The Journal of Obstetrics and Gynecology of India (May–June 2023) 73(3):248–253
https://doi.org/10.1007/s13224-022-01731-x

ORIGINAL ARTICLE

Comparison of HPV 16/18 Genotyping and p16/Ki67 Dual Staining for Detection of High-Grade Cervical Lesion in Patients with Low-Grade Cervical Smears

Saloni Chadha[1] · Gauri Gandhi[1] · Suresh T. Hedau[2] · Ruchika Gupta[2]

Received: 5 April 2022 / Accepted: 30 October 2022 / Published online: 9 January 2023

Abstract

Objective To triage low-grade cervical smears (ASCUS/LSIL) by HPV 16/18 genotyping and dual staining with p16/Ki67 and to compare the sensitivity and specificity of these two triage methods for detection of high-grade cervical intraepithelial neoplasia (HGCIN).

Methods In this prospective cross-sectional study, we evaluated a total of 89 women with low-grade smears (54 ASCUS, 35 LSIL) recruited from a tertiary care hospital. All patients underwent colposcopy guided cervical biopsy. Histopathology was used as gold standard. All samples were subjected to HPV 16/18 genotyping (excluding 9) using DNA PCR and p16/Ki67 dual staining (excluding 4) using Roche® kit. We then compared the two triage methods to detect high-grade cervical lesions.

Results Overall, in all low-grade smears sensitivity, specificity and accuracy of HPV 16/18 genotyping, was found to be 66.7%, 77.1% and 76.2% respectively ($p=0.03$). In low-grade smears sensitivity, specificity and accuracy of dual staining, was found to be 66.7%, 84.8% and 83.5% respectively ($p=0.01$).

Conclusions Overall, in all low-grade smears the sensitivity of the two tests was comparable. However, dual staining had a higher specificity and accuracy than HPV 16/18 genotyping. It was concluded that both are effective triage methods but dual staining had a better performance than HPV 16/18 genotyping.

Keywords Colposcopy · ASCUS · LSIL · Pap smear · HPV testing · HPV16 · 18 Genotyping · Dual staining · Cervical intraepithelial neoplasia

Introduction

Cervical cancer is the fourth most common cancer affecting women worldwide, with an estimated 604,000 cases and 342,000 deaths in 2020 worldwide [1]. There are 122,844 new cases and 67,477 deaths every year in India [2].

The evolution of precancerous lesions of cervix after persistent infection with human papillomavirus (HPV) to becoming invasive cervical cancer often takes 10–15 years to develop, it thus provides many opportunities for detection and treatment of precancerous lesions. These are asymptomatic lesions which can be easily detected by use of effective screening methods. If detected early, there is almost complete cure with simple procedures, while advanced cancers have less than one-third survival rates.

Pap smear is an established method for cervical cancer and pre-cancer screening. CIN, a precursor lesion of cervical squamous cell carcinoma, is seen in almost 10–20% of individuals with ASCUS (Atypical squamous cells of

Dr. Saloni Chadha (MS) is a Senior Resident, Department of Obstetrics and Gynecology, Maulana Azad Medical college and LNJP Hospital, New Delhi; Gauri Gandhi MD is a Director Professor, Department Obstetrics and Gynecology, Maulana Azad Medical college and LNJP Hospital, New Delhi; Dr. Suresh T. Hedau (Ph. D) is a Scientist E, ICMR-National Institute of Cancer Prevention and Research, Noida; Dr. Ruchika Gupta (MD) is a Scientist C, ICMR-National Institute of Cancer Prevention and Research, Noida.

