HPV Detection and Clinical Implications

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6.1 Background

Human papillomaviruses (HPV) are known as the primary cause of cervical cancer. Most HPV infections resolve spontaneously, but those that persist may lead to the development of precancerous abnormalities and, if left untreated, may progress to cancer. Papillomaviruses are members of large family of viruses known as *Papovaviridae*. HPV is a relatively small virus containing non-enveloped double-stranded (ds) DNA. HPV genome is functionally divided into three regions: early region (E), late region (L), and long control region (LCR) [1] (Fig 6.1).

Early Region (E): It constitutes about 50 % of the viral genome and is one of the protein coding regions for early viral life cycle. E1 and E2 encode proteins for viral DNA replication and regulate the transcription of E6 and E7. E4 helps in the release of virions from infected cell. E5 interacts with various transmission proteins which promote cell growth. *E6 and E7 are viral oncogenes, which induce cell immortalization and transformation of the host cell.*

Late Region: It forms about 40 % of the viral genome and is expressed late in the viral life cycle. This region encodes two structural proteins of the viral icosahedral capsid. L1 is responsible for the formation of major capsid proteins and L2 for minor capsid proteins.

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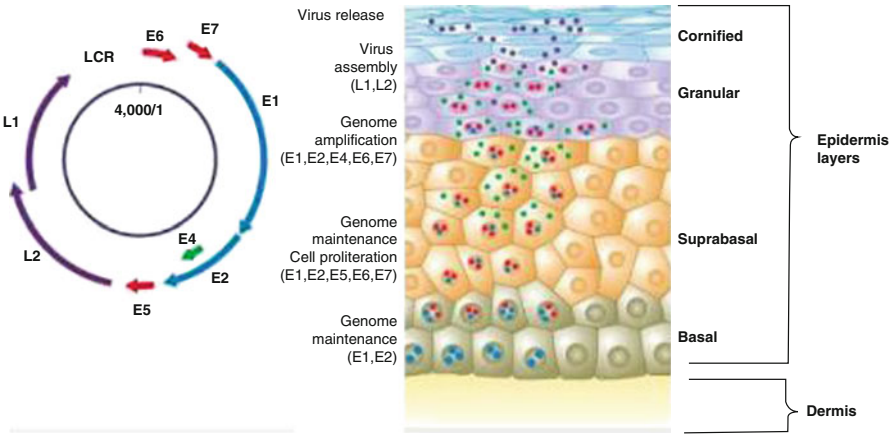


Fig 6.1 HPV genome and life cycle of the virus [2]

Long Control Region (LCR): It's a noncoding regulatory region which constitutes approximately 10 % of the HPV genome. It controls DNA replication and transcription by protein coding regions, i.e., early and late regions.

The life cycle of HPV begins with the entry of virus into the basal epithelium of the host. The entry of virus requires mild abrasion or microtrauma. The virus replicates in the basal cell and gradually migrates upward to the surface epithelium. Late viral genes appear at this stage, and virions are released to restart the cycle. The viral genome remains extrachromosomal during replication in normal life cycle, in benign lesions, and in early dysplasia, but for development of precancer and invasive malignancies, viral DNA integrates into the host genome.

Approximately 190 different types of HPV viruses have been known. There are 30 types of HPV which target the genital mucosa; out of which 15 are high-risk or oncogenic types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) [3]. HPV types 16 and 18, together, account for more than 70 % of cervical precancers and cancer cases, followed by HPV 45, 31, and 33. Non-oncogenic low-risk types especially HPV 6 and HPV 11 account for 90 % of benign genital warts. Many HPV strains are structurally and functionally similar. HPV 16 is closely related to HPV 31, 33, 35, 52, and 58, and HPV 18 is related to HPV 45.

