Increased HPV L1 gene methylation and multiple infection status lead to the difference of cervical epithelial cell lesion in different ethnic women of Xinjiang, China

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Abstract

Human papillomavirus (HPV) L1 gene methylation deeply involved in the progression and heterogeneity of cervical cell epithelial lesions. The DNA ploidy also represented the early lesions of cervical cell, and it was associated with different HPV infection status in different ethnic women. So, the research was to explore whether it was possible that HPV L1 gene methylation and HPV infection status as the risk factors to lead to the differences of cervical epithelial cells’ lesions in different ethnic women.

The flow-through hybridization and gene chip for HPV genotypes test, general characteristics, and cervical exfoliated cell samples were collected from 94 Uygur and 79 Han women with HPV-16 infection. The cases were divided into the single HPV-16 (sHPV-16) infection group and multiple HPV-16 (mHPV-16) infection group in each ethnic women. The DNA ploidy was analyzed by flow cytometry, and the methylation-sensitive high resolution melting (MS-HRM) was used to test the HPV-16 L1 gene methylation, the results of methylation was segmented into mild methylation, moderate methylation, and severe methylation groups. Multifactor logistic analysis explored the relation between DNA heteroploid and HPV-16 infection status, HPV-16 L1 gene methylation in different ethnic women.

The higher proportion of mHPV-16 infection in Uygur than Han women (61.7% vs 38.0%). L1 gene methylation had statistic difference between single and mHPV-16 infection under the same ethnic women. The proportion of DNA heteroploid had statistic difference between different HPV-16 infection status or different L1 gene methylation grades in Han or Uygur women. Both L1 gene methylation and HPV infection status were the risk factors of DNA heteroploid. Compared to the sHPV-16 infection, the odds ratio (OR) of mHPV-16 infection were 4.409 (CI: 1.398–13.910) and 3.279 (CI: 1.069–10.060) in Han and Uygur women. Compared the mild L1 gene methylation, the OR of moderate L1 gene methylation were 3.313 (CI: 1.002–10.952) and 5.075 (CI: 1.385–18.603) in Han and Uygur women, the OR of severe L1 gene methylation were 20.592 (CI: 3.691–114.880) and 63.634 (CI: 10.400–389.368) in Han and Uygur women.

The study first reported that HPV L1 gene methylation and HPV infection status were the risk factors to the DNA heteroploid of cervical cell in different ethnic women, HPV L1 gene methylation and infection status should be recommended to the existing system of cervical lesion screening in order to provide better serves for the HPV infected women, especially for the ethnic women with high proportion of severe L1 gene methylation and multiple infection status.

Abbreviations: DI = DNA index, HPV = human papillomavirus, mHPV-16 = multiple HPV-16, MS-HRM = methylation-sensitive high resolution melting, OR = odds ratio, sHPV-16 = single HPV-16, SPF = S-phase cells’ peak percentage.

Keywords: cervical epithelial cell, DNA ploidy, human papillomavirus, L1 gene, methylation
1. Introduction

Cervical cancer occurs in the womb malignant tumors of the vagina and cervix tube. In the developing countries, cervical cancer has the highest incidence in gynecological tumors.\(^{[1]}\) It was the 8th high incidence cancer for women in the People’s Republic of China, the general trend is higher incidence in rural than urban area, and the prevalence shows younger trend.\(^{[2]}\) Xinjiang region had the highest incidence of cervical cancer in China, the incidences of cervical cancer were different in 2 major ethnic of Xinjiang, including Han and Uygur ethnic.\(^{[3]}\)

Human papillomavirus (HPV) infection especially high-risk type HPV infection was a major cause of cervical lesions. There were numerous studies of HPV about cervical lesions, which focused on the relationship between cervical lesions and HPV-related gene and protein, such as L1 protein, L2 protein, E6, and E7 gene.\(^{[4-7]}\) The L1 protein as the major capsid protein of HPV played an important role to recognize the host cell and keep persistent infection, which was a good index to evaluate the infection state in host cell.\(^{[8]}\) Previous studies showed that the quantity of L1 protein was declining with aggravate of cervical cell lesion, L1 gene was the coding gene of L1 protein, its methylation was the major reason of L1 protein decreasing, which showed positive correlation to the degree of cervical lesions.\(^{[9,10]}\) So, L1 gene methylation deeply involved in the progression and heterogeneity of cervical lesions, which was the potential clinical molecular target of cervical lesions to early diagnose and monitor the prognosis.\(^{[11]}\)