✉ Saloni Chadha
Chadha.saloni@gmail.com

[1] Department of Obstetrics and Gynecology, Maulana Azad Medical College and LNJP Hospital, New Delhi 110002, India

[2] ICMR-National Institute of Cancer Prevention and Research, Noida, India

undetermined significance) [3]. The rates of CIN2/CIN3 for LSIL (Low grade squamous intra epithelial lesion)/ASC-H (atypical squamous cells—cannot rule out high-grade lesion) vary from 29 to 40.7% [4]. Worldwide 71% of cervical cancer is associated with HPV 16–18 [5].

p16, a cyclin-dependent kinase inhibitor, is a tumor suppressor protein. It regulates the cell cycle by decelerating progression into S phase by facilitating rebinding of Rb and E2F transcription factor. High-risk HPV genes E6, E7 cause multiple interferences with such cellular proteins involved in cell cycle regulation and result in oncogenesis [6]. E7 oncoprotein causes disruption of Rb/E2F pathway, which results in over expression of p16 in HPV transformed cervical epithelial cell but this does not indicate proliferation [7].

Ki67 is a nuclear protein marker of cellular proliferation and coexpression of p16/Ki67 in the same cell indicates deregulation of cell cycle induced by HR HPV (High Risk). When both p16 and Ki67 are positive, it is considered as dual stain positive [8].

Thus, this present study was undertaken to compare HPV16/18 genotyping and p16/Ki67 dual staining for detection of high-grade cervical lesions in patients with low-grade cervical smears.

Materials and Methods

This study was carried out in the Department of Obstetrics and Gynecology, in a tertiary care hospital from November 2017 to March 2019. The sample size for our study was based on a study by Bergeron et al. [8] who reported prevalence of HGCIN as an average of 9.75% for ASCUS and LSIL. The calculated sample size with 95% confidence interval (CI) and 5% margin of error was 136, but we could not recruit that many patients as it was a time-bound study. Eighty-nine women in the age group of 30–65 years attending the Gynecology OPD or admitted in the Gynecology ward at the hospital with low-grade cervical lesions, i.e., LSIL and ASCUS, were included. Pregnant women, women with active bleeding per vaginum, known cases of carcinoma cervix and endometrium, women who have been previously treated for CIN or carcinoma cervix, women with history of pelvic irradiation and those with a frank growth on cervix were excluded from the study.

An informed consent was taken. Detailed history, general physical examination followed by abdominal, local per speculum and per vaginal examination was done. After this samples were taken for HPV16/18 genotyping and dual staining cytology p16/Ki67. In the same setting, patients were subjected to Visual Inspection by Acetic Acid (VIA) and colposcopy followed by directed biopsy. Pap smear processing, reporting, HPV detection, HPV 16/18 genotyping and dual staining were carried out at a medical research facility. Cervical biopsies were processed and diagnosed in the department of Pathology of the tertiary care hospital. It was a cross-sectional prospective study.

The initial HPV diagnosis was performed by using a pair of L1 consensus degenerate primers. The samples which were positive for HPV testing by DNA PCR were further processed for HPV 16/18 genotyping using type-specific primers. The basic steps were DNA extraction and amplification followed by visualization of the amplified product by gel documentation. For dual staining, the alcohol fixed cervical smear was stained for p16/Ki67 by CINtec ® Plus cytology kit (Roche mtm laboratories AG, Mannheim, Germany) using manufacturer's protocol. The kit included epitope retrieval solution (10×), peroxidase blocking reagent, primary antibody solution, visualization reagent HRP, visualization reagent AP, DAB buffered substrate, DAB chromogen, Naphthol phosphate substrate, Fast red chromogen and CINtec® Plus mount. A positive result was interpreted as brown cytoplasmic staining for p16 expression and red nuclear staining for Ki 67 expression.

After sending the above samples, per vaginal examination was done and then *Visual Inspection by Acetic Acid* was done in all patients by smearing the cervix with a cotton swab dabbed in 5% acetic acid solution and the findings were reported after one minute. A distinct acetowhite area within the transformation zone was considered VIA positive.