Most infections resolve spontaneously but may persist in some women leading to persistence and progression to precancerous lesions and invasive cancer in untreated women over a period of 5–15 years. The prevalence of HPV infection peaks at the age of 18–28 years, after which it declines. Approximately 90 % of lesions regress spontaneously within 12–36 months. The prevalence of hrHPV infections in women above 30 years of age is around 10 %. Older women with persistence are more likely to be at risk of invasive cancer.

Other factors influencing the progression towards cervical cancer are immunosuppression, long-term use of oral contraceptives, multiple sexual partners, early onset of sexual activity, and smoking.

6.2 Tests for HPV Detection

Detection of HPV in human cells has been strenuous because of two main reasons: the early proteins being expressed in low amounts and lack of specific antibodies against the viral proteins. Since HPV cannot be cultured, diagnostics rely mainly on the detection of viral nucleic acids in cervical smear samples. There has been constant evolution in detecting the presence of HPV in cervical smears. These techniques evolved from scoring of koilocytes to indicate the presence of HPV in the specimen to the most recently advanced signal and target-amplified nuclide acid hybridization tests.

For genome analysis, non-amplified nuclide acid hybridization tests, such as *Southern blot* for DNA molecules and *Northern blot* for RNA molecules, were used, but these tests are time-consuming and require well-preserved and full-sized molecules and hence cannot be done with specimens particularly those derived from fixed tissues containing degraded nucleic acid. Therefore, these tests cannot be used for large population studies.

6.2.1 In Situ Hybridization

It is based on the complementary pairing of a labeled probe to HPV antigens or nucleic acids in cells of cervical smear sample [4]. It demonstrates the localization of viral genome in individual cells by using chromogenic or fluorescence technique. The INFORM HPV [5] assay includes a low-risk (6, 11, 42, 43, and 44) and high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66) assay. The advantage of ISH is that it can be applied to tissues that have been fixed and processed. However, the clinical sensitivity of this technique is limited due to probe cross-hybridization.

6.2.2 Polymerase Chain Reaction (PCR)

It can detect HPV in samples with few cells containing few viral copies with poor DNA quality. PCR can produce one billion copies from a single dsDNA molecule after 30 cycles of amplification. Also the reaction mix includes internal controls to decrease false-positive and false-negative results. Since integration of the HPV genome into the human chromosomes may result in loss of the L1 region, PCR tests can have false-negative results. Furthermore, as PCR can produce millions of copies of a DNA target from a single molecule, hence the environment is extremely vulnerable to contamination with HPV sequences from aerosolized reaction mixtures [6]. The size of the amplified product remains the same regardless of the HPV type; therefore, electrophoresis cannot detect the actual type of HPV. Studies have shown the sensitivity and specificity for detecting CIN 3 or higher with PCR testing to be 88.2 % (78.9–93.8 %) and 78.8 % (77.9–79.7 %), respectively [7]. Recently Roche Diagnostics developed the AMPLICOR HPV kit test that amplifies a smaller

fragment of the L1 gene; this short PCR fragment (SPF)-PCR can discriminate a broad spectrum of HPV types and is considered to be more sensitive and usable for less-preserved specimens.

6.2.3 Hybrid Capture

Digene Corporation (now known as Qiagen Corporation) developed signal amplification technique that detects nucleic acid targets directly [8]. It has developed two tests:

- Hybrid capture tube (HCT) test: It is a US Food and Drug Administration (FDA)-approved semiquantitative measure of viral load relative to 10 pg/ml and uses RNA probes that react with nine high-risk HPV types (16, 18, 31, 33, 35, 45, 51, 52, and 56).
- HPV hybrid capture test (HCII): In 1999, FDA approved the second generation of HCT. It detects viral load up to 1 pg/ml and four additional hrHPV (39, 58, 59, 68). The test is based on hybridization, in a solution of long synthetic RNA probes complementary to the genomic sequence of 13 high-risk (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and 5 low-risk (6, 11, 42, 43, 44) HPV types. It takes around 6–7 hours for detection of HPV; about 90 patients' samples can be processed simultaneously on one microtiter plate. The test result is expressed as relative light units (RLU). The FDA recommended the cutoff value for test-positive results to be 1.0 RLU (equivalent to 1 pg of HPV DNA per 1 ml of sampling buffer). As HCII is based upon signal amplification, it is less prone to cross specimen contamination as compared to PCR. However, there are false-negative and false-positive results because of the absence of internal control for the amount of input of DNA and inability to identify specific HPV types.

