Clinical Evaluation of DNA Ploidy for the Triage of HPV-Positive Chinese Women During Cervical Cancer Screening

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ABSTRACT

Quantification of DNA aneuploidy has great potential as a prognostic marker of cervical precancerous lesions. We aim to evaluate the performance of DNA ploidy analysis for the triage of HPV-positive women. 523 HPV-positive women ages 25–64 undergoing HPV and pap cytology testing with valid cervical biopsies in Renji Hospital were enrolled in a prospective observational study from June 2018 to June 2019. The clinical performances of DNA ploidy, with or without HPV16/18 genotyping, were evaluated for all HPV-positive women to detect histologic high-grade squamous intraepithelial lesion or worse ($HSIL^+$). For $HSIL^+$ detection, DNA ploidy had statistically higher specificity (83.89%) than Pap cytology (75.50%, $P = 0.002$) and HPV16/18 genotyping (77.92%, $P = 0.023$). Although the sensitivity of DNA ploidy (58.57%) remained similar with pap cytology (65.71%, $P = 0.461$) and HPV16/18 genotyping (55.71%, $P = 0.734$). A comparable sensitivity (84.29% vs. 84.29%, $P = 1.000$) and a higher specificity (66.00% vs. 58.94%, $P < 0.001$) compared with combination with Pap cytology. DNA ploidy triage strategy required fewer colposcopies per detection of $HSIL^+$ compared with pap cytologic testing, with a 13.1% (34 of 258) reduction of colposcopies compared with routine triage strategy of HPV screening with Pap cytologic testing. HPV16/18-negative women with negative DNA ploidy results had the lowest risk of $HSIL^+$ among HPV-positive women (3.55%). Automated DNA ploidy analysis, alone or in combination with HPV16/18 genotyping, shows the potential as a triage strategy of cervical cancer screening for HPV-positive women.

Prevention Relevance: Results from this study indicate that DNA ploidy analysis has good performance in early detection of high-grade precancerous and cancerous lesions of the cervix. This strategy could be used in the triage of HPV-positive women in cervical cancer screening.

Introduction

Infections with high-risk human papillomaviruses (HR-HPV) are a necessary cause of almost all cervical cancers, which led to the development of cervical cancer–screening strategies based on HPV testing (1). In 2014, the FDA approved the cobas HPV test for primary screening for cervical cancer in women over 25 years (2). HPV16 and HPV18 have the highest carcinogenic potential and account for 70% of cervical cancers and pre-cancerous lesions (3). Although HPV infections are common in the population, most HPV infections are transient and pose very limited cancer risk. Success of HPV-based screening depends on the effective triage strategies of HPV-positive women. In accordance with FDA-approved use of the cobas HPV Test, women positive for HPV16/18 genotypes (16 and/or 18) should receive colposcopy and women testing positive for other high-risk HPV genotypes should receive a Pap cytology test for routine triaging. Papanicolaou stain has been widely used in the Pap cytology test to identify nuclear and cytoplasmic features, which is subjective and its sensitivity relies on well trained and highly skilled pathologists. The limitations of Pap cytology have helped shift guidelines from morphology-based screening to other objective triage strategies.

One theory of cancer holds the view that aneuploidy, resulting from an error in the division of a normal cell and representing the instability of chromosomes, is not only the drive of cancer, but also an early event of carcinogenesis (4, 5). Aneuploidy has been proved to be significantly associated with progression of cervical carcinoma and serve as a promising prognostic marker of cervical malignancies (6, 7). Therefore, quantification of chromosomal aneuploidy can help to distinguish benign and malignant cervical cell cytopathy. Different from Papanicolaou stain, the Feulgen stain is a stoichiometric stain that has a linear relation to the amount of DNA in the cell nucleus. The Feulgen stain can be used to detect DNA ploidy

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using computer-assisted image cytometry in an automated manner and applied in the diagnosis of cervical lesions (8). Collective evidence has also suggested that DNA ploidy analysis is capable of a stand-alone testing method in cervical cancer primary screening (9, 10).

