46. Sherman ME, Schiffman MH, Lorincz AT, Herrero R, Hutchinson ML, Bratti C, Zahniser D, Morales J, Hildesheim A, Helgesen K, Kelly D, Alfaro M, Mena F, Balmaceda I, Mango L, Greenberg M: Cervical specimens collected in liquid buffer are suitable for both cytologic screening and ancillary human papillomavirus testing. *Cancer* 1997, 81:89-97
47. Hall S, Wu TC, Soudi N, Sherman ME: Low-grade squamous intraepithelial lesions: cytologic predictors of biopsy confirmation. *Diagn Cytopathol* 1994, 10:3-9
48. Lee KR, Minter LJ, Crum CP: Koilocytotic atypia in Papanicolaou smears: reproducibility and biopsy correlates. *Cancer Cytopathol* 1997, 81:10-15
49. Genest D, Dean B, Galvanek E, Lee K, LaGuerre E, Sheets EE, Crum CP, Cibas ES: Qualifying the diagnosis of "atypical squamous cells of undetermined significance" affects the frequency of SIL on subsequent biopsy. *Arch Pathol* 1998, 122:338-341
50. Collins LC, Wang HH, Abu-Jawdeh GM: Qualifiers of atypical squamous cells of undetermined significance help in patient management. *Mod Pathol* 1996, 9:677-681
51. Crum CP, Genest DR, Hogan C, Krane J, Bellerose BA, Sun D, Lee KR: Subclassification of atypical squamous cells (ASCUS): reproducibility and human papillomavirus correlates in Thin-prepR smears (meeting abstract). *American Society of Cytopathology, Nashville, 1998, p 14*