

Incidence of HPV infection in the uterine cervix in HIV positive pregnant women seen at the Hospital de Base in São José do Rio Preto, Brazil

Incidência de HPV em colo do útero de gestantes HIV positivas atendidas no Hospital de Base de São José do Rio Preto, SP

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ABSTRACT

Objective: To evaluate the incidence of HPV-HIV co-infection among pregnant women and to relate it to CD4+ cell count in peripheral blood, to the expression of protein p16 in the epithelium of the uterine cervix and to the presence of HPV DNA in cervical biopsies. **Methods:** Pregnant women at the same age were selected and distributed into two groups with 70 patients each: HIV positive (Study Group) and HIV negative (Control I). Another group (Control II) comprised 36 HIV negative patients at different ages and with the diagnosis of cervical intraepithelial neoplasia, in the period from 2000 to 2007. Colpocytology and/or cervical biopsy of these patients were analyzed for the occurrence of morphological characteristics, suggesting HPV infection; for the CD4 count in peripheral blood; and expression of protein p16 in the cervix epithelium and compared the findings with p16 expression in a group of 36 women with cervical epithelial neoplasm. PCR was performed in the samples of the Study Group to confirm the presence of HPV DNA. **Results:** The 70 HIV positive pregnant patients were aged from 15 to 45 years (mean of 28 years, median of 28.2 years). Twenty-two (31.4%) of them presented morphological changes consistent with cervical HPV infection; of these, 16 presented CD4 count lower than 500 cells ($p=0.03$). In the HIV negative Group, one patient (1.4%) had cervical intraepithelial lesion. Ten cervical biopsies in the Study Group presented HPV DNA in the PCR. The Control Group II showed similar results to the Study Group. **Conclusions:** The incidence of HPV-HIV co-infection in

pregnant women was relevant compared to the HIV negative Group. In the HIV-positive Group, the incidence of HPV was directly related to low immunity. The p16 expression was stronger among the cases diagnosed as having higher grade intraepithelial cervical lesions (CIN 2 and 3). The presence of HPV DNA was confirmed through PCR in cervical lesions of HIV positive pregnant women. This study points to the importance of having periodic gynecological exams and routine cytology screening tests for the early detection of HPV infection during antenatal care of HIV-positive pregnant women.

Keywords: HIV Seropositivity; Pregnancy complications; HIV; Papillomavirus infections

RESUMO

Objetivo: Avaliar a incidência de coinfeção HPV-HIV em gestantes e relacioná-la à quantidade de células CD4+ no sangue periférico; à expressão da proteína p16 no epitélio do colo uterino; e, finalmente, à presença de DNA de HPV nas biópsias cervicais. **Métodos:** Foram selecionadas gestantes da mesma idade em dois grupos com 70 pacientes em cada um: um HIV soropositivo (grupo de estudo) e outro soronegativo (controle I). Outro grupo (controle II) foi formado por 36 pacientes HIV negativas de idades variadas e com diagnóstico de neoplasia intraepitelial cervical, no período de 2000 a 2007. Foram analisadas colpocitologias e/ou biópsias dessas pacientes

Study carried out at Hospital de Base de São José do Rio Preto – São José do Rio Preto (SP), Brazil.

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Received on Feb 1, 2009 – Accepted on Aug 13, 2009

quanto à ocorrência de características morfológicas sugestivas de infecção pelo HPV; contagem de células CD4+ no sangue periférico; a expressão da proteína p16 no epitélio do colo uterino, elas foram comparadas com a mesma expressão em um grupo de 36 mulheres HIV soronegativas com neoplasia intraepitelial cervical; e PCR das amostras do grupo de estudo para confirmar a presença de DNA de HPV. **Resultados:** As 70 pacientes gestantes HIV positivas, tinham idade entre 15 e 45 anos, com média etária de 28 anos (mediana = 28,2 anos). Delas, 22 (31,4%) apresentaram alterações morfológicas compatíveis com infecção cervical pelo HPV; dessas pacientes, 16 apresentaram menos de 500 células CD4 positivas ($p=0,03$). No grupo HIV soronegativo, uma paciente (1,4%) apresentou lesão intraepitelial cervical. Dez biópsias cervicais do grupo de estudo apresentaram DNA de HPV pela PCR. O Grupo Controle II mostrou resultados semelhantes ao de estudo. **Conclusões:** A incidência de coinfeção HPV-HIV foi relevante em relação ao grupo de gestantes HIV negativas. Nas gestantes HIV positivas, a incidência de HPV mostrou-se diretamente relacionada com baixa imunidade. A expressão da proteína p16 mostrou índices de marcação maiores nos casos com diagnóstico de lesões do colo do útero mais graves (NIC 2 e 3). A presença de DNA de HPV foi confirmada em lesões cervicais de gestantes HIV positivas por meio de PCR. O presente estudo pôde constatar que existe necessidade de avaliações ginecológicas e colpocitológicas periódicas no pré-natal de pacientes HIV positivas, visando o diagnóstico precoce da infecção pelo HPV.

