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ORIGINAL RESEARCH

Usefulness of P16 and KI67 Immunostaining in Cervical Smear Cytology in Guatemalan Women: A Cross-Sectional Study

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Abstract

Background: Conventional cytology is an economic method for screening abnormalities in cervical smears, but with implications in false positive and false negative as compared to better detection of transformed cells with the Dual staining P16/KI67 proteins in cervical smears, therefore, we evaluate the validity and reproducibility of cytology with double staining of P16 and KI67 as compared to conventional cytology in cervical smears.

Patients and methods: Participants were 210 Guatemalan women with abnormalities in conventional cytology (ASCUS, AGUS, ASCH, LSIL, HSIL, and cervical cancer) that consulted from 2013-2014 to the colposcopy Unit of Liga Nacional Contra El Cáncer e Instituto de *Cancerología* y Hospital Dr. Bernardo del Valle S. in Guatemala, from whom smear cytology was processed with P16 and KI67 immunostaining, and histological sections with P16 immunostaining as gold standard. They were evaluated by three blinded pathologists and one independent cytotechnologist.

Results: The sensitivity and specificity of Dual staining P16/KI67 for detecting abnormalities in cervical smears was 78.95%, 95% confidence interval (CI) 71.03-86.87 and 94.74%, 95%CI 89.06-100 respectively, as compare to conventional cytology in which it was 78.64% and 75.85%, respectively; positive predictive value (PPV) and negative predictive value (NPV) of Dual staining P16/KI67 were 95.74%, 95%CI 91.13-100.0 and 75%, 95%CI 65.82-84.18, as compare to conventional cytology in which it was 79.41% and 75% respectively. In women older than 30 years the sensitivity and PPV of Dual staining P16/KI67 was 78.43% and 95.24%, as compare in women less than 30 years in which it was 41.67% and 83.33% respectively. Interobserver agreement weighted Kappa indices for cervical smear cytology with double staining ranged between 0.66 to 0.83.

Conclusion: Dual staining P16/KI67 in women older than 30 years showed better sensitivity and PPV values, suggesting greater utility in this age group, although the sensitivity was like conventional cytology, but with greater specificity and PPV.

Keywords

Immunostaining, P16, KI67, Cervical intraepithelial neoplasia, Cervical smear cytology, Women, Guatemala

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Introduction

Globally, including in Latin America, low- and middle-income countries (LMICs) are experiencing an epidemiologic transition from infectious diseases to cancer and chronic diseases, and in Latin America cancer is the second leading cause of death [1,2].

Cervical cancer ranks second in incidence and mortality behind breast cancer in lower human development index setting, with an estimated 570,000 cases and 311,000 deaths in 2018 worldwide [3]. In Latin America Cervical cancer remains the number one cause of mortality due to malignant neoplasm among 20 to 40-year-old women [4]. Virtually all cervical cancers (99%) are liked to genital infection with human papilloma viruses (HPV), and persistent genital HPV infection causes cervical cancer in women. A large majority (around 85%) of the global burden occurs in the less developed regions, where it accounts for almost 12% of all female cancers [5].

In Guatemalan general population (GP) and among a group of female sex workers (SW) the HPV prevalence estimates were 38.1% (95% CI = 32.5-43.8) and 67.3% (95% CI = 61.7-72.6) [6]. It is well known that high risk HPV is responsible for causing preinvasive lesions of the cervix in 36%, 63% and 80% of the cervical intraepithelial neoplasia (CIN)I, II and III [7].

In Guatemala, by 2018, the age-standardized incidence rate of cervical cancer was 21.1, and the mortality 11.7 per 100,000, ranked as the third cancer, and fourth as a cause of death [8], and remains as a major public health problem [9].

The sensitivity and specificity of pap smear has being reported as 57% and 76%, and for colposcopy 92% and 67% respectively in detecting premalignant and malignant cervical lesion [10].

It has been described the capacity of detecting cells that are in the process of transforming to neoplasia with Dual stain testing P16 and KI67, which is a useful surrogate biomarker of cervical neoplasia with higher specificity and increases the precision of conventional cytology. The Sensitivity of Dual-stain testing for the detection of biopsy-confirmed CIN2+ during preliminary follow-up within the group of Pap negative/HPV positive women was 91.9% for CIN2+ (34/37 cases), and 96.4% for CIN3+ (27/28 cases). Specificity was 82.1% for CIN2+ on biopsy, and 76.9% for CIN3+, respectively [11-13].

A variety of studies have shown a sensitivity of 93.43% and specificity of 78.96% which is higher when compare to women younger than 30 years for detecting cervical neoplasia using the Dual stain testing P16 and KI67, and the diagnostic accuracy improved when both the stains were used in conjunction, of note, there were no studies in which the Dual stain has being done using cervical smears, but only in liquid base [11,13-16]. P16 and Ki-67 have emerged as important biomarkers for the detection of high-risk human papilloma virus and in confirming the histopathological diagnosis [17]. Therefore, we aim to evaluate the validity and reproducibility of cytology with double staining of P16 and KI67 in cervical smears.