For automated DNA ploidy analysis to be considered as an alternative for the screening of cervical cancers, our study presented a comparative analysis of DNA ploidy analysis, Pap cytology, HPV16/18 genotyping, and their combination in terms of triaging HPV-positive Chinese women for detection of HSIL$^+$. Our results may have an impact on the choice of cost-effective strategies for large-scale screening programs.

Materials and Methods

Study population and clinical procedures

From June 2018 to June 2019, 612 women ages ≥25 years undergoing routine cervical screening were enrolled in the study at Renji Hospital of Shanghai Jiao Tong University. Women with negative HPV results are advised to return for regular screening after 3 years and positive ones were required for Pap cytology and DNA ploidy tests within the next 4 weeks. Women with positive HPV results and valid pap cytology and DNA ploidy results were then referred to undergo colposcopy, which entailed cervical biopsy to confirm the cervical histological results 2–4 weeks after the cytology tests had been done. Females were excluded from the study in case of: (i) negative HPV results; (ii) no valid Pap cytology results; (iii) no valid DNA ploidy results; (iv) no cervical biopsy histopathological diagnosis; (v) having uterine or cervical resection; (vi) pregnancy. We excluded a total of 89 women without evaluable results or lost to follow-up (**Fig. 1**). The study was approved by the institutional review board of Renji Hospital, School of Medicine, Shanghai Jiao Tong University. The written informed consent was obtained from all the participants.

HPV testing and pap cytology

A liquid-based cervical specimen with endocervical brush was collected from all the participants by experienced clinicians. The specimen was then processed on the cobas 4800 instrument (Roche) by licensed and trained personnel per the manufacturer's protocol. Results from cobas testing included HPV negative, HPV 16/18 positive, and 12 other high-risk HPV subtypes positive. HPV-positive women were required to collect a second liquid-based cervical specimen for Pap cytology and DNA ploidy, whereas women with negative HPV test results were excluded from the study and returned to routine screening. Half of the second cervical specimen were processed using the Thinprep method. The results were analyzed by the Bethesda system: Negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of undetermined significance (ASC-US), low-grade squamous intraepithelial lesion (LSIL), and high-grade squamous intraepithelial lesion (HSIL). ASC-US and the above grade were defined as Pap cytology-positive.

DNA ploidy analysis

The residual second cervical sample was prepared for routine slide. After Feulgen staining the samples were first scanned by MoticEasyscan and then analyzed using MotiCytometer, an imaging cytometry system. This system automatically classified the cells on the basis of more than 100 features for each object. A positive DNA ploidy result was defined according to 2 parameters based on Guillaud's study (11): (i) DNA ploidy index (DI): The cell was considered as aneuploid when DI was above 2.5; (ii) the number of aneuploid cells: The specimen was called aneuploid in the presence of no less than 3 aneuploid cells. Specimens with negative DNA aneuploidy results were considered invalid if the analyzed cells were less than 800.

Histopathologic diagnostic criteria

All women that underwent colposcopy had at least one biopsy taken, with the majority of women receiving the multi-point biopsies to improve ascertainment of cervical precancerous lesions. The pathology reading was performed by two experienced gynecological pathologists who were blinded to the HPV, Pap cytology, and DNA ploidy results. In cases of ambiguous readings, the slide was read a third time by a study pathologist to provide a final consensus diagnosis. Histological results were classified as normal, LSIL, HSIL, and cervical carcinoma. HSIL and cervical carcinoma are collectively named HSIL$^+$.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of different triage strategies were calculated using MedCalc Software (version 18.0). The sensitivity and specificity between groups were compared using the McNemar's test. The differences in PPV and NPV were investigated using the R package, DTComPair (R Foundation for Statistical Computing). Statistical significance was defined as $P < 0.05$. All statistical tests performed were two-sided.

Results

Study population

In the current study, a total of 523 HPV-positive women were enrolled and the mean age of the 523 HPV-positive women was 43.73 ± 11.83 years. There were 70 cases of pathologically diagnosed HSIL$^+$, including 3 cases of cervical carcinoma. There were 81 cases of LSIL and 372 cases of normal pathology. Among the 523 HPV-positive women, 139 were HPV16/18 positive and 384 were 12 other HPV genotyping positive (**Fig. 1**).