Descritores: Soropositividade para HIV; Complicações na gravidez; HIV; Infecções por Papilomavirus

INTRODUCTION

HIV, AIDS and CD4 positive blood levels

Little is known about HPV infection in pregnant women with HIV. In pregnant women, the consequences of this infection are significantly more severe and they may be lethal⁽¹⁾. The identification, in 1983, of HIV as the agent causing AIDS became a hallmark in the description of the pathophysiogenic mechanisms, as well as in the development of a specific therapy. Two molecularly distinct types of HIV are known: HIV-1, of universal distribution; and HIV-2, restricted to Western Africa. Approximately 75% of infections in Brazil are caused by the B subtype, followed by subtypes F (20%) and C (5%)⁽²⁾.

HIV, an essentially lymphocytotropic virus, with RNA nucleic acid, when in its free form (virion) and by means of the enzyme reverse transcriptase, integrates into cell nuclei, especially of helper lymphocytes (CD4) in the form of DNA (provirus). After the provirus is formed, HIV perverts the cell machinery to its own benefit, replicating and destroying the host cell.

The CD4 molecule, at the surface of helper T lymphocytes (T4), seems to function as the major cell receptor for HIV, allowing its entrance into the host cell. Currently, it is known that the relative or, especially, the absolute reduction of T CD4 lymphocytes is associated

with the appearance of opportunistic infections. Helper lymphocytes above 500/mm³ in peripheral blood reflect a good immunity level; levels between 200 and 500 cells/mm³, moderate immunodeficiency; and levels below 200 cells/mm³ indicate severe immunodeficiency⁽²⁾.

HIV-1 replication is massive during any phase of the viral infection. About 10 billion viral strains are produced and eliminated every day in an infected person. Likewise, two million T CD4 lymphocytes are produced and eliminated every day. Due to the constant struggle between the virus and immune system, the lymphocytes and thymus are wasted, and CD4 cell replenishment is difficult in the circulation. This immune disturbance will characterize the surge of AIDS, with risk of developing opportunistic infections and tumors⁽³⁾.

HPV-HIV co-infection and pregnancy

The fact of the increased incidence of invasive cervical cancer related to HPV infection is recognized by the US Center for Disease Control and Prevention, since 1992⁽⁴⁾.

Disease progression is affected by other factors such as host immunocompetence. Thus, the person with HIV is susceptible to secondary infections, including by HPV. In addition, the progression of an HPV lesion into tumor, is affected by the presence of HIV, considering that, in this case, the surveillance for cancer cells is hindered⁽⁴⁾.

Aside from the higher incidence, infection persistence is significantly higher among HIV positive patients when compared to women not infected by it. Also, the prevalence of HPV genotypes associated with genital warts is four to five times higher among HIV positive women and the presence of warts is three times higher in this group of women⁽⁵⁾.

During pregnancy, a characteristic immunomodulation occurs, increasing the HPV cases, and when pregnancy is associated with HIV infection, the pregnant woman becomes extremely susceptible to HPV infection⁽⁶⁾.

Transformation mechanism induced by HPV and progression to cancer

Cervical cancer is the first cancer 100% attributed to viral infection by the World Health Organization (WHO)⁽⁷⁾. HPV type 16 is the one mostly associated with cervical intraepithelial neoplasia (CIN) and cervical cancer, in Southern Africa and other developing countries, in which 50% of women with cervical disease are infected by HPV-16⁽⁸⁻⁹⁾.

