Performance of P16 INK4a immunocytochemical stain in facilitating cytology interpretation of HSIL for women aged 50 and above

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Research Article

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Abstract

Background

Few articles have focused on cytological misinterpretation of the high-grade squamous intraepithelial lesion (HSIL) in older women. Due to estrogen deficiency, cervical epithelial cells in postmenopausal women tend to show atrophic change that looks like HSIL, resulting in a higher rate of cytological misinterpretation. P16\textsuperscript{INK4a} immunocytochemical staining (P16-cytology) can effectively differentiate diseased cells from normal atrophic ones with less dependence on cell morphology.

Objective

To evaluate the performance of P16-cytology in differential analysis of cytology high-grade squamous intraepithelial lesions and benign atrophy in women aged 50 years and above.

Methods

Included in this analysis were the women who were positive of hr-HPV in a cervical cancer screening project in central China and returned back for triage, with complete data of primary hr-HPV test, Liquid-based cytology, P16 immuno-stained cytology, and patholosgy diagnosis. Included patients were divided into $\geq$ 50 (1,127 cases) and < 50 years (1,430 cases) of age groups. The accuracy of LBC in the diagnosis of $\geq$ HSIL was compared between the two groups, and detailed analysis was conducted to demonstrate how many cases with cytology $\geq$ HISL and pathology $\leq$ LSIL were P16 negative.

Results

The accuracy rate of LBC for detection of pathology $\geq$ HSIL was 86.9% (93/107) in age group of $\geq$ 50, significantly lower than that of 95.5% (105/110) in age group of < 50 years ($P = 0.026$). P16 immunocytochemical stains on cases with cytology $\geq$ HISL and pathology $\leq$ LSIL were all negative in both groups.

Conclusion

The misinterpretation of LBC-$\geq$HSIL was rated higher in older women. P16 immunocytochemical stain works well in differentiating atrophic changes from LBC-$\geq$HSIL.

BACKGROUND
Diagnostics and treatment of cervical precancers for postmenopausal women has become important work of the cervical cancer prevention because of the social-aging tendency in many countries. Cervical cytology remains the standard cervical cancer screening test worldwide for either primary or secondary screening. However, evidence shows that cytology may misinterpret some atrophic changes on the squamous and columnar epithelium as HISL when analyzing the exfoliated cervical cells from postmenopausal women whose obviously decreased estrogen has caused such changes [1]. On the other hand, many studies have evidenced that over-expression of P16 protein is positively related to transformative hr-HPV infection and grade of cervical cell proliferation, and can be an objective indicator to reflect the lesion grade. As a tumor suppressor that is highly related with HSIL and cervical cancer, P16 over expression can be a biomarker for early diagnosis of squamous intraepithelial lesion (SIL) and evaluation of the lesion prognosis [2]. A P16 immunocytochemical stain technology was developed by Senying Biotechnology Co., LTD., which uses P16\textsuperscript{INK4a} monoclonal antibodies (sy-a01) to stain the exfoliated cervical cells that was diluted at a ratio of 1:4000 on PathCIN®p16\textsuperscript{INK4a} automatic staining system (P16 immuno-stained cytology). This technology has been demonstrated to be more sensitive than and equal specific with liquid bases cytology (LBC) in detection of grade II and above cervical intraepithelial neoplasia (CIN2+) [3]. As it provides the cytopathologists with more objective marker for cytology interpretation, it reduced the subjective diversity in cytology interpretations from different cytologists and degrades the reliance of cytology on cytologists’ experiences. This study is aimed to demonstrate the performance of P16 immuno-stained cytology (or P16-cytology) in facilitating differentiation of HISLs from atrophic lesion through comparing the concordance of P16-immuno-stained cytology with the LBC and histopathology diagnoses between the groups of women ≤ 50 and > 50 years of age.

**MAETRIALS AND METHOD**

**Study design, participants, and procedures**

The subjects of the study are 73,624 women living in central China, who were screened for cervical cancer by primary HPV testing in a population-based municipal cervical cancer screening program in November 2019. Those women are enrolled for screening because they were eligible of 30–64 years of age, unpregnant, without uterine or cervical resection, and consented for participation of the screening and this study through signing an electronic version of informed consent form when they registered for participation on a website (www.curekeys.com).