6.2.4 RNA-Based Amplification Techniques

Of late, HPV RNA is considered as an important target for molecular diagnosis of HPV infections. Unlike HPV DNA assays that detect only the presence of viral genomes, testing for viral RNA evaluates the HPV genome expression and viral activity in the infected cells. Detection of HPV E6/E7 mRNA in cervical smear samples can be performed by reverse transcription (RT)-PCR or by nucleic acid sequence-based amplification (NASBA) [9] (PreTect HPV-Proofer; Norchip). It detects E6/E7 transcripts from the five common hrHPV types in cervical carcinoma (16, 18, 31, 33, and 45). In this, single-stranded nucleic acids (viral genomic RNA, mRNA, or rRNA) are amplified in a background of dsDNA.

Gen-Probe has developed the APTIMA HPV [10] Assay, targeting E6/E7 mRNA from 14 carcinogenic HPV genotypes. A prototype of this assay was evaluated in 536 women with histological outcomes. Detection of E6/E7 mRNA was strongly correlated with severity of the lesion; all five carcinomas and 90 % of CIN 3 cases revealed E6/E7 mRNA [10].

A Norwegian hospital-based, cross-sectional study has shown that PreTect HPV-Proofer [11] is positive in 89 % of cervical cancer and in 77 % of high-grade precursor lesions. High-grade histology (CIN 2+) was found in 83 % of women with normal cytology and positive PreTect HPV-Proofer. Though the predictive value of HPV testing was not calculated in this study, the specificity of mRNA testing seems to be better compared to HPV DNA testing.

6.2.5 Newer Tests

Khan et al. [12] reported that 21 % of cytology-negative, HPV 16-positive women developed CIN 3+ over a period of 10 years, while 18 % who were cytology negative and HPV 18 positive developed CIN 3+ during this period; for all other high-risk HPV types combined, only 1.5 % developed CIN 3+, reinstating the importance of HPV genotyping. The FDA has approved two tests for HPV genotyping: Cervista HPV 16/HPV 18 (Hologic, Bedford, MA) and cobas HPV Test (Roche Diagnostics).

Cervista HPV 16/HPV 18 [13] is a qualitative, in vitro diagnostic test for the detection of DNA from high-risk HPV types: 16 and 18. The CervistaTM HPV 16/HPV 18 test uses signal amplification method for detection of specific nucleic acid sequences. It uses two types of isothermal reactions: a primary reaction that occurs on the targeted DNA sequence and a secondary reaction that produces a fluorescent signal. A final positive, negative, or indeterminate result for any particular sample is generated based on the analysis of two separate reaction wells.

The cobas® HPV Test [14] is based on automated specimen preparation to simultaneously extract HPV and cellular DNA followed by PCR amplification of target DNA sequences using both HPV and beta-globin-specific complementary primer pairs. The amplified signal from 12 high-risk HPV types (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) is detected using a common fluorescent dye, while HPV 16, HPV 18, and beta-globin signals are each detected with their own dedicated fluorescent dye. The results are determined as "positive," "negative," or "invalid" in each channel based on predefined parameters and Ct ranges. The ultimate result is determined as a combination of results from all four detection channels according to a predefined table.

careHPV test [15]*:* This is a new test which has been developed by Qiagen to detect high-risk HPV DNA. It is a screening test that is accurate and affordable. It can detect DNA from 14 high-risk types of HPV with the test results being available in about 2.5 hours. The test is based on the same principle of signal amplification as hybrid capture 2 and is only slightly less sensitive than it. As the test requires no electricity, no running water, and only 2.5 h to conduct, it is cheap and is a promising option in the low-resource settings.