DNA ploidy of cervical epithelial cells was contributed to monitor the lesion of HPV infected cervical cells and the prognosis of treatment.\(^{[12-14]}\) Our previous studies had proved that single and multiple HPV infection status could influenced on the DNA ploidy of cervical exfoliated cells in Xinjiang women.\(^{[15]}\) Meanwhile, we also found that, when the proportion of DNA heteroploid had no difference between Uygur and Han women in Xinjiang when they were in the same HPV infection status, but DI and S-phase cells’ peak percentage (SPF) as quantitative index of DNA ploidy had differences,\(^{[16]}\) which was contradictory. We speculated that the persistent/ transient infection and single/multiple infection primary lead to the contradictory. Because L1 gene methylation reflected the persistent or transient infection of HPV infection in the host cell, so it was speculated that, the L1 gene methylation and single/multiple infection should explain the contradictory.

In conclusion, the research was to explore whether it was possible that HPV L1 gene methylation and HPV infection status as the risk factors to lead to the differences of cervical epithelial cell lesions in different ethnic women.

2. Methods

2.1. Patients

The sample cases sourced from Xinjiang Uygur and Han women, who initially visited the gynecology department of the Tumor Hospital Affiliated to Xinjiang Medical University from July 2015 to October 2016. The chosen cases must not accept any HPV-related treatment and HPV vaccine. A total of 173 HPV-16 genotype infected cases were collected, including 94 Uygur women and 79 Han women. At the same time, their general case characteristics were also collected. The ethics committee of the tumor hospital affiliated to Xinjiang Medical University approved the study and the consent procedure. The samples of flow cytometry DNA ploidy analysis, HPV genotype test, and HPV-16 L1 gene methylation were cervical exfoliated cells, which were collected as required by clinicians. The insufficient or polluted samples were ruled out. The research related to human had been complied with all the relevant national regulations, institutional policies, and in accordance with the tenets of the Helsinki Declaration, and had been approved by The Tumor Hospital Affiliated to Xinjiang Medical University institutional review board.

2.2. Reagents and instruments

The HPV genotype test used the 21 HPV GenoArray Diagnostic Kit from ChaoZhou Hybridbio Biological Chemical Co. Ltd. (People’s Republic of China). The method of HPV genotype test was flow-through hybridization and gene chip, the equipments for the test such as Thermal Cycler and HybriMax devices (Flow-through Hybridization HybriMax).

The DNA ploidy analysis kit was from the Beckman Coulter; the flow cytometer was Beckman CytomicsTM FC500. The DNA cell cycle analysis software was also from Beckman Coulter.

The L1 gene methylation level was tested by the methylation-sensitive high resolution melting (MS-HRM). The major instrument was Roche LightCycler type 480 sensitivity analyzer. The completely methylated and unmethylated HPV-16 L1 gene standards of MS-HRM were synthesized (Genscript, Nanjing, China), the specific primers also were synthesized (Genscript, Nanjing, China). The EpiTect Bisulfite Kit and EpiTect HRM PCR Kit were bought from Germany QIAGEN company.

2.3. Experimental procedure

2.3.1. Flow cytometry DNA ploidy analysis.

1. Collected the exfoliative cytology specimens, which were in cell preservation solution. Then through the 300mesh nylon mesh filter, 1500 r/s centrifugal for 10 minutes, discarded the liquid supernatant, and then repeated this process by adding PBS fluid to the sediment, finally, suspended the exfoliated cells with 1mL phosphate buffered saline (PBS) solution.

2. Added 200μL DNA-Prep LPR reagent into the above solution that blended immediately, and placed it for 5 seconds; then added 2mL of the DNA-Prep Stain reagent into it. Incubated for 20 minutes in dark place. Last, tested the specimen by Flow Cytometry DNA Ploidy Analysis System of FC500 flow cytometer.

3. Applied the DNA ploidy analysis software (DNA cell cycle analysis software) to analyze the results and obtained the DNA index (DI) and SPF of each specimen.