DNA ploidy positivity by Pap cytologic and histologic results

Of the 523 women included in our study, 366 (69.98%) had NILM, 67 (12.81%) had ASC-US, 55 (10.52%) had LSIL, 10 (1.91%) had ASC-H, and 25 (4.78%) had HSIL according to Pap cytologic results. Positive DNA ploidy results increased from 8.74% (32 of 366) in women with NILM to 88.00% (22 of 25) in women with HSIL cytologic findings. The DNA ploidy

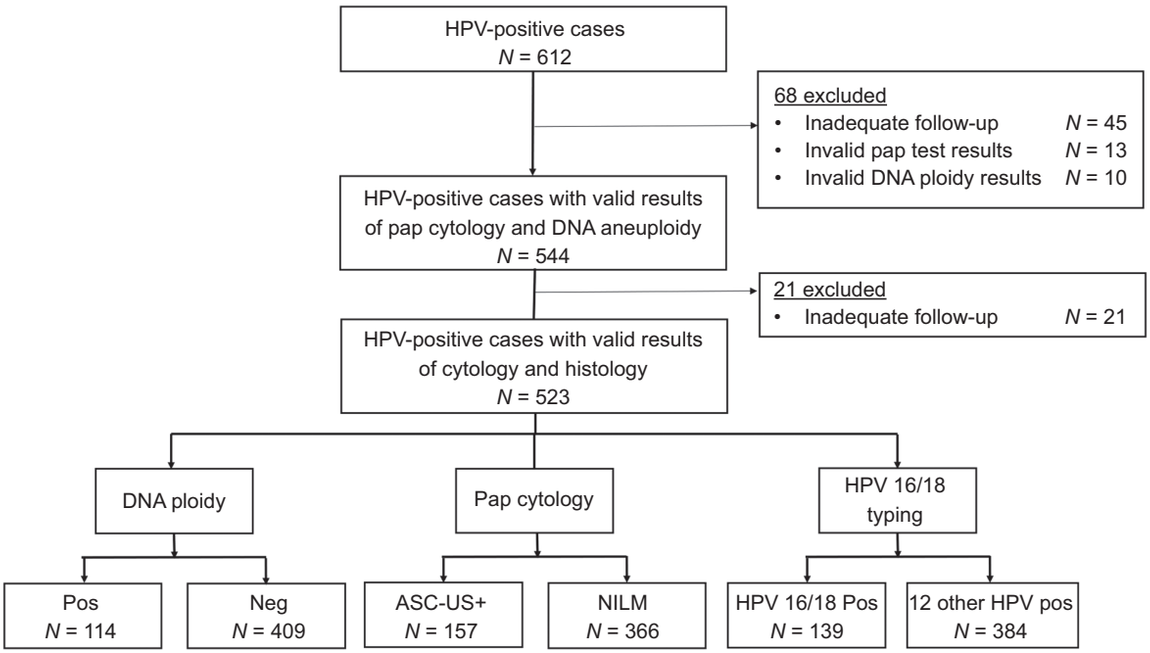

Figure 1.

Flow Diagram. Women with positive HPV results were required to take the pap cytology and DNA ploidy analysis afterwards. The HPV-positive women with valid DNA aneuploidy and pap cytology results were then referred to colposcopy and obtained the cervical histological results.

positivity rate (114, 21.80%) was lower than that of positive Pap cytology results at an ASC-US threshold (157, 30.02%, $P = 0.002$; **Table 1**).

Diagnostic performance of DNA ploidy for HSIL$^+$ detection

The sensitivity, specificity, PPV and NPV of DNA ploidy, Pap cytology and HPV16/18 genotyping for detection of HSIL$^+$ are shown in **Table 2**. For detection of HSIL$^+$, the specificity of d DNA ploidy was 83.89%, which was significantly higher than that of Pap cytology (75.50%, $P = 0.002$) and HPV16/18 genotyping (77.92%, $P = 0.023$). The sensitivity of DNA ploidy, Pap cytology, and HPV16/18 genotyping were comparable, respectively (58.57%, 65.71%, and 55.71%, $P > 0.05$). The PPV of DNA ploidy was 35.96%, which was significantly higher than Pap cytology (29.30%, $P = 0.024$) and HPV16/18 genotyping

Table 1. DNA ploidy positivity by Pap cytology and histology results.