6.3 Sample Collection

Studies have shown that HPV testing of self-collected vaginal swabs is less sensitive but as specific as HPV DNA for detecting high-grade cervical disease [16], with provider-collected cervical sample resulting in highest HPV DNA sensitivity of

84–100 % and sensitivity of 66–88 %. A cross-sectional mixed method study was conducted within the context of a cervical cancer screening demonstration project, the Screening Technologies to Advance Rapid Testing-Utility and Program Planning (START-UP) [17] project in India, Nicaragua, and Uganda, with the objective to generate evidence comparing screening options implemented by public health systems in regionally representative developing country settings. The studies show that self-sampling was highly acceptable and that a majority of women preferred self-sampling because it was more comfortable and less painful.

6.4 Clinical Application of HPV Testing

Initially in 1999, FDA approved HCII to be used as an adjunctive test for the triage of patients with equivocal cytology results (ASCUS) so as to determine the need for referral to colposcopy and later in 2003 approved it as primary screening together with cytology in women aged 30 years and older. Presently there are three clear indications for HPV testing:

1. Primary screening modality
   (a) Co-testing with cytology
   (b) hrHPV DNA testing alone
2. Triage of minor cytological abnormalities
3. Follow-up after treatment of CIN

6.4.1 Primary Screening Modality

Strategies of HPV testing for primary screening include:

6.4.1.1 Co-testing with Both HPV and Pap Smear

This is the most acceptable screening method at present. If both tests are negative, there is a very strong negative predictive value, and the tests need to be repeated after 5 years. As more data becomes available, this testing interval might be increased to 10 years.

The International Agency on Research on Cancer (IARC) recommends that the age at which screening begins should aim to maximize the detection of cervical pre-cancer cases while avoiding the large number of transient HPV infections. ACOG and ASCCP both reinforce the same and recommend the following (level A evidence):

- Cervical cancer screening should begin at age 21 years.
- Pap cytology screening is recommended every 3 years for women between the ages of 21 years and 29 years.
- For women aged 30–65 years, co-testing with cervical cytology screening and HPV testing is preferred and should be performed every 5 years.

Pap smear has been the gold standard for screening of cervical cancer for half a century. Its greatest advantage lies in its simplicity and ease of sample collection. But the advantage of the HPV test in comparison to the Pap smear lies in greater

sensitivity. This difference allows the screening interval to be increased. However, specificity is an important parameter in screening because it involves healthy women and positive results require a follow-up colposcopy which is costly and time-consuming. Specificity takes on more importance in low-resource settings where colposcopy is not available, and women who are screen positive would be treated in a "screen and treat" approach. Cuzick et al. [18] found HPV testing to be on average 25 % more sensitive than cytology (at an ASCUS threshold) but 6 % less specific for the identification of high-grade CIN. Agorastos et al. also found that HCII is 23 % more sensitive and 6 % less specific than cytology [19].

However, testing for high-risk types of HPV DNA has a very high negative predictive value. In a number of cross-sectional studies, the NPV of HPV DNA testing was consistently greater than 97 % using either the HCII or PCR-based assays, with most studies reporting values around 99 % and some even reporting 100 %. Castle and colleagues [20] analyzed a subcohort of 2020 women with negative results on cytologic evaluation but positive results with HC II assay who were followed up for a period of 57 months. It was found that 15 % of these women had an abnormal cervical smear within 5 years. Thus, HPV DNA testing identifies women who require closer surveillance, and over time the specificity of HPV DNA tests increases. The longest follow-up is for the Hammersmith Study, where only 0.42 % of women who were HPV negative developed CIN 2 or worse after 5 years compared to 0.83 % for women with negative cytology results.