2.3.2. Flow-through hybridization and gene chip for HPV genotype test. HPV genotype test was carried out by the steps of HPV GenoArray Diagnostic Kit, which could detect 21 HPV genotypes, including 6, 11, 16, 18, 31, 33, 35, 39, 42, 43,44, 45, 51, 52, 53, 56, 58, 59, 66, and 68 types, and CP8304 types.

1. Extracted the HPV viral DNA by DNA extraction kit.

2. Took 1μL of extracted DNA solution and then did PCR amplification according to the instructions in the reaction system by PCR amplification.

3. Made diversion hybridization amplification for amplified DNA samples.

4. Made hybridization results analysis of hybrid membrane after coloration; corresponding color parts’ classification is the result.
2.3.3. MS-HRM analysis of the HPV-16 L1 gene. The sequence HPV-16 L1 localized from nucleotide (nt) 5576 to (nt) 5636 (NCBI accession no. NC_001526.2), which contains 4 CpG sites (nt 5602, nt 5608, nt 5611, and nt 5617) were tested.

1. Mixed the completely methylated and unmethylated HPV-16 L1 gene standards in 0%, 10%, 25%, 50%, 75% and 100% methylated to unmethylated template ratios, which served as the methylation standards for MS-HRM.

2. Extracted the HPV viral DNA by DNA extraction kit.

3. The methylation standards and all extracted HPV viral DNA were bisulfite modified, the detailed steps referred to the instruction book of EpiTect Bisulfite Kit.

4. The specific PCR primers used were that, forward primer: 5′ GCCGCACTAAACAAC-CAAAAAACATCTAAAAAATA 3′, reverse prime: 5′ GCCGCATTATGGTGGTAGTGGATTATTTTATTTATA-TTTTAG3′, reverse primer: 5′ GCCGCACTAAACAAC-CAAAAAACATCTAAAAAATA 3′. The detailed steps of MS-HRM PCR referred to the handbook of EpiTect HRM PCR.

5. The HRM data were analyzed using the Genescanning Software (Roche).

2.4. Statistical analysis

The result was showed by mean ± standard, if the data were normally distributed; the statistical analysis was processed by SPSS 18.0 software. Comparison of count data models was by chi-square test. Multivariate logistic regression analysis was used to test the risk factors, α = 0.05 is the inspection level, and P < 0.05 was received as having statistical differences.

Table 1
The general characteristics of 173 cases.

|                | Han (n=79) | Uygur (n=94) | χ² value | P     |
|----------------|------------|--------------|----------|-------|
| Age            |            |              |          |       |
| <35 years old  | 7          | 17           | 6.485    | 0.039 |
| 35–55 years old| 41         | 55           |          |       |
| >55 years old  | 31         | 22           |          |       |
| Marriage status|            |              |          |       |
| Married        | 77         | 90           | 0.381    | 0.685 |
| Unmarried      | 2          | 4            |          |       |
| Childbearing history | |          |          |       |
| 0 time        | 3          | 3            | 14.410   | 0.002 |
| 1–2 times     | 39         | 21           |          |       |
| 3 times       | 20         | 39           |          |       |
| >3–times      | 17         | 31           |          |       |
| Abortion history |         |              |          |       |
| Yes           | 9          | 19           | 2.462    | 0.117 |
| No            | 70         | 75           |          |       |

3. Results

3.1. The general characteristics of 173 cases

The 4 characteristics factors between 2 ethnics were collected and compared such as age, marriage status, childbearing history, and abortion history, which was related to the HPV infection. The detail result is given in Table 1.

3.2. The HPV infection situation of 173 cases

The HPV-16 infected women were divided into 2 groups in each ethnic women, including single HPV-16 (sHPV-16) infection (only HPV-16 infection) and multiple HPV-16 (mHPV-16) infection (existing HPV-16 infection and other HPV genotype infection at the same time). Then, the differences of infection status between 2 ethnics were compared. The results are shown in Table 2.

3.3. Comparison of HPV-16 L1 gene methylation in different HPV infection status between Han and Uygur women

The result of L1 gene methylation was divided into 3 grades, including mild methylation group (L1 gene methylation less than 25%), moderate methylation group (L1 gene methylation between 25% and 50%), and severe methylation group (L1 gene methylation more than 50%). The differences between 2 ethnics and 2 HPV-16 infection status were compared. The results are shown in Table 3.