Patients and Methods

Study design

A cross sectional study from 2013 to 2014 of 210 Guatemalan women referred to the colposcopy unit of Instituto de *Cancerología* y Hospital Dr. Bernardo del Valle S. in Guatemala.

Inclusion criteria

Women referred with abnormal conventional cytology as Atypical squamous cells of undetermined significance (*ASCUS*), atypical glandular cells of undetermined significance (AGUS), Atypical squamous cells (ASCH), low-grade squamous intraepithelial lesion (LSIL), high-grade squamous intraepithelial lesion (HSIL), and cervical cancer (CC).

Exclusion criteria

Patients treated by any oncological disease of the cervix, as well as any pregnant woman.

Sample size calculation

With a confidence level of 97%, to be able to detect a difference of 5%, we were interested in PPV (probability that the disease is present when the test is positive) of the Dual staining P16/KI67 and we wanted it to be 90%, considering 12% of missing data, the total sample size required was 190 participants.

Patient management

For each patient, new samples of cervical smear for cytology were obtained as part of their routine standard of care, another for histology that included cervical biopsies and in 45 cases cervical conization tissue. According to the consensus of the evaluating doctors, of the 210 samples, 20 were excluded from the study due to poor quality for interpretation. In total there were 190 cases, each patient read and signed the informed consent, all required permission were obtained.

Procedural step process

Prior to the colposcopy examination, a sample of cervical and endocervical exfoliation was obtained with Ayre's plastic palettes and cytobrush brushes, placing the rub on lamellae with a load and preserved with buffered alcohol. Subsequently, the evaluation was carried out with aleisegang optik1-02 brand colposcope, the cervix was previously prepared with 5% acetic acid; documenting the findings, capturing the images and completing the institutional card of colposcopy, in which the observed findings are placed, number of biopsies obtained and colposcopic impression. Obtaining samples of the cervix by means

of a Kevorkian biopsy forceps and in cases where no transformation zone was observed, curetting was performed with endocervical legra.

Sample processing

The processing of both cytological and histological samples was carried out in the molecular pathology laboratory of the Biomedical Research Center of the Faculty of Medicine of the University of San Carlos de Guatemala. All step process of sample packaging and transportation where consider to be appropriate from the hospital to the laboratory which is located about three blocks from the hospital.

For the cytologies, Dual staining P16/KI67 test was used following the manufacturer's instructions (Laboratories mtm Roche from Germany); subsequently they are stained with hematoxylin and Eosin.

For cervical biopsies and cones, 4 µm thick cuts were made for immunostaining of P16, using the in Vision (Dako), P16INK4a system was detected using the CINtec histology kit (clone E6H4, mtm Laboratories, Heidelberg, Germany), following the manufacturer's instructions. Histological sections for both P16 Immunostaining and for staining hematoxylin and Eosin were obtained from the same paraffin block.

Each cytology was previously evaluated by a cytotechnologist with more than 15 years of experience, dictating the quality of the rub. Subsequently, each of the three independent pathologists ruled the cytomorphological findings, as well as whether the double staining test was positive or not, when observing in the same cell red nucleus and brown cytoplasm.

In the case of histology samples, histomorphology findings were ruled, as well as whether P16 staining was positive or not, being positive if the tissue is stained with diffuse brown color.

Statistical analysis

A 2 times 2 table was established to calculate the sensitivity and specificity of both the conventional cytology and the Dual P16 and KI67 staining test in less than 30 years and older than 30 years, as well as PPV and NPV with its 95% confidence interval. Kappa coefficient was calculated between pathologist from Guatemala and Spain using Epidat version 3.0.

Samples interpretation

Each cytology was previously evaluated by a cytotechnologist with more than 15 years of experience. Subsequently, each of the three independent pathologists ruled the cytomorphological findings. Each pathologist and cytotechnologist report their findings with the Bethesda system of the year 2001, independently and blindly as reported by the others.

Results

Of the 210 participating patients, 20 were excluded due to poor sample quality. Of the 190 patients, the mean age and standard deviation was 41 ± 13.9 years. The most numerous age range was between 26 to 35 years (29.5%), followed by the range 36 to 45 years (26.8%). According to previous pregnancies, 43 patients (24%) have 3 children, followed by 33 patients (18.75%) with 1 child and 32 patients (18.18%) with more than 6 children.

Table 1: Demographic characteristics of patients.

Age	**N (190)**	**%**
< 20	7	3.7
21 a 25	16	8.4
26 a 35	56	29.5
36 a 45	51	26.8
46 a 55	29	15.3
56 a 65	16	8.4
> 66	15	7.9
Pregnancias	**n**	**%**
0	6	3.2
1	34	17.9
2	29	15.3
3	48	25.3
4	24	12.6
5	13	6.8
> 6	36	18.9
Contracpetive methods	**n**	**%**
None	123	64.7
Pomerooy or rings	42	22.1
Oral contraceptives	12	6.3
Quaterly	8	4.2
Jadell	3	1.6
Intrauterine device	2	1.1

Table 2: Cervical cytologies according to conventional and Dual P16/KI67 staining.