6.4.1.2 Primary Screening with HPV Testing Followed by Triage of the Positive HPV Test by the Pap Smear

There is now growing evidence that cervical cancer screening needs to move away from cytology as a first-line screening test. A number of prospective follow-up studies have clearly shown that co-testing offers minimal benefit over HPV alone as the first-line screening test. For example, a review of co-testing results from over 300,000 women enrolled in Kaiser Permanente [21] found a minimal difference in the cumulative incidence of ≥CIN 3 after 5 years of follow-up among women who were co-test negative (incidence of 0.16 %) compared to women who were HPV negative (incidence of 0.17 %). After 6 years of follow-up, the cumulative incidence of >CIN 3 in HPV-negative women was 0.28 % compared to 0.27 % for women who were co-test negative.

A number of clinical trials have clearly documented the potential of using HPV for primary screening. One of the first studies, Canadian study, demonstrated the superior sensitivity of HPV as a primary screening test over cytology [22]. The study enrolled over 10,000 women, and it found that the sensitivity of HPV testing for ≥CIN 2 was 94.6 %, whereas it was only 55.4 % for cytology. Two additional large studies from Scandinavia found similar findings when they compared HPV alone versus cytology.

The NTCC [23] trial from Italy has also proven the safety and effectiveness of using HPV alone as the first-line screening test. This randomized screening trial included nearly 100,000 women with median age of 41 years and compared a cytology only arm with an HPV only arm and followed for up to 6 years with an additional round of screening. In the first round of screening, the relative detection of CIN 3 for HPV alone versus cytology alone was 2.08 in women 35–60 years.

Although there were a similar number of invasive cancers detected in the first round of screening in both arms, in the second round, nine additional women were diagnosed with cervical cancer women in the cytology arm versus none in the HPV arm; this difference presumably was because HPV testing identified more women at risk for developing invasive cancer in the first round of screening than did cytology.

In the ATHENA trial [24] that included more than 47,000 women, results showed that the HPV test used in the study performed better than the Pap test at identifying women at risk of developing cervical cancer precursor lesions. The greater assurance against future cervical cancer risk with HPV testing has also been demonstrated by a cohort study of more than a million women, which found that after 3 years women who tested negative on the HPV test had an extremely low risk of developing cervical cancer.

The ARTISTIC trial (A randomized trial of HPV testing in primary cervical screening) which involved 8873 women and median follow-up for 72 months also concluded that HPV testing as an initial screen was significantly more protective than cytology and the use of primary HPV screening could allow a safe prolongation of the screening interval. Following negative cytology at entry into the study, the cumulative rate of CIN 2+ was significantly higher than women who were HPV negative at baseline (1.41 % vs. 0.87 %) at 6 years. HPV as the sole primary test was also found to be cost-effective in both the vaccinated and non-vaccinated cohort [25].

Therefore, the Society of Gynecologic Oncology (SGO) and ASCCP issued an Interim Guidance Report in 2014 after the US FDA approved the cobas HPV test as a "primary" or first test performed for cervical cancer screening.

The Interim Guidance Report recommends [26]:

- Primary HPV testing can be considered for women starting at age 25.
- Women under age 25 should continue to follow current guidelines that recommend cytology alone beginning at age 21.
- Women with a negative primary HPV test result should not be retested again for 3 years. This is the same screening interval recommended under current guidelines for a normal cytology test result.
- An HPV test positive for HPV 16 and HPV 18 types should be followed with colposcopy.
- A test that is positive for HPV types other than 16 and 18 should be followed by reflex cytology testing.