3.4. Comparison of DNA ploidy in different HPV-16 infection status between Han and Uygur women

The result of DNA ploidy was shown as DI and SPF, DI = 1.10 was the threshold of the DNA ploidy results; if a sample’s DI was more than 1.10, it was seen as positive of DNA ploidy analysis, which meant heteroploid. If not, the sample was seen as negative of DNA ploidy analysis. The differences between 2 ethnics and 2 HPV-16 infection status were also compared. The results are shown in Table 4.

Table 2
Comparison of the HPV infection status in Han and Uygur women.

|                | Han (n=79) | Uygur (n=94) | χ² value | P     |
|----------------|------------|--------------|----------|-------|
| sHPV-16 infection | 49/79 (62.0%) | 36/94 (38.3%) | 9.669 | 0.002 |
| mHPV-16 infection | 30/79 (38.0%) | 58/94 (61.7%) |      |       |

HPV = human papillomavirus, mHPV-16 = multiple HPV-16, sHPV-16 = single HPV-16.

Table 3
Comparison of L1 methylation status between 2 ethnics and 2 HPV-16 infection status.

| Ethnic | HPV infection status | Mild methylation | Moderate methylation | Severe methylation |
|-------|----------------------|------------------|----------------------|--------------------|
| Han   | sHPV-16 infection    | 30/49 (61.2%)    | 13/49 (26.5%)        | 6/49 (12.3%)       |
|       | mHPV-16 infection    | 8/30 (16.7%)     | 10/30 (33.3%)        | 12/30 (40.0%)      |
| Uygur | sHPV-16 infection    | 17/36 (47.2%)    | 12/36 (33.3%)        | 7/36 (19.5%)       |
|       | mHPV-16 infection    | 9/56 (16.1%)     | 23/56 (41.1%)        | 26/56 (46.8%)      |

L1 gene methylation had no statistic difference in Han or Uygur women when the same HPV-16 infection status (P=0.452 and 0.414). For Han women, L1 gene methylation had statistic difference between single and multiple HPV-16 infection (x² = 11.207, P=0.004). For Uygur women, L1 gene methylation also had statistic difference between single and multiple HPV-16 infection (x² = 12.388, P=0.002).
Table 4
Comparison of DNA ploidy between 2 ethnics and 2 HPV-16 infection status.

| Ethnic      | HPV infection status | DNA ploidy |       |       |
|-------------|----------------------|------------|-------|-------|
|             |                      | Di value   | SPF value | Heteroploid |
| Han         | sHPV-16 infection    | 1.005±0.076| 5.900±0.863| 14/49 (28.6%) |
|             | mHPV-16 infection    | 1.151±0.084| 6.928±1.074| 22/30 (73.3%) |
| Uygur       | sHPV-16 infection    | 1.107±0.083| 6.908±1.016| 12/36 (33.3%) |
|             | mHPV-16 infection    | 1.208±0.135| 8.256±1.730| 42/58 (72.4%) |

For HPV-16 infection, Di and SPF had statistic difference between Han and Uygur women (P=0.041 and 0.032). For mHPV-16 infection, Di and SPF also had statistic difference between Han and Uygur women (P=0.038 and 0.029). But rate of heteroploid had no statistic difference between Han and Uygur women under same HPV-16 infection status (P=0.658 and 0.714). Di, SPF, and rate of heteroploid had statistic difference between single and multiple HPV-16 infection in same ethnic (P<0.01). Di=DNA index, HPV=human papillomavirus, mHPV-16=multiple HPV-16, shHPV-16= single HPV-16, SPF=S-phase cells’ peak percentage.

Table 5
Comparison of Di, SPF in different L1 gene methylation grades.

| L1 gene methylation | Han ethnic | Di | SPF | Uygur ethnic | Di | SPF |
|---------------------|------------|----|-----|--------------|----|-----|
| Mild methylation    | 1.042±0.061| 5.938±0.849 | 1.054±0.039 | 6.349±0.869 |
| Moderate methylation| 1.120±0.077| 6.374±1.051 | 1.141±0.095 | 7.404±1.164 |
| Severe methylation  | 1.189±0.065| 7.170±0.972 | 1.290±0.099 | 9.246±1.252 |

For the Han women, Di and SPF exist differences among the 3 groups (P<0.001). Tamhane pairwise comparison, Di had differences between mild, moderate, and severe methylation (P<0.001), moderate and severe methylation (P=0.011). SPF also had differences between severe, mild, and moderate methylation (P<0.001 and P=0.049). For the Uygur women, Di and SPF existed differences among the 3 groups (P<0.001). Tamhane pairwise comparison, Di or SPF existed differences between any 2 methylation status (P<0.001). Di=DNA index, SPF=S-phase cells’ peak percentage.