Cytology results	Total No. (%)	Normal No. (%)	LSIL No. (%)	HSIL No. (%)	Cancer No. (%)
NILM	366 (69.98)	291 (78.23)	51 (62.96)	22 (32.84)	2 (66.67)
DNA ploidy+	32 (8.74)	23 (7.90)	5 (9.80)	4 (18.18)	0 (0.00)
HPV16/18+	88 (24.04)	60 (20.62)	15 (29.41)	11 (50.00)	2 (100.00)
ASC-US	67 (12.81)	47 (12.63)	11 (13.58)	8 (11.94)	1 (33.33)
DNA ploidy+	19 (28.36)	9 (19.15)	4 (36.36)	5 (62.50)	1 (100.00)
HPV16/18+	17 (25.37)	9 (19.15)	3 (27.27)	4 (50.00)	1 (100.00)
LSIL	55 (10.52)	29 (7.80)	16 (19.75)	10 (14.93)	0 (0.00)
DNA ploidy+	34 (61.82)	19 (73.31)	8 (50.00)	7 (70.00)	0 (0.00)
HPV16/18+	13 (23.64)	5 (17.24)	4 (25.00)	4 (40.00)	0 (0.00)
ASC-H	10 (1.91)	2 (0.54)	1 (1.23)	7 (10.45)	0 (0.00)
DNA ploidy+	7 (70.00)	1 (50.00)	0 (0.00)	6 (85.71)	0 (0.00)
HPV16/18+	2 (20.00)	0 (0.00)	0 (0.00)	2 (28.57)	0 (0.00)
HSIL	25 (4.78)	3 (0.81)	2 (2.47)	20 (29.85)	0 (0.00)
DNA ploidy+	22 (88.00)	2 (66.67)	2 (100.00)	18 (90.00)	0 (0.00)
HPV16/18+	19 (76.00)	3 (100.00)	1 (50.00)	15 (75.00)	0 (0.00)
Total	523 (100.00)	372 (71.13)	81 (15.49)	67 (12.81)	3 (0.57)
DNA ploidy+	114 (21.80)	54 (14.52)	19 (23.46)	40 (59.70)	1 (33.33)
HPV16/18+	139 (26.58)	77 (20.70)	23 (28.40)	36 (53.73)	3 (100.00)

Table 2. Clinical performance of DNA ploidy, Pap cytology and HPV16/18 typing for detection of HSIL$^+$.

	DNA ploidy		Pap cytology		HPV16/18 typing			
	NO. of positive/*N*	Estimate (95% CI)	NO. of positive/*N*	Estimate (95% CI)	NO. of positive/*N*	Estimate (95% CI)	*P*	*P′*
Detection of HSIL$^+$ ($N = 70$)								
Sensitivity	41/70	58.57% (46.2%–70.2%)	46/70	65.71% (53.4%–76.7%)	39/70	55.71% (43.3%–67.6%)	0.461	0.734
Specificity	380/453	83.89% (80.2%–87.2%)	342/453	75.50% (71.3%–79.4%)	353/453	77.92% (73.8%–81.7%)	0.002	0.023
PPV	41/114	35.96% (29.6%–42.8%)	46/157	29.30% (24.7%–34.4%)	39/139	28.06% (22.9%–33.8%)	0.024	0.011
NPV	380/409	92.91% (90.8%–94.6%)	343/367	93.46% (91.1%–95.2%)	353/384	91.93% (89.7%–93.7%)	0.543	0.501

Note: *P* value indicates the comparison between DNA ploidy and Pap cytology; *P′* value indicates the comparison between DNA ploidy and HPV16/18 typing
Abbreviation: CI, confidence interval.

(28.06%, $P = 0.011$). The NPV of DNA ploidy was 92.91%, and no significant difference was observed between of Pap cytology (93.46%, $P = 0.543$) and HPV16/18 genotyping (91.93%, $P = 0.501$; **Table 2**).