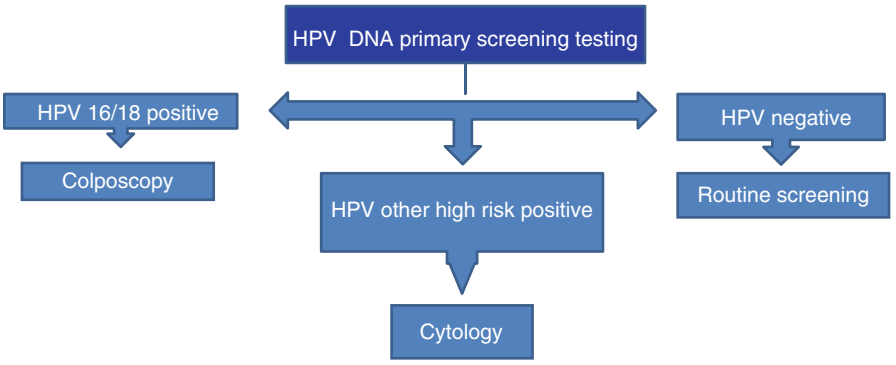


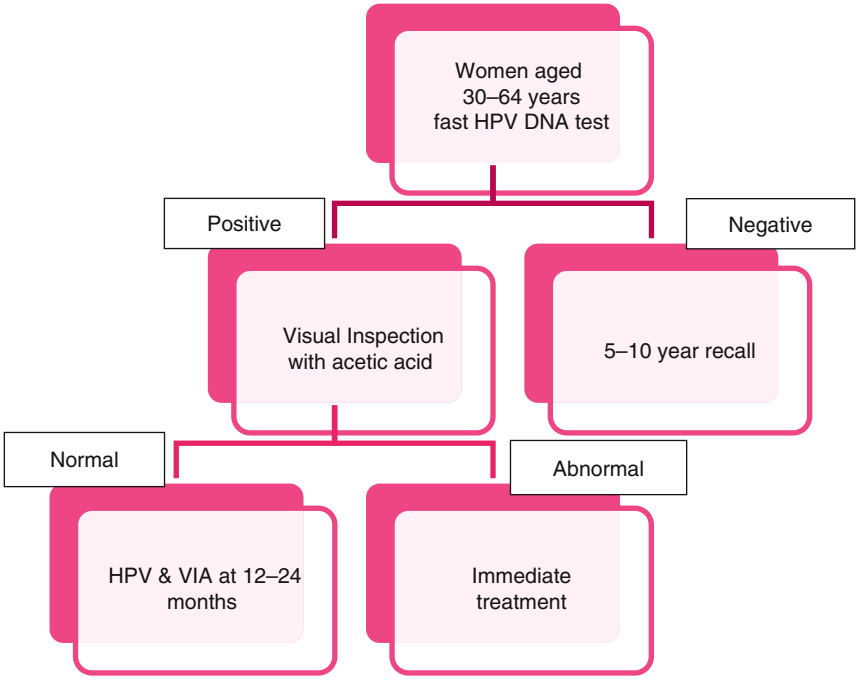
Table 6.1 Comparison of HPV testing, co-test, and cytology alone for primary screening [8]

	Sensitivity (%)	Specificity (%)
HPV	71.7	87.5
Co-testing	72.5	96.5
Cytology	63.8	97.4

Using a highly sensitive test such as HPV test as the primary test helps in picking up the suspicious cases, and applying a more specific cytology test reduces the number of referrals to colposcopy and biopsy. Women found to be HPV positive, but, with a negative or ASCUS cytology result, can be safely managed with repeated testing 12 months later.

Co-testing and LBC had higher positive predictive values for CIN 2+ (97.8 and 98.9 %) than primary HPV screening alone (91 %), whereas primary screening alone and co-testing demonstrated higher negative predictive values (63.6 and 62.5 %) than LBC alone (43.2 %) (Table 6.1).

Also the greater sensitivity of HPV DNA testing allows it to be used as a primary screening test followed by immediate "screen and treat" algorithm based on visual inspection tests in those who are HPV positive.