Table 6
Comparison of HPV-16 L1 gene methylation and HPV-16 infection status in different DNA ploidy status for Han women.

| DNA ploidy | shHPV-16 infection | mHPV-16 infection |       |       |       |       |
|------------|--------------------|--------------------|-------|-------|-------|-------|
|            | Mild methylation   | Moderate methylation| Severe methylation | Mild methylation | Moderate methylation | Severe methylation | Total |
| +           | 4                  | 5                  | 5     | 4     | 7     | 11    | 36    |
| –           | 26                 | 8                  | 1     | 4     | 3     | 1     | 43    |
| Total       | 30                 | 13                 | 6     | 8     | 10    | 12    | 79    |

The proportion of DNA heteroploid had statistic difference between different HPV-16 infection status (χ²=15.031, P<0.001); the proportion of DNA heteroploid also had statistic difference in different L1 gene methylation grades (χ²=23.231, P<0.001). HPV16 status = human papillomavirus, mHPV-16=multiple HPV-16, shHPV-16= single HPV-16.

Table 7
Comparison of HPV-16 L1 gene methylation and HPV-16 infection status in different DNA ploidy status for Uygur women.

| DNA ploidy | shHPV-16 infection | mHPV-16 infection |       |       |       |       |
|------------|--------------------|--------------------|-------|-------|-------|-------|
|            | Mild methylation   | Moderate methylation| Severe methylation | Mild methylation | Moderate methylation | Severe methylation | Total |
| +           | 2                  | 4                  | 6     | 2     | 15    | 25    | 54    |
| –           | 15                 | 12                 | 7     | 9     | 23    | 26    | 94    |

The proportion of DNA heteroploid had statistic difference between different HPV-16 infection status (χ²=13.878, P<0.001); the proportion of DNA heteroploid also had statistic difference in different L1 gene methylation grades (χ²=36.938, P<0.001). HPV16 status = human papillomavirus, mHPV-16=multiple HPV-16, shHPV-16= single HPV-16.

3.6. Comparison of HPV-16 L1 gene methylation and HPV-16 infection status in different DNA ploidy status

Because Di or SPF had statistic difference in different L1 gene methylation grades; therefore, respectively further compared the HPV-16 L1 gene methylation and HPV-16 infection status in different DNA ploidy status, the results show in Tables 6 and 7.

3.7. Multifactor analysis between HPV-16 infection status, HPV-16 L1 gene methylation, and heteroploid of DNA ploidy

Because both L1 gene methylation and HPV-16 infection status had statistic differences in different DNA ploidy status for Uygur women or Han women, so respectively discussed the relationship between HPV-16 infection status, HPV-16 L1 gene methylation, and heteroploid of DNA ploidy by logistic regression analysis in Han or Uygur women. The corresponding logistic regression expression was:

For Han women:

\[
\text{Logit P} = -1.735 + 1.484 \times \text{HPV16 status} + 1.198 \\
\times \text{L1 gene methylation (moderate)} + 3.025 \\
\times \text{L1 gene methylation (severe)}
\]

For Uygur women:

\[
\text{Logit P} = -2.229 + 1.188 \times \text{HPV16 status} + 1.624 \\
\times \text{L1 gene methylation (moderate)} + 4.153 \\
\times \text{L1 gene methylation (severe)}
\]
Tables 8 and 9 show the results of multivariate logistic regression analysis. Therefore, HPV-16 infection status, HPV-16 L1 gene methylation were the risk factors which were significantly associated with increased risk of DNA heteroploid. The odds ratio (OR) is displayed in Figs. 1–3.