Diagnostic performance of combining HPV16/18 genotyping with DNA ploidy

The utility of DNA ploidy analysis and Pap cytology as triage options for HPV-positive women was further assessed combined with HPV16/18 genotyping. The combination triage strategies were implemented as follows: Women positive for HPV16/18 were referred to colposcopy, whereas women with 12 other HPV genotyping positive results were further detected with DNA aneuploidy and Pap cytology. Compared with the currently routine primary HPV screening strategy with HPV 16/18 genotyping and Pap cytologic triage, HPV16/18 genotyping combined with 12 other HPV genotyping followed with a further triage of DNA ploidy analysis showed a higher specificity (66.00% vs. 58.94%, $P < 0.001$), PPV (27.70% vs. 24.08%, $P = 0.002$), whereas the sensitivity and NPV were equal for both combination tests (**Table 3**).

Colposcopy referral and HSIL$^+$ detection using different triage strategies

Next, we compared the efficiency of different triage strategies in colposcopy referral and detection for HSIL$^+$. The DNA ploidy alone required the fewest referral colposcopies and the lowest number of colposcopies detected per HSIL$^+$ ($n = 2.78$). For triage of HPV-positive women with HPV16/18 genotyping, DNA ploidy showed fewer colposcopies detected per HSIL$^+$ compared with Pap cytologic testing ($n = 3.61$ vs. $n = 4.15$; **Table 4**).

Cervical precancerous lesion risk in combinations of DNA ploidy, HPV16/18 genotyping, and Pap cytology

Figure 2 showed the risk of HSIL$^+$ among HPV-positive women for different combinations of screening tests. The strategy of HPV16/18 genotyping in combination with DNA ploidy yielded better risk stratification compared with HPV16/18 genotyping and Pap cytology triage: More women had a very low risk (310/523, 59.3% vs. 278/523, 53.2%). HPV16/18-negative women with negative DNA ploidy results had the lowest risk of HSIL$^+$ among all combinations (11/310, 3.55%), and women with positive DNA ploidy results and positive HPV16/18 findings are faced with the highest risk of HSIL$^+$ (21/40, 52.5%).

Discussion

Cervical cancer screening based on primary HPV testing has been successfully applied to clinical practice (12). However, most HPV infections are transient and have little association with cervical precancerous lesions, leading to unnecessary referral to colposcopies. This highlights the need for effective triage tests that reduce colposcopy referral and maintain high specificity of the primary screening for HPV-positive women.

Table 3. Performance of combing HPV16/18 genotyping with DNA ploidy compared with Pap cytology in HPV-positive women.

	HPV16/18 to colposcopy and triage 12 other HR-HPV(+) with DNA ploidy		HPV16/18 to colposcopy and triage 12 other HR-HPV(+) with Pap cytology		
	NO. of positive/*N*	Estimate (95% CI)	NO. of positive/*N*	Estimate (95% CI)	*P*
Detection of HSIL$^+$ ($N = 70$)					
Sensitivity	59/70	84.29% (73.6%–91.9%)	59/70	84.29% (73.6%–91.9%)	1.000
Specificity	299/453	66.00% (61.4%–70.4%)	267/453	58.94% (54.3%–63.5%)	<0.001
PPV	59/213	27.70% (24.5%–31.1%)	59/245	24.08% (21.5%–26.9%)	0.002
NPV	299/310	96.45% (93.0%–97.9%)	267/278	96.04% (93.3%–97.7%)	0.546

Note: *P* value indicates the comparison between DNA ploidy and HPV16/18 typing.
Abbreviations: CI, confidence interval; HR-HPV, high-risk human papillomavirus.

Table 4. Colposcopy referral and detection of HSIL$^+$ for different combined triage strategies.