Most HPV infections are transient and clinically not evident, with 70 to 90% of cases with spontaneous resolution in 12 to 30 months⁽¹⁰⁻¹¹⁾. However, high

risk HPVs (types 16, 18 and 31, for instance) need, often, to be integrated into the host genome⁽⁴⁾. The integration status of the viral genome is not sufficient for the viral life cycle, but it enables advanced growth by the increased expression of E6 and E7⁽¹²⁾, which are sequences of the viral DNA that cause functional inactivation of p53 and pRb, which are tumor suppressing proteins⁽¹³⁻¹⁵⁾. The p53 degradation, which is CDK-cyclin dependent, occurs in the transition from G1 to the S phase of the host cell cycle. On the other hand, pRb degradation, which is CDK2-cyclin E dependent, occurs in the S phase⁽⁴⁾.

The E6 gene is also known for inducing the expression of human telomerase, increasing the life span of infected squamous cells and, in contrast, there is an increase in the number of cancer cells due to cell death suppression. E7 along with proteins of the pRb family causes eventual degradation and release of E2F, activating the expression of genes required for cell DNA synthesis. Even if the DNA is damaged, the lack of p53 allows cell survival by means of an E7 induced S phase and replication of the viral genome⁽⁴⁾.

In addition to releasing E2F, the HPV E7 protein, when degrading the pRb protein, interferes in the p16^{ink4a}-CDK4-6/cyclinD-pRb pathway, releasing p16^{ink4a}. In the normal cell, the p16^{ink4a} is a negative regulator of cell cycle progression by means of the activity's verification of pRb tumor suppressor. The p16^{ink4a} inhibits the pRb phosphorylation by the cyclin D-CDK4/CDK6 complex. Hypophosphorylation of the pRb union to the transcription factor E2F inhibits them, in the G0 and early G1 phase of the cell cycle. In proliferating cells, pRb phosphorylation releases E2F and induces genes that mediate entry in the S phase⁽¹⁶⁻¹⁷⁾. The p16^{ink4a} overexpression does not inhibit cell cycle progression in these tumor cells when pRb is absent⁽¹⁶⁻¹⁷⁾.

The p16^{ink4a} overexpression is an indicator of the aberrant expression of E7 viral oncogenesis and of the transformation induced by HPV in epithelial cells. It was demonstrated, by means of immunohistochemistry, that there are several copies of p16^{ink4a} expressed in cervical dysplasia and carcinoma, when compared with normal epithelium. Therefore, the detection of p16^{ink4a} overexpression is, especially, used for the early diagnosis of high-risk HPV associated with cervical cancer⁽¹⁶⁻¹⁷⁾.

Pathophysiology of the HPV-HIV co-infection

HPV infection in the immunocompromised host is latent, controlled by immunological activity, tending to manifest itself clinically when there is immunological deficit. Thus, there is a frank association between HPV and HIV infections, and seropositive HIV

patients have higher prevalence of HPV induced lesions, especially those with helper lymphocytes (CD4 positive) below 500 cells/mm³⁽¹⁸⁾. HIV infection seems to induce HPV replication, intensifying the infectious process instigated by it and promoting reactivation and its persistence. Likewise, the cervical invasive lesions have a more aggressive and poorer prognosis. When HIV and HPV are concomitant, changes in the HPV genetic material seem to occur. This would render it more aggressive and, when associated with immunosuppression, it could cause lesions with greater carcinogenic potential⁽¹⁸⁾. From the biomolecular standpoint, the TAT-1 gene, coding for an HIV-1 regulatory protein, would be associated with magnified expression of HPV. The TAT-1 gene, combined with the E2 protein of the HPV DNA, like the 16 and 18, would enhance transactivation of the HPV's region regulatory sequence, thus stimulating the expression of the papillomavirus genes, even in patients without severe immune depression (CD4+ count higher than 500 cells/mm³)⁽¹⁸⁾. HPV infection may manifest itself at any HIV viral load or immune deficiency, both factors considered as viral replication activating agents. Tumoral response, on the other hand, is strong in the early stages of infection, becoming unstable with time⁽¹⁹⁻²⁰⁾.