Eligible women were primarily tested for hr-HPV with SeqHPV assay on their self-collected samples. Women with HPV-negative result were advised to regular screening by HPV assay after 3 years, while those positive of hr-HPV were called-back for triages following a protocol that required a cervical sample be collected by the physician for LBC and P16-cytology analysis for all positive women followed by multiple biopsies on women who were positive of HPV-16 and/or -18, positive of hr-HPV type other than HPV-16 and -18 (other hr-HPV type) plus positive of acetic acid test, or positive of other hr-HPV types,
negative of acetic acid test, but positive of LBC (≥ ASCUS). Endocervical curettage (ECC) was performed on patients if the squamocolumnar conjunction zone (T-zone) could not be completely visible. Pathology analysis was conducted on the biopsied and ECC specimens. Included in this analysis are 2557 women who were had completed data on the primary HPV testing result, liquid-based cytology (LBC) analysis, P16\textsuperscript{INK4a} immune-stained cytology (P16-Cytology) analysis, and the pathology diagnosis on the multi-biopsies and ECC. Women who were positive of HPV 12 types other than HPV-16/-18 and normal of both LBC and P16-cytology or women who were positive of any type but failed to have results of LBC, P-16-cytology, histopathology due to sample reasons were excluded from this study (Fig. 1). The study was approved by Institutional Review Board (IRB) of BGI Institute and Ethnic Committee of Peking University Shenzhen Hospital (PUSH, No.2018035).

**Sampling and HPV testing**

After successful registration, which means eligible for participation in the primary screening, women can be screened in the sampling sites in the communities temporarily setup according to the number of registered women in the relevant communities or in a nearby medical facilities (the screening sites). At the screening site, eligible women were guided to collect vaginal sample for themselves in sampling rooms in referring the self-sampling instruction with texts and graphs. According to the instruction, a conical-shaped brush was used to collect samples from the vagina, which included exfoliated cervical cells. If any women had problem in self-sampling, an on-site medical provider would give personal instruction. Samples collected were applied on an FTA-Illusion-card (GE) for HPV testing on SeqHPV (BGI-Shenzhen) by a reference lab of BGI-Shenzhen. SeqHPV is Next Generation Sequencing (NGS) based HPV testing assay that uses multi-plex PRC to amplify DNA and NGS for HPV genotyping \[3\]. This assay can detect and report 14 high-risk HPV (hr-HPV) genotypes, including HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and - 68. SeqHPV had been validated to be equal sensitive and specific with Cobas4800 when tested on either provider-collected or self-collected samples \[4\] and to work well with FTA cards (a hard sample processing card). It has been licensed by China Food and Drug Administration (CFDA) for clinic use.

**LBC and p16 immuno-stained cytology**

A liquid-based cytology and the P16\textsuperscript{INK4a} immuno-stained cytology (Senying Biotechnology Co., Ltd., Shenzhen, China) was used for research purpose and as the secondary screening in triage of the women who positive of 12 HPV other types plus negative of acetic acid test. Cervical sample was collected by the provider and was put into a vial containing cell preservation liquid provided by Senying. The samples were shipped to Senying lab for processing: part of each sample was processed with P16\textsuperscript{INK4a} immunocytochemical stain, and the remains was processed for standard HE stains. Both the P16\textsuperscript{INK4a} immuno-stained and HE stained cytology slides were reviewed and interpreted by 2 senior cytopathologists who were blinded of each other's interpretation.
Following TBS classification standards\[^5\], LBC was reported as Negative for Squamous Intraepithelial Lesion (NILM), Atypical Squamous Cells of Undetermined Significance (ASCUS), Atypical Glandular Cells (AGC), Low grade Squamous Intraepithelial Lesion (LSIL), Atypical Squamous Cells-cannot exclude HSIL (ASC-H), High grade Squamous Intraepithelial Lesion (HSIL), or Squamous Cell Carcinoma (SCC). P16-cytology positive was reported when at least one cell under microscope was found to have $\text{P16}^{\text{INK4a}}$ immune-stained substance in the nuclear or the cytoplasm. A quality control was conducted after the two cytopathologists completed their reviewing on the slides, on which each of the slides with unmatched interpretations were picked up for agreed interpretation via discussion between the 2 cytopathologists.

**Colpo/biopsy and Histopathology diagnostics**

To women who needed biopsies according to the triage protocol, multiple biopsies were obtained at the site with suspected lesions and the site opposite the lesion, or randomly at the squamocolumnar conjunction zone in four quadrants of the cervix if no lesion was suspected. ECC were performed on patients whose squamocolumnar conjunction zone could not be completely visible under colposcope.