	Number of colposcopies	Number of HSIL$^+$ detected	Number of colposcopies per HSIL$^+$ detected (95% CI)
Triage all HPV(+) with DNA ploidy	114	41	2.78 (2.29–3.34)
Triage all HPV(+) with Pap cytology	157	46	3.41 (2.90–3.99)
Triage all HPV(+) with HPV 16/18 type	139	39	3.56 (3.00–4.21)
HPV16/18 to colposcopy and triage 12 other HR-HPV(+) with DNA ploidy	213	59	3.61 (3.14–4.13)
HPV16/18 to colposcopy and triage 12 other HR-HPV(+) with Pap cytology	245	59	4.15 (3.65–4.71)

The technique of DNA ploidy analysis was first put forward by Sandritter by measuring the DNA content of cells. In such measurements, an atypical DNA distribution pattern of aneuploid are indicative of abnormal cells (13). Later, this new approach was applied clinically and detection of DNA aneuploidy was proven to be an effective marker of neoplastic cells (14). Detecting cervical cancer cells using DNA ploidy analysis was adopted in past 15 years. Sun reported the sensitivity and specificity of imager for detecting HSIL$^+$ to be 82% and 71%, respectively, compared with 52% and 92%, respectively, for conventional cytology (9). Another study evaluated a DNA image cytometry system with a higher specificity (96.9%) but moderate sensitivity (54.4%; ref. 15).

We evaluated the clinical performance of DNA ploidy analysis for detection of HSIL$^+$ among the HPV-positive women. DNA ploidy showed a lower positivity rate compared with Pap cytology (**Table 1**), and this would signify a lower referral to colposcopy (**Table 4**). The specificity of DNA ploidy test was significantly higher than that of Pap cytology and higher than that of HPV16/18 genotypes as well (**Table 2**). The PPV of DNA ploidy was also higher than Pap cytology and HPV16/18 genotypes, whereas the sensitivity of DNA ploidy remained comparable with the other two triage tests (**Table 2**). Previous literature has proved that DNA ploidy triage showed comparable sensitivity, specificity, PPV, and NPV values with conventional cytology and HPV16/18 genotyping as a primary screening test (11). Our data indicated that DNA ploidy test could achieve equal or superior performance compared with Pap cytology as a triage test for HPV-positive women in China, with lower program cost and higher speed of analysis.

DNA ploidy has demonstrated potentiality an ideal test for primary cervical screening, whether it could be used for "second-line" triage in HPV-positive women has not been studied so far. Therefore, our study further analyzed a combination of HPV16/18 genotyping and DNA ploidy for triage of HPV-positive women. DNA ploidy combined with HPV16/18 genotyping had a significantly higher sensitivity for HSIL$^+$ detection than three tests alone. Furthermore, HPV16/18 genotyping combined with DNA ploidy showed a higher specificity and PPV compared with combination with Pap cytology, whereas the sensitivity of both combination strategies was similar (**Table 3**).

Colposcopy is a centerpiece of cervical cancer prevention programs. In the United States, the only FDA-approved protocol for primary HPV screening includes HPV genotyping and cytology for triage. In this strategy, women positive for HPV16/18 and women testing positive for other high-risk subtypes and ASC-US or greater are referred to colposcopy (16). Within a clinical study by Kaiser Permanente Northern California, current primary HPV screening would lead to two