4. Discussion

The study researched 173 HPV-16 infected women in Xinjiang region by completely randomized design, including 94 Uygur and 79 Han women. It was found that there were differences in the risk factors of age and childbearing history by analyzing the general characteristics. The age of Uygur infected HPV-16 women was younger than Han women, meanwhile, the Uygur women showed more childbearing time than Han women, all of these fit on the common characteristics of Uygur and Han women in Xinjiang.\(^{[17]}\)

The infection status of HPV-16 genotype included sHPV-16 and mHPV-16 infection. In this study, the proportion of mHPV-16 infection in Uygur women was much higher than Han women (61.7% vs 38.0%). So, the mHPV-16 infection was more common in Uygur, because high-risk multiple HPV infection was easy likely to cause the lesion of cervical epithelial cells in the previous report.\(^{[15]}\) So, the cervical lesion because of HPV infection was more common in Uygur women.

### Table 8

| Risk factors                | β    | Wald x² value | P     | OR value | OR (95% CI) |
|----------------------------|------|---------------|-------|----------|-------------|
| Constant                   | 1.735| 14.176        | <0.001| 0.176    |             |
| Infection status           | 1.484| 6.407         | 0.011 | 4.409    | 1.398–13.910|
| L1 methylation status      |      |               |       |          |             |
| Methylation status (moderate) | 1.198| 3.854         | 0.049 | 3.313    | 1.002–10.952|
| Methylation status (severe) | 3.025| 14.176        | 0.001 | 20.592   | 3.691–114.880|

CI = confidence interval, OR = odds ratio, sHPV-16 = single HPV-16.

\(\ast\) Compared to the sHPV infection.

\(\dagger\) Compared to the methylation status (mild).

### Table 9

| Risk factors                | β    | Wald x² value | P     | OR value | OR (95% CI) |
|----------------------------|------|---------------|-------|----------|-------------|
| Constant                   | 2.229| 12.282        | <0.001| 0.108    |             |
| Infection status           | 1.188| 4.311         | 0.038 | 3.279    | 1.069–10.060|
| L1 methylation status      |      |               |       |          |             |
| Methylation status (moderate) | 1.624| 6.008         | 0.014 | 5.075    | 1.385–18.603|
| Methylation status (severe) | 4.153| 20.195        | <0.001| 63.634   | 10.400–389.368|

CI = confidence interval, OR = odds ratio, sHPV-16 = single HPV-16.

\(\ast\) Compared to the sHPV infection.

\(\dagger\) Compared to the methylation status (mild).

Figure 1. The odds ratio of mHPV-16 infection status compared to sHPV-16 infection status for DNA heteroploid in Han and Uygur women. mHPV-16 = multiple HPV-16, sHPV-16 = single HPV-16.

Figure 2. The odds ratio of moderate L1 gene methylation compared to mild L1 gene methylation for DNA heteroploid in Han and Uygur women.
methylation. According to the results of MS-HRM, the MS-HRM was a feasible method to detect HPV-16. It also be reported that the gene methylation, which could make semiquantitative measurement of specimens by DI value and SPF value, meanwhile, the phenomenon proved that DNA methylation could effect the DNA heteroploid, so logistic multifactors regression analysis was respectively done for Han and Uygur women. DNA heteroploid as dependent variable, with infection status and L1 gene methylation as potential influence factors, it was explored the risk level of infection status and L1 gene methylation to generate the DNA heteroploid. Infection status and L1 gene methylation were the risk factors to cause DNA heteroploid. When L1 gene methylation unchanged, it was 4.409 times to appear DNA heteroploid in multiple infection than single infection in Han women, in Uygur women, the OR was 3.279, the results accord with previous studies. Compared to the Uygur women, HPV-16 infection status seemed to be more influence on Han women (OR: 3.297 vs 4.409). And L1 gene methylation created larger influence on Uygur women than Han women, in the same HPV infection, with a mild degree of L1 gene methylation as reference, Uygur women increase 5.075 and 63.634 times to appear DNA heteroploid when L1 gene methylation changed to moderate and severe grade, but the 2 OR values were 3.313 and 20.592 in Han women, which was smaller than Uygur women.

In a word, the research explored the influence of HPV-16 infection status and HPV-16 L1 gene methylation on DNA ploidy of cervical cell, discovered that both HPV-16 infection status and L1 gene methylation should be the risk factors of DNA ploidy. Especially L1 gene methylation had the greatest influence on the DNA heteroploid of cervical cell in Uygur women, it should be recommended to introduce the L1 gene methylation as a potential index to the existing system of cervical lesion screening and treatment standard of HPV infection in order to provide better serves for the masses of HPV-16 infected women.

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