All the pathology slides were analyzed by a senior pathologist from PUSH who performed pathology analysis for several internationally cooperative clinical trials. Pathology analysis was conducted in blind of the results from both the P16-cytology and LBC tests. Histological diagnoses for cervical lesions were reported following a two-grade classification system, according to which the cervical lesions were classified as LSIL and HISL. We adopt this system because many studies demonstrated that different grades of cervical interepithelial neoplasm (CIN) are not the different stage of a cervical lesion development but the two obviously distinguishable pathological processes, and the two-grade-classification matches with the bio-behavior of HPV that causes pathology changes in human cells and is with better duplicability \[^7,8,9\].

**Statistics**

Results from LBC and Pathology were compared to demonstrate the bias of LBC on interpretation of HSILs in women $\geq 50$ years of age, and P16-cytology results of the cases with LBC -LSIL and -HSIL were analyzed using the relevant pathology diagnosis as the endpoint (Path-LSIL and Path-HSIL). SPSS 26.0 statistical software was used for data analysis. Chi-square test was used to compare the differences in various rates, and a $p$ value of $< 0.05$ was considered as statistical significance for all analyses.

**RESULTS**

Among the 73,624 primary screening participants, 73,462 had valid results for HPV primary testing after excluding 162 for failure of HPV testing. Of the 73,462 participants, 5,768 were positive of primary HPV testing and 2,557 of those positives had complete data of HPV testing, LBC, P16-cytology, and histopathology results and was included in the analysis for the purpose of this study (the analytic cases).
Patients who were primarily positive of HPV other-type but normal of cytology and P16-cytology, or abnormal of the 2 cytology tests but did not return for colposcopy were excluded from this analysis.

Of the 2,557 analytic cases, 1,127 were aged 50 and above and are included in ≥ 50 age group, while other 1,430 were younger than 50 and are included in < 50 age group for analysis. HSIL cases from LBC (LBC-HSIL) and pathology analysis (Path-HSIL) are 107 (9.5%, 107/1,127) and 167 (14.7%, 167/1,127), respectively in ≥ 50 group (Table 1, A). While cases of LBC- and Path- HSIL were 110 (7.7%, 110/1,430) and 255 (17.8%, 255/1,430), respectively, in < 50 group (Table 1, B). Comparison shows that the two groups are not significantly difference in LBC detection of HSILs (p = 0.105) but is in pathology detection of HSIL (p = 0.042), with more Path-HSIL cases (55.7%) detected from < 50 age group. One point five percent (1.5%, 4/960) of the Path-LSIL cases in ≥ 50 group were reported to be LBC-HSILs but the same rate was only 0.4% (5/1,175) in < 50 group. This indicates that more Path-LSIL in ≥ 50 group were incorrectly upgraded by LBC analysis.

<table>
<thead>
<tr>
<th></th>
<th>LBC- ≤LSIL</th>
<th>LBC- ≥HSIL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATH- ≤LSIL</td>
<td>946(98.5)</td>
<td>14(1.5)</td>
<td>960(100)</td>
</tr>
<tr>
<td>PATH- ≥HSIL</td>
<td>74(44.3)</td>
<td>93(55.7)</td>
<td>167(100)</td>
</tr>
<tr>
<td>Total</td>
<td>1,020(90.5)</td>
<td>107(9.5)</td>
<td>1,127(100)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>LBC- ≤LSIL</th>
<th>LBC- ≥HSIL</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PATH- ≤LSIL</td>
<td>1,170(99.6)</td>
<td>5(0.4)</td>
<td>1,175(100)</td>
</tr>
<tr>
<td>PATH- ≥HSIL</td>
<td>150(58.8)</td>
<td>105(41.2)</td>
<td>255(100)</td>
</tr>
<tr>
<td>Total</td>
<td>1,320(92.3)</td>
<td>110(7.7)</td>
<td>1,430(100)</td>
</tr>
</tbody>
</table>

Comparison of Table 2. A and B: $X^2 = 2.635$, $P = 0.105$ for LBC and $X^2 = 4.155$, $P = 0.042$ for Pathology

Detailed analysis of cases of Path- ≤LSIL and LBC- ≥HSIL show that all those cases in the both age-groups were tested negative by P16-Cytology, including 18 cases normal of pathology and 1 Path-LSIL, suggesting that P16-Cytology facilitates identification of the incorrectly upgrade LBC-HSIL cases (Table 2).
Table 2
P16-Cyto performance in detection LBC-HSIL + cases in the 2 age groups.

<table>
<thead>
<tr>
<th>Pathology:</th>
<th>Normal</th>
<th>LSIL</th>
<th>HSIL+</th>
</tr>
</thead>
<tbody>
<tr>
<td>P16-Cyto:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P16+</td>
<td>0</td>
<td>13</td>
<td>90</td>
</tr>
<tr>
<td>P16-</td>
<td>0</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>≥ 50 (n = 107)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBC-HSIL+</td>
<td>0</td>
<td>5</td>
<td>104</td>
</tr>
<tr>
<td>&lt; 50 (n = 110)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The pathological results of the above four cases were CIN2, Path-P16 were negative.