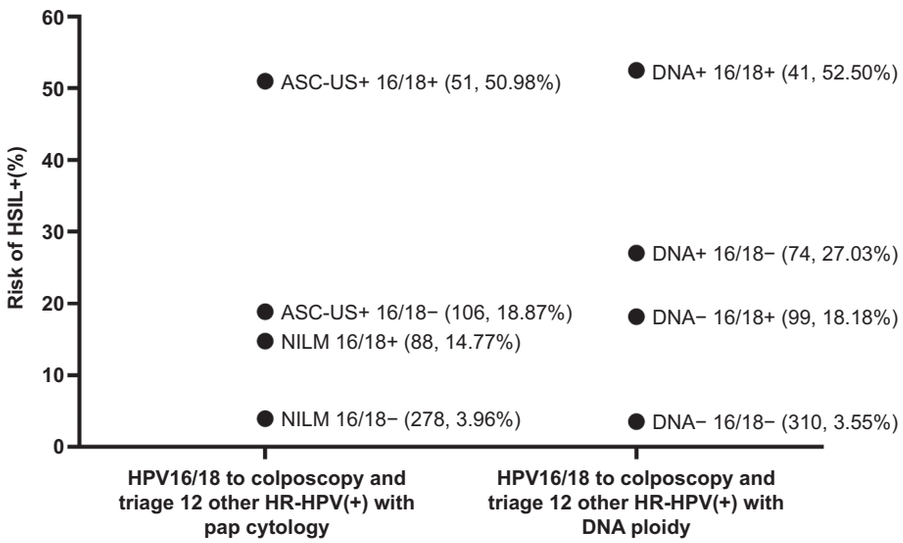

Figure 2.
Risk of HSIL$^+$ in Strata of Pap Cytology, DNA ploidy, and HPV16/18. The risk of HSIL$^+$ for combinations of DNA ploidy with HPV16/18 genotyping, Pap cytology with HPV16/18 genotyping, is plotted on the *y*-axis, with number and risk of women for particular test combinations indicated.

thirds of HPV-positive women being referred to undergo colposcopy immediately (17). Colposcopy practice should be shifted to a more conservative management approach, reducing colposcopy referrals. We evaluated the colposcopy referral and HISL$^+$ detection using different combined triage strategies. We found that on the basis of HPV16/18 genotyping, DNA ploidy showed lower numbers of colposcopies needed per detection of HSIL$^+$ compared with Pap cytologic testing (**Table 4**), which indicated this strategy to be more efficient.

In our current study, we found that performing DNA ploidy would identify most women at a very low risk of cervical precancerous lesions (negative HPV16/18 results and negative DNA ploidy results) who may remain safe before retesting at extended intervals. Among HPV16/18-negative women, lower risk for HSIL$^+$ was observed in women negative for DNA ploidy than those with normal Pap cytologic results, which was below the risk threshold for referral to colposcopy recommended by ASCCP (3.55% vs. 4.00%; ref. 18), suggesting that DNA ploidy could be a good triage option for populations negative for HPV16/18.

Our study evaluated a considerable population with uniform and well-organized screening and good disease ascertainment. We first found that HPV16/18 genotyping following DNA ploidy analysis could perform similarly or better compared with Pap cytology for triage of HPV-positive women. Meanwhile, DNA ploidy also reduces superfluous colposcopies and may reduce unnecessary cervical treatment. Recent research evaluated the cost-effectiveness of DNA ploidy analysis for cervical screening compared with Pap cytology and considered that DNA ploidy strategy appeared to be less costly and comparably effective (19). The technology of DNA ploidy analysis has become well-established for diagnostic use in several tumors such as endometrial cancer and lung cancer (20, 21). Inexpensive and semiautomated features of DNA ploidy measurement made it an ideal alternative for triaging HPV-positive women in China and other developing countries. The potential weakness of our study should also be noted. The sample size of our study is relatively limited. We have calculated the sample size prior to the beginning of our study and the sample size should be 600. The number of participants included in our study was actually 523, then we used this number to calculate the power of this study. The final power was 0.73, which could prove the results to be credible. In addition, the applicability of our results cannot fully be determined without specific information about the epidemiology of cervical cancer in such particular setting. Additional follow-up procedures also needed to be completed to confirm whether women with negative DNA ploidy results do have a lower risk of cervical precancerous lesions.

In summary, we evaluated the clinical performance of DNA ploidy for HSIL$^+$ detection in triaging HPV-positive women. DNA ploidy, either alone or combined with HPV16/18 genotyping, represents a potential triage strategy for HPV-positive women.

Authors' Disclosures

No disclosures were reported.

Authors' Contributions

W. Cang: Acquisition of data, analysis of data, writing–review and revision of the manuscript. **Q. Li:** Acquisition of data, writing–review and revision of the manuscript. **L. Gu:** Acquisition of data, writing–review and revision of the manuscript. **Z. Hong:** Acquisition of data, writing–review and revision of the manuscript. **Y. Hu:** Acquisition of data, writing–review and revision of the manuscript. **W. Di:** Conception and design, writing–review and revision of the manuscript, study supervision. **L. Qiu:** Conception and design, writing–review and revision of the manuscript, study supervision.

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