Persistent HPV infection in the genital mucosa is an important predictive factor of intraepithelial neoplasia in seropositive patients. In these patients, the incidence of HPV infection is high, characterized by frequent relapses and disease progression, associated with higher therapeutic failure. There is also a higher prevalence of the high oncogenic risk types, such as HPV 16 and 18, along with the multiplicity of other viral subtypes when compared to women seronegative for HIV. Seropositive HIV patients usually present high viral load of HPV DNA. High viral load of oncogenic HPV, especially the 16, predisposes the appearance of high grade cervical lesion and invasive disease^(5,17-19).

Several reports of the outcome of invasive carcinoma in seropositive women demonstrate that the invasive cervical neoplasia is rapidly progressive to death. In a comparative study of 16 women infected with HIV and 68 seronegative women with invasive cervical cancer, 100% of HIV positive women had grade 2 or 3 carcinoma and 70% had grades III/IV compared to only 28% in HIV negative women. The occurrence of metastasis was significantly higher in these co-infected patients, mean survival was of only nine months compared to two years in the group of women not infected by HIV. This observation demonstrates the need for early diagnosis of HIV seropositive patients, as well as more aggressiveness in the HIV treatment of these co-infected women. Early detection of pre-invasive lesions is mandatory⁽²¹⁻²²⁾.

OBJECTIVES

To assess the incidence of HPV-HIV co-infection in pregnant women and relate it to the number of CD4 positive cells in peripheral blood; to the expression of p16 protein in the cervical epithelium; and to the presence of HPV DNA in cervical biopsies.

METHODS

Selection of research subjects

A group with 70 HIV positive pregnant women (Study Group) registered in the Pharmacy of the Outpatients Clinic of Hospital de Base de São José do Rio Preto, São Paulo state, in the period between January 2000 and December 2007.

Another group was randomly selected among the pregnant women delivering at the Department of Gynecology and Obstetrics of the same hospital, in the same period (2000 to 2007), which consisted of 70 HIV negative pregnant women with ages similar to those of the pregnant women of the Study Group (Control Group I).

The Control Group II was made up of 36 HIV negative women of different ages and with the diagnosis of CIN grades 1 (6 patients), 2 and 3 (22 patients) and invasive squamous carcinoma (8 patients).

The patients selected for the two groups underwent colposcopy and/or cervical biopsy before, during or right after pregnancy. The patients of Control Group II were randomly chosen among the patients who underwent cervical biopsy to confirm the routine colposcopy diagnosis, which revealed the diagnosis of squamous intraepithelial low or high grade lesion.

Diagnoses and studies performed

From the charts of pregnant women, the age during pregnancy and when the CD4 positive cells were counted in peripheral blood were obtained. These patients were sorted into two groups: CD4 positive < 500 cells and CD4 positive \geq 500.

Samples of the selected patients uterine cervix, obtained for cytological processing (Pap smear) or histological, biopsies for paraffin inclusion with histological slices 4 micra thick were used in the present study. The specimens were stained with hematoxylin-eosin (for the diagnosis of the cervical lesion), with immunohistochemistry using biomarker for the p16 protein or subjected to the polymerase chain reaction (PCR) for HPV DNA identification.

When present, the cervical lesions were classified according to the Bethesda nomenclature system

for colposcopy 2001 review. For the biopsies, the WHO classification was used: CIN 1, 2 or 3. No cases of invasive squamous carcinoma were detected in the selected patients in the study and Control I Groups.

The study of the smears and histological slices consisted in the assessment of the presence of morphological characteristics suggestive of HPV infection: koilocytosis (major sign) or keratinized scales, parakeratosis, dyskeratosis, bi or multinucleation and nuclear atypia (minor signs), which must be present in the same sample.

Histological slices subjected to immunostaining with p16 protein biomarker were used to confirm the presence of molecular cell cycle alteration of the epithelial cells, produced by HPV, which after releasing the protein p16, causes it to accumulate in the cell and be detected in an antigen-antibody reaction. For the quantitative assessment of p16, the relationship between the number of p16 immunolabeled cells among the counted cells were calculated, which were always \geq 100 epithelial cells per sample. This represents the positivity index for p16. We have also performed the qualitative analysis by observing the predominant localization of the labeled cells (lower 1/3, lower 2/3 or full epithelial thickness), the type of distribution (uniform and diffuse or uneven and multifocal) and the labeled sector in the cell (only the nucleus, the cytoplasm or nucleus and cytoplasm at the same time).