DISCUSSION

Our analysis shows that the rate of the LBC-reported false HSILs is significantly higher in ≥ 50 group than in < 50 group. This result is consistent with many studies reporting that the average age of cases Path-Normal was higher than Path-HSIL after LBC-HSIL \[10,11,12\]. In a study on LBC-ASC-H cases \[13\], Halford and coauthors reported that the CIN-2 rates were 55.8% and 37.5% among patients aged < 50 and ≥ 50 of years, respectively, with significant difference. Other literatures attributed the higher rate of inconsistence between LBC-HSIL and Path-Normal among women aged ≥ 50 to estrogen-dropdown resulted cervical atrophic changes, which often led to many parabolic basal cells and basal cells be dark stained on cytology views \[14,15,16\]. LBC may possibly misinterpret cervical atrophic exchange as HSIL or even cancer \[17\]. The atrophy changes on squamous epithelium makes the Pap test less precisive and specific in detection of HISL \[18,19\]. Recent studies found that P16/Ki67 double-stained cytology performed high profiles for CIN2 + in postmenopausal women cytologically reported with ASC-US \[20\]. As misinterpretation of HSIL + would not only bring heavy psychological pressure to women, but lead to unnecessary biopsies in subsequent colposcopy, the performance of P16 immunocytochemical stain in facilitation of the cytology interpretation for women at age of 50 or above is worth of address in its clinical application.

In our study, P16\[^{INK4a}\] stained cytology (P16-cytology) performs well in differentiating Path- ≤ LSIL from LBC- ≥ HSIL. Those findings are important for further studies and clinic services since P16-cytology helps avoiding the cervical atrophic changes to be misinterpreted as ≥ HSIL by LBC for aged women and indicating potential HSIL + that is invisible under colposcopy \[21\]. Data analysis on several large-scale cervical cancer screening projects showed that the sensitivity of colposcopy-directed biopsy for detection of HSIL is 57.1–74.7\% \[22,23\]. In our study, LBC- ≥ HSIL cases positive of P16 were all pathology diagnosed as ≥ HSIL, showing that and P16 immuno-stain can potentially avoid either overdiagnosis and miss-diagnosis. For many years, investigators endorsed to find proper technology for secondary screening that can keep enough sensitivity for detection of HSIL but avoid unnecessary biopsies.
Our analysis in this study suggests that P16-cytology can be a proper technology for secondary screening before biopsy. Previous studies demonstrated that the positive rate of P16-cytology is as same as that of HPV testing and LBC analysis for Path-HSIL and above, indicating that P16-cytology effects as well as Cytology in finding abnormal cells those may potentially progressed to be carcinomas and is better than HPV testing in indicating precancers\textsuperscript{[24]}. P16-cytology can be tested at the same time with LBC on a same sample and is advantageous in the secondary screening for patients who are primarily positive of Hr-HPV testing and facilitate to improve the accuracy of cytology\textsuperscript{[25]}.

In conclusion, our study finds that P16-Cytology facilitates differentiation of Path- $\leq$LISL from LBC-$\geq$HSIL, especially in aged women and can be used as the secondary screening test for women aged $\geq$ 50 to avoid unnecessary biopsies from misinterpretation of LBC primary or secondary screening.

This study is one of the few retrospective study on triage in women with LBC-$\geq$HSIL with P16\textsuperscript{INK4a} immunocytochemical stain. It contributes a basis for further studies in the relevant area. However, our study has limits: patients were grouped by age other than by menstruation status, thus it lack of proof for the histological atrophy changes; and it could be more evident is the LBC and P16-cytology results were from primary screening.

**Declarations**

**Author contributions**

RW, HD, and JH conceived and designed the study; JH analysis and interpreted the data and drafted the article. HD, JH, FS, CW contributed to the data collection and quality control; RW, XQ made the critical revision of the manuscript. All authors gave their comments on the article and approved the final version before submission.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this article. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Ethics approval and consent to participate**

The study protocol and the digital informed consent was approved by the Ethics Committee from Peking University Shenzhen Hospital (No.2018035). All methods were carried out in accordance with the rules of the Declaration of Helsinki. The informed consent was obtained from every participant.
Consent for publication

The authors agree to publication.

Competing interests

The authors declare that they have no competing interests.

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Figures
Figure 1 A chart flow of the study

Figure 1

See image above for figure legend