Colposcopy was done for all patients. It was performed using a video colposcope (Digital Colposcope with workstation, Goldway). The cervix was inspected in good light. Mucus or any vaginal discharge was removed with saline and any area suggestive of leucoplakia was noted. Green filter was used to look for abnormal vessels. Then, 5% freshly prepared acetic acid solution was liberally applied over the cervix and vaginal walls using a cotton tipped applicator. After 1 min of acetic acid application, the entire cervix was closely examined under magnification ranging from 5 to 25×. The cervix was then examined after application of Lugol's iodine solution so that any abnormal areas of iodine non-uptake could stand out as mustard/canary yellow against the mahogany brown color of the normal squamous epithelium. Swede scoring was done for all patients. Cervical biopsies were taken from the abnormal areas noted on the colposcope and if the colposcopy was normal, random four-quadrant biopsy was taken. Biopsy was taken with a punch forceps, wedge biopsy or a loop biopsy was taken using electrosurgical unit or cone biopsy was done if indicated. The specimen was fixed in 10%. Loop formalin and sent to the Pathology department for processing. Biopsy with CIN 2 or worse was taken as positive.

Data were entered in excel and analyzed using SSPS version 22.

The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the two triage tests were calculated with histopathology as a gold standard.

Observation and Results

Out of 89 women, 54 were ASCUS and 35 were LSIL. Mean age of patients in our study group was 37.75 ± 8.07 years, while the median age was 35 years. Most, i.e., 82.0% of the patients in our group got married after the age of 18 years. The majority (87.6%) of patients belonged to the lower socioeconomic status according to the modified Kuppuswamy scale. The history of smoking passive/active was found in 15.7%. Commonly, the parity in our patient group was up to 3 children (83.1%), while majority of the subjects were para 2–3 (65.1%), among the cases with biopsy proven CIN 2 or worse, the majority (83.2%) were para 2 or 3. Most common risk factor among CIN 2 or worse lesions was history of reproductive tract infections (16.7%). The commonest presenting complaint overall was discharge per vaginum (62.3%), while in cases with CIN 2 or worse lesion was discharge per vaginum (75%) followed by pain in lower abdomen (50%). On per speculum examination in cases of CIN 2 or worse lesion, the most common finding was chronic cervicitis with or without ectropion (66.7%).

Overall, out of 89 patients recruited 21 (23.5%) patients were VIA positive. On colposcopy, Swede score showed that 88.7% had score < 4 and 11.2% had score > 4.

CIN 2 or worse lesions were found in 6 out of 89 (6.7%) cases. Among 54 cases with ASCUS cytology there were 2 CIN 2 cases (3.7%), and among 35 LSIL cases there were 3 CIN 2 cases (8.6%) and 1 CIN 3 case (2.9%), i.e., 4 (11.4%) CIN 2 + lesions out of 35.

Overall, 21 out of 80 (26.2%) cases were positive for HPV 16 or 18 genotyping (9 were excluded due to lack of DNA). The rate of HPV 16/18 positivity was higher in the LSIL group (33.3%) than in ASCUS group (21.2%), but this difference was not statistically significant (*p* value 0.22) (Table 1).

Overall, 16 out of 85 (18.8%) study subjects tested positive for dual staining (4 were excluded due to lack of cellularity). The rate of dual stain positivity was higher in the LSIL group (27.2%) than in ASCUS group (13.4%) and this difference was statistically significant (*p* value 0.02) (Table 2).

When the two triage tests were compared for detection of high-grade CIN (HGCIN) in ASCUS both had equal sensitivity (50% in both), but specificity of dual staining was higher at 88% versus 80% for 16/18 genotyping. The accuracy of dual stain test was also higher at 86.5% versus 78.7% for HPV 16/18 genotyping. When the two triage tests were compared for detection of HGCIN in LSIL both had equal sensitivity (75%), but specificity of dual staining was higher at 79.3% versus 72.4% for 16/18 genotyping. The accuracy of dual stain test was also higher at 78.8% versus 72.7% for HPV 16/18 genotyping (Table 3).

When the two triage tests were compared for detection of HGCIN in all low-grade smears including ASCUS and LSIL both had equal sensitivity (66.7%), but specificity of dual staining was higher at 84.8% versus 77.1% for 16/18 genotyping. The accuracy of dual stain test was also higher at 83.5% versus 76.2% for HPV 16/18 genotyping (Table 4).