The PCR allowed for the retrieval of the HPV DNA from the cervical epithelial cells, revealing the presence or absence of the virus in the samples. For the DNA quality control, the retrieval of the glutathione S-transferase P1 gene (GSTP1) was done.

Presentation of results and statistical study

The data were shown in an Excel spreadsheet. It was, then, presented as a "database" of the Epi Info 2000 (version 3.4.1) packet, with a significance level of $p \leq 0.05$.

RESULTS

The 70 pregnant HIV positive patients of the Study Group were 15 to 45 years old (mean = 28 years, median = 28.2 years), of which six patients (8.6%) were younger than 20, 55 (78.6%) were 21 to 35 years and 9 (12.9%) were older than 36 years. HPV was found in 22 pregnant women (31.4%). In Control Group I, the age of the 70 pregnant women selected ranged from 16 to 43 years (mean = 27 years and median = 27.9 years). In this group, only one pregnant woman (1.4%) had HPV. When the incidence of HPV in the two groups was compared in regards to being HIV positive or not, $p=0.0015$ was found, which indicates the higher

incidence of cervical HPV as directly related to HIV positive patient.

Regarding CD4 positive cell count in peripheral blood, according to HPV infection, $p = 0.03$ was observed, showing that this relation was statistically significant. That is, of 40 patients (57.1%) with less than 500 CD4 positive cells, 16 (72.7%) had HPV. The association between the colposcopy diagnosis and the CD4 positive cell count was statistically valid, with $p = 0.0062$; the same was observed in the association between the colposcopy diagnosis and the biopsy of the same patient ($p = 0.02$).

It was observed that CD4 positive cell count in relation to the age of patients was not statistically significant, with $p = 0.24$. Of the 70 HIV positive patients, 34 (61.8%) who aged 21 to 35 years had less than 500 CD4 positive cells in the peripheral blood.

The relation between the incidence of infective agents, in colposcopy, during pregnancy and the number of CD4 positive cells in peripheral blood was not statistically valid ($p = 0.27$). However, there was a higher prevalence of infective agents in the pregnant women with less immunity (< 500 CD4 positive cells) (67.1%), with *Candida sp* present in ten pregnant women (14.3%), *Gardnerella vaginalis* in 14 patients (21.4%) and HPV in 22 (31.4%).

The frequency of biopsy diagnosis of the 22 pregnant women with HPV-HIV association was: 13 (59.1%) were CIN 1, and 9 (40.9%) CIN 2 and 3. Regarding the association of biopsy diagnosis and the persistence of the cervical lesion, of the 22 biopsies performed, four (18.2%) were persistent CIN 1 and two (9.1%), persistent CIN 2 and 3 ($p = 0.00001$).

The quantitative analysis of the biopsies with the p16 protein biomarker revealed the following positivity indices: in CIN 1 biopsies, 0.53 and 0.51, respectively, for Study and Control Group II; in CIN 2 and 3 biopsies, 0.76 (Study Group) and 0.69 (Control Group II), and; in invasive cervical squamous carcinoma biopsies, 0.89 (Control Group II).

When the indices were compared, one could see they were similar among themselves concerning the diagnosis ($p < 0.0005$) and showed evidences of statistically significant differences from one diagnosis to the other, that is, in CIN 1 the positivity index was smaller than CIN 2 and 3, which was smaller than that in invasive squamous carcinoma ($p < 0.0001$).

The qualitative analysis revealed:

- localization of the p16 expression in the cells: from the 13 cases with CIN 1, 38.5% were strongly (3 to 4+) only in the cytoplasm and in the other 30.8% it highlighted (3 to 4+) nuclei and cytoplasm; of the nine CIN 2 and three cases, 88.9% was positive (3 to 4+) only in the cytoplasm;

- localization of epithelial labeling of the 13 CIN 1 cases, 23.1% showed positive cells for p16 in the lower 1/3 of epithelial thickness and 38.5% in epithelial thickness lower 2/3; of the nine CIN 2 and 3 cases, 22.2% of epithelial thickness lower 1/3 and 44.4% in the lower 2/3;
- type of distribution of the p16 expressing cells: uniform and diffuse in all nine CIN 2 and three cases and 53.8% of 13 CIN 1 cases.

In Control Group II the proportions were similar.