6.4.2 Triage of Minor Cytological Abnormalities

Initially, the US FDA accepted clinical use of HPV DNA testing only for the triage of women found to have ASCUS. Studies have shown that 5–20 % of cases with low-grade cytologic findings (ASCUS or LSIL) may have undetected high-grade

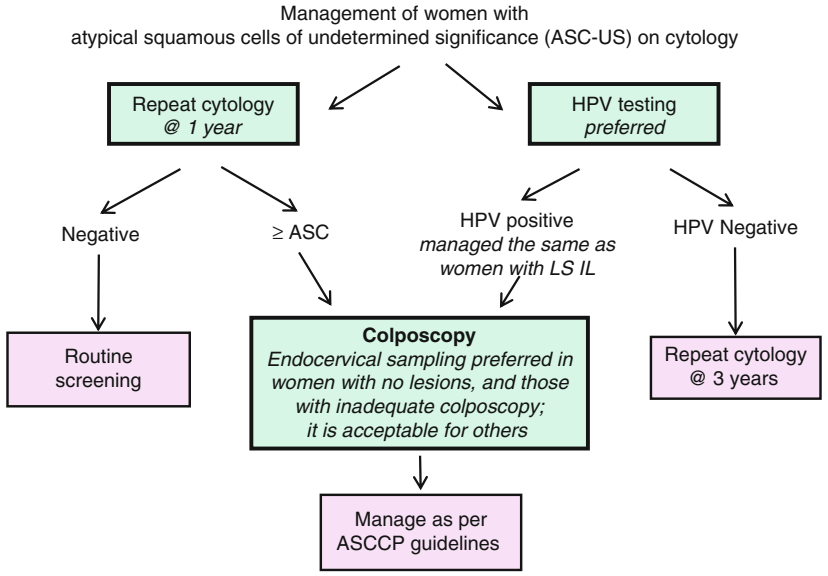
lesions. Thus, management of low-grade cytologic has been controversial, and options have included immediate colposcopy or repeated cytologic assessment at 6–12-month intervals. The ASCUS/LSIL Triage Study (ALTS) trial [27] is a large, randomized trial specifically designed to evaluate three methods of managing women with cytologic findings of ASCUS and LSIL. The three methods compared were immediate colposcopy, HPV testing, and referral for colposcopy if the results were positive and repeated cytologic assessment with referral for colposcopy if the smear showed the presence of HSIL. The study concluded that HPV testing was not useful in the management of women with LSIL on cytologic evaluation, and ASCCP recommends that these women should undergo colposcopy instead of HPV testing. With regard to ASCUS on cytologic evaluation, the trial found that HPV triage was at least as sensitive as immediate colposcopy for detecting grade 3 CIN and it also helped to decrease the number of colposcopy referals by 50 %.

The Cochrane Review (2013) also recommends triage with HCII for women with ASCUS as it yields higher accuracy, significantly higher sensitivity; relative sensitivity of 1.27 (95 % CI 1.16 to 1.39; *P* value <0.0001), and similar specificity; relative specificity: 0.99 (95 % CI 0.97 to 1.03; *P* value 0.98) than repeat cytology. When triaging women with LSIL, HCII gives a significantly higher sensitivity but a significantly lower specificity (relative specificity 0.66; 95 % CI 0.58 to 0.75, *P* <0.0001) compared to repeat cytology [28].