Table 1 Results of HPV 16/18 genotyping (*n* = 80)

HPV genotype finding	Overall (*n* = 80)		ASCUS (*n* = 47)		LSIL (*n* = 33)	
	No	%	No	%	No	%
HPV16/18 positive	2	2.5	0	0.0	2	6.06
HPV 16 positive	19	23.7	10	21.2	9	27.2
Negative for 16/18	59	73.7	37	78.7	22	66.6
No DNA[a]	9	10.1	7	12.9	2	5.7

[a]Not included in analysis

Table 2 Results of dual staining (*n* = 85)

Dual staining results	Overall (*n* = 85)		ASCUS group (*n* = 52)		LSIL group (*n* = 33)	
	No	%	No	%	No	%
Negative	69	81.1	45	86.5	24	72.7
Positive	16	18.8	7	13.4	9	27.2%
Inadequate sample[a]	4	4.4	2	3.7	2	6.06

[a]Not included in analysis

Discussion

As it takes a long time for the evolution of precancerous lesions of the cervix after persistent infection with HPV to becoming invasive cervical cancer, there is scope of their early detection. But there is lack of consensus regarding the management of low-grade cervical smears. These cases may be left to follow up or referred for colposcopy, but it leads to anxiety and loss to follow up. The rates of CIN 2 and worse in ASCUS ranged from 4 to 8% and in LSIL 12–15% [9]. Hence, there is a need to find effective triage tests for low-grade smears for *risk stratification* and to reduce referrals to colposcopy.

It is known that cervical cancer is a long-term consequence of persistent infection of the lower genital tract by one of the 15 high risk HPV types, which are the causative agents of cervical cancer. Among the different types of HR HPV, 71% of cervical cancer is associated with HPV 16, 18 [5]. Therefore, specific detection of these two genotypes, i.e., *HPV 16/18 Genotyping* provides useful information to triage women for colposcopy.

Another method of triaging is by the detection of biomarker *p16/Ki67 dual Staining* which is a surrogate marker of E7 oncoprotein-induced HPV transformed cells and cellular proliferation.

We compared these two methods for detection of high-grade cervical lesions in patients with low-grade cervical smears.

The prevalence of high-grade lesions in low-grade smears like ASCUS and LSIL ranges between 3.1 and 11.9% according to a study by Bergeron et al. [8] and this was found comparable to our study which showed prevalence of 3.7% for ASCUS and 11.4% for LSIL.

In another study by Lin et al. [10], similar rates were found at 3% prevalence of HGCIN for ASCUS and 11.5% for LSIL (Table 5).

In present study, the sensitivity of HPV 16/18 genotyping for detection of CIN 2 or worse in all low-grade smears was found as 66.7% and specificity as 77.1%. The *p* value was found to be 0.03 which makes it statistically significant test for this group (< 0.05).

In present study, the *sensitivity* of dual staining was *66.7%* and *specificity 84.8%* for CIN 2+ lesions for all low-grade

Table 3 Comparison of HPV 16/18 genotyping vs dual staining for detection of CIN 2+ in ASCUS and LSIL

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
ASCUS					
HPV16/18 genotyping	50	80	10	97.3	78.7
Dual stain	50	88	14.3	97.8	86.5
LSIL					
HPV16/18 genotyping	75	72.4	27.3	72.4	72.7
Dual stain	75	79.3	33.3	79.3	78.8

Table 4 Comparison of HPV 16/18 genotyping vs dual staining for detection of CIN 2+ in all low-grade smears

Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Low-grade smears					
HPV 16/18 genotyping	66.7	77.1	10.4	90.1	76.2
Dual stain	66.7	84.8	25.0	97.1	83.5

Table 5 Performance of HPV 16/18 genotyping to detect CIN 2+ lesion in ASCUS/LSIL