Comparing the biopsy diagnoses with the presence of HPV as seen by PCR, it was observed that in nine CIN 1 biopsies and three CIN 2 and 3 biopsies, HPV DNA could not be retrieved. In 16 (72.3%) of the 22 biopsies, the GSTP1 could be retrieved and it was done as a quality control, and in ten (45.5%) of the cases the HPV DNA was found. To not find HPV DNA and GSTP1 gene in all 22 biopsies was a study bias caused by the small size of the epithelium with intraepithelial lesion in the biopsies, which were minimal. Even so, such associations were statistically significant with $p < 0.00001$.

DISCUSSION

In the present study an association between HPV-HIV was found there. The higher incidence of cervical HPV is directly related to seropositive HIV patients, with low immunity during pregnancy, since most HPV cases occur in patients with less than 500 CD4 positive cells, while in Control Group I (HIV negative) only one HPV case was found.

According to Quintana and Duarte⁽⁶⁾, during pregnancy, the activity and number of helper T lymphocytes and suppressor T cells are reduced. This improves until the sixth puerperium month in normal women, but this is not observed in women harboring HIV. On the other hand, G and A class immunoglobulins (IgG and IgA) decrease in the cervical mucus, HPV replication is enhanced because of the high levels of steroids secreted during pregnancy, which stimulate the viral estrogen receptors; the ease of HPV genome integration to the host cell, enhanced by progesterone and by diminished synthesis of macrophages and lymphocytes because of the high steroid hormone levels. All these factors render the HIV pregnant women extremely susceptible to HPV infection⁽⁶⁾.

Some studies, such as the Women's Interagency HIV Study (WIHS), which analyzed 2,015 HIV positive and 577 HIV negative women (paired controls) demonstrated that 58% of women had co-infection compared to 26% of women with HPV among the HIV seronegative. Data from this study showed that in HIV seropositive patients there is an increased prevalence

of HPV infection, in women with greater immune deficiency, as well as a higher incidence of types 16 and 18 HPV, of higher oncogenic power⁽²³⁾.

A higher prevalence of infectious agents in pregnant women with lower immunity, which agrees with other authors⁽²⁴⁾ regarding the findings of *Candida sp*, *Gardnerella vaginalis* and HPV, was observed. However, the potential for the immune system to enhance the immune response with specific antigens for HPV requires other studies, in order to better understand the role of HIV in pregnancy and on how to prevent other infections during this period, especially, caused by HPV⁽²⁵⁾. The use of antiretroviral drugs seems to improve the immune response and then allowing better response against HPV during co-infection⁽²⁶⁾. This should be our next step in the future.

HPV-induced morphological changes were identified first by colposcopy, confirmed by cervical biopsy, with concordant diagnosis. Less than 20% of CIN 1 and less than 10% of CIN 2 and 3 became persistent after pregnancy. The Pap-smear screening for HIV positive women should be performed more often during and after pregnancy, aiming at diagnosis of lesions as early as possible. Kitchener et al.⁽²⁷⁾ stated that cytology seems enough for cervical surveillance. Levi et al.⁽²⁸⁾, analyzing 208 HPV infected women, found virtually all HIV seropositive (98%) by using PCR, 80% of them were infected by several HPV genotypes (in average 3.1 genotypes per patient) and however, 90% of these women showed inflammatory cytology.

Immunohistochemistry for the p16 protein showed the presence of HPV in the cervical epithelium. In the present study, results were similar to those of a previous one and those of the literature⁽²⁹⁻³⁰⁾. The presence of HPV DNA was confirmed by PCR. This reaction is used in most epidemiological and molecular studies and is becoming a helpful and sensitive tool for detection of oncogenic HPV⁽³⁰⁾. In the follow-up of this study we will attempt to identify the HPV types found in HIV positive pregnant women.

CONCLUSIONS

The incidence of co-infection was relevant compared with HIV negative pregnant women. In the HIV positive women, the incidence of HPV was directly related to low immunity. The p16 protein expression was greater in cases with the most severe cervical lesions (CIN 2 and 3). The presence of HPV DNA was confirmed in cervical lesions in the HIV positive pregnant women by means of PCR. Hence, there is the need for gynecological and colposcopic assessments periodically in the prenatal period in HIV positive patients aiming at early diagnosis of HPV infection.

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