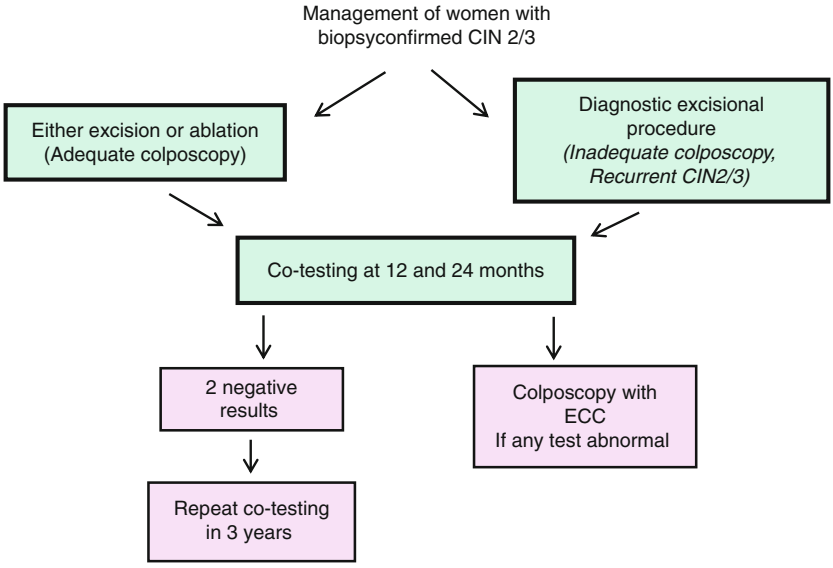
Consensus management guidelines developed by ASCCP for the follow-up of women with ASCUS include repeated cytologic assessment or HPV testing. However, if LBC was used for the cervical smear, then reflex HPV testing using the residual fluid is the preferred option, as it makes a second clinic visit unnecessary (Flow Chart 6.1) [29].

6.4.3 Follow-Up After Treatment of CIN

Ablative or excisional techniques for the treatment of cervical cancer precursors are reported to achieve more than 90 % cure rates. However, the precursor lesions will persist or recur in 5–15 %; thus, they need close follow-up and re-treatment once lesions have been identified again. Also, treated women remain at increased risk of cervical cancer for at least next 8 years. Earlier, a combination of cytologic and colposcopic assessment was used to follow up women posttreatment. But studies have shown that likelihood of posttreatment persistence or recurrence of disease is negligible in the absence of HPV DNA; HPV testing has recently been investigated as an alternative for "test of cure" of high-grade lesions following treatment. Paraskevaidis et al. in their review of literature on the role of HPV testing in follow-up period after CIN treatment concluded that the sensitivity of HPV testing in detecting treatment failures was very good and reached 100 % in few studies, but the specificity ranged from 44 to 95 % in various studies. Also in women who were treated successfully, 84.2 % had a negative postoperative HPV DNA test as compared to 17.2 % in treatment failures [29]. Zielinski et al. combined the result of 11 studies and estimated the NPV of hrHPV testing for recurrent/residual disease as 98 % and that of cytology as 93 %. When HPV was combined with cytology, the sensitivity was 96 % and NPV was 99 % [30]. The information gathered so far suggests HPV testing to be significantly more reliable than colposcopy and cytology.



Flow Chart 6.1 ASCCP recommendation (2013) for management of ASCUS



Flow Chart 6.2 ASCCP recommendation (2013) for management of biopsy-proven CIN 2/CIN 3

ASCCP also recommends co-testing at 12 and 24 months following excision or ablative procedure for CIN 2/CIN 3 as described in Flow Chart 6.2 [31].

Conclusion

HPV infection is necessary for development of cervical cancer, and HPV 16 and HPV 18 are responsible for 70 % of cervical precancers and cancers. Detection of

persistent HPV infection with high-risk types can be used for screening women with highest risk of developing cancer. The high negative predictive value of high-risk HPV detection tests can help in using this test as the stand-alone test for screening purposes. ASCCP has also recommended using HPV testing as a primary screen in women aged more than 25 years. With careHPV test becoming available soon, such testing will become a commercially viable option.

Key Points

- Persistence of hrHPV infection is the primary cause of cervical precancers and cancer.
- The detection of HPV in human cells is difficult as the viral proteins are expressed in low amounts and there is lack of specific antibodies against these proteins.
- The newer RNA-based amplification tests detect not only the presence of viral genomes but also the viral activity in infected cells.
- The addition of careHPV test for detection of hrHPV DNA will provide impetus to cervical cancer screening in low-resource settings. It is affordable and accurate, and the results are available in 2.5 h.
- Self-sampling for HPV testing is preferred by women, and it is as specific as HPV DNA testing.
- HPV testing is indicated as primary screening modality, for triage of minor cytological abnormalities and for follow-up of women treated for cervical dysplasia.

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