Study name	Year	Sensitivity		Specificity		PPV		NPV	
		ASCUS (%)	LSIL	ASCUS	LSIL	ASCUS	LSIL	ASCUS	LSIL
Zhang et al. [11]	2018	59.3	–	91.4%	–	59.3%	–	91.4%	–
Arbyn et al. [12] (meta-analysis)	2016	54.6–62.9	52.4–58.5%	79.6–85.7%	73.5–78.9%	–	–	–	–
Lin et al. [10]	2014	81.8	66.66%	90.8%	83.8%	23.6%	35.2%	99.31%	95%
Gage et al. [13]	2013	46.3	51.7%	92.3	85.5%	35.7%	44%	90.4%	91.3%
Present study		50	75%	80%	72.4%	10%	27.3%	97.3%	95.5%

Table 6 Performance of dual stain to detect CIN 2 + in ASCUS/LSIL

Study name	Year	Sensitivity		Specificity		PPV		NPV	
		ASCUS	LSIL	ASCUS (%)	LSIL (%)	ASCUS (%)	LSIL (%)	ASCUS	LSIL
Schmidt. et al. [14]	2011	89.1%	92.7%	85.5	70.7	4.2	5.5	93.6%	90.8%
Bergeron et al. [8]	2015	87.5%	86.5%	81.1	56.0	12.5	29.4	99.5%	95.1%
White et al. [15]	2015	71.9%	77.8%	87.9	88.6	17.8	15.7	96.5%	97.3%
Wu et al. [16]	2017	–	–	89.8	83.3	33.3	31.8	–	–
Magkana et al. [17]	2021	90.4%	95%	97.2	95.2	90.4	96.6	97.2%	93%
Present study		50%	75%	88	79.3	14.3	33.3	97.8%	95.8%

smears including ASCUS and LSIL, which is comparable to the previously published data (Table 6). The *p* value was found to be 0.01 which makes it a statistically significant test for this group (< 0.05).

Thus, overall, in all low-grade smears it was seen that both triage tests had a comparable sensitivity which was on the lower side. However, the specificity and accuracy of both tests were higher than sensitivity. Both triage tests are effective for the detection of high-grade CIN and can help in risk stratification, but *dual Staining was seen to be more specific and accurate than HPV16/18 genotyping*.

The prospective design of our study and utilization of histopathology as gold standard formed the core strength, whereas the small sample size and selection bias (hospital based) were our limitations.

Conclusion

Overall, in all low-grade smears the sensitivity of the two tests was comparable. However, dual staining had a higher specificity and accuracy than HPV 16/18 genotyping. It was concluded that both are effective triage methods, but dual staining had a better performance than HPV 16/18 genotyping. Thus, unnecessary colposcopy referrals and negative biopsies can be avoided in women who are negative for HPV 16/18 genotype and dual stain. This can bring down the burden of cost on the healthcare system.

Acknowledgements A part of this paper entitled "P16/KI67 dual Staining In Low-Grade Cervical Smears for Detection of High-Grade Lesions." was presented at 17th World Congress of the International Federation of Colposcopy and Cervical Pathology-IFCPC 2021. It was awarded as the first prize in oral paper presentation category and published as abstract in "Abstracts of 17th World Congress of the International Federation of Colposcopy and Cervical Pathology-IFCPC 2021. Indian J Gynecol Oncol 2021; 19(4):65." (Available from: https://doi.org/10.1007/s40944-021-00548-2).

Author Contributions All authors whose names appear on the submission: SC made substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data; used in the work. GG drafted the work or revised it critically for important intellectual content; STH approved the version to be published; and RG agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding None to make.

Declarations

Conflict of interest All authors declare that there is no conflict of interest.

Ethical Clearance The study was reviewed and approved by the Institutional Ethics Committees of Maulana Azad Medical College and associated hospitals as well as ICMR-NICPR, Noida. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments.

Research Involving Human Participants Yes.

Informed Consent Written informed consent for participation in the study was obtained for all patients included in a language understood by the participant.

Consent for Photographs Written informed consent taken from all patients included.

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