	Conventional n (%)	**P16 and KI67 n (%)**
Negative	0 (0)	65 (34.21)
[a]AGUS	4 (2.1)	1 (0.53)
[b]ASCUS	27 (14.21)	4 (2.11)
[c]ASCH	3 (1.58)	2 (1.05)
[d]LSIL	54 (28.42)	24 (12.63)
[e]HSIL	75 (39.47)	72 (37.89)
[f]ISC	22 (11.58)	14 (7.37)
[g]IAC	5 (2.63)	8 (4.21)

[a]AGUS: Atypical Glandular Cells Of Undetermined Significance; [b]ASCUS: Atypical Squamous Cell Of Undetermined Significance; [c]ASCH: Atypical Squamous Cell Of Undetermined Significance; [d]LSIL: Low-Grade Squamous Intraepithelial Lesion; [e]HSIL: High-Grade Squamous Intraepithelial Lesion; [f]ISC: Invasive Squamous Carcinomas; [g]IAC: Invasive Adenocarcinoma.

To the question of the use of contraceptive methods used, 123 patients (64.7%) report not having used any, the most commonly used method was Pomeroy or rings, 42 patients (22.1%), see Table 1. Table 2 shows the distribution of conventional cytology versus Dual staining P16/ KI67, and Table 3 shows the distribution according to histology diagnosis with P16 staining.

From each patient, a cytology sample with double staining P16 and KI67 and one or two histology (biopsy and cervical cone) with P16 staining were evaluated. There were 4 evaluators; 3 pathologists of which one was from Spain (A) and two from Guatemala (B and C); and a cytotechnologist (D). The concordance index was obtained with Kappa weighted with 95%CI. It is observed that the index for cytology is between 0.66 to 0.83, being significant with $p < 0.0001$, being the largest agreement between the expert pathologist and the cytotechnologist. In the biopsy samples they obtained a better concordance index, which was between 0.80 and 0.89, considered very well with $p < 0.0001$. With respect to the interpretation of cervical cones, there was a concordance index between 0.76 and 0.90, respectively

Table 3: Cytology with Dual P16/Ki67, according to histology diagnosis with P16 staining.

		Morphology in cytology with immunostaining P16/Ki67			
Final diagnosis, staining P16	No. cases	Negative No. (%)	ASC-US, LSIL[x] No. (%)	HSIL No. (%)	CC[h] No. (%)
Negative	53	45 (84.90)	4 (7.55)	4 (7.55)	0 (0)
LSIL	23	8 (34.78)	15 (65.22)	0 (0)	0 (0)
HSIL	78	12 (15.38)	11 (14.10)	52 (66.67)	3 (3.85)
Carcinoma[y]	36	0 (0)	1 (2.78)	16 (44.44)	19 (52.78)

[x]Includes 1 case of AGUS, 4 of ASCUS, 2 of ASCH and 24 of LSIL; [y]Includes 6 cases of Invasive adenocarcinoma; [a]AGUS: Atypical Glandular Cells Of Undetermined Significance; [b]ASCUS: Atypical Squamous Cell Of Undetermined Significance; [c]ASCH: Atypical Squamous Cell Of Undetermined Significance; [d]LSIL: Low-Grade Squamous Intraepithelial Lesion; [e]HSIL: High-Grade Squamous Intraepithelial Lesion; [f]ISC: Invasive Squamous Carcinomas; [h]CC: Cervical Cancer.

Table 4: Interobserver agreement.

Type of sample interpreted						
	Cervical cytology, double staining P16/Ki67		**Cervical biopsy, staining P16**		**Cervical cone, staining with P16**	
	***Kappa index*[a]**	**95%CI**	***Kappa index*[a]**	**95%CI**	***Kappa index*[a]**	**95%CI**
A-B	0.77*	0.69-0.84	0.83*	0.77-0.89	0.81*	0.60-1.0
A-C	0.74*	0.65-0.84	0.89*	0.85-0.93	0.90*	0.81-0.99
A-D	0.83*	0.77-0.89	-----	-----	-----	-----
B-C	0.66*	0.57-0.75	0.80*	0.74-0.87	0.76*	0.53-0.98
B-D	0.75*	0.66-0.81	-----	-----	-----	-----
C-D	0.75*	0.67-0.84	-----	-----	-----	-----

A: Expert Pathologist; B y C: Guatemalan Pathologists; D: Cytotechnologist; *K:* Weighted Kappa*; CI:* Confidence Interval*;* *P value < 0.0001.

Table 5: Results of histology interpretation with H&E and with P16.

	Cervical bioposies		**Cervical cones**	
	H&E staining n (%)	**P16 n (%)**	**H&E staining n (%)**	**P16 n (%)**
Negative	53 (27.9)	53 (27.9)	4 (8.9)	4 (8.9)
[d]LSIL	34 (17.9)	23 (12.1)	4 (8.9)	0 (0)
[e]HSIL	68 (35.9)	78 (41.1)	31 (68.9)	35 (77.8)
[f]ISC				
Microinvasive	6 (3.1)	3 (1.6)	3 (6.7)	1 (2.2)
Invasive	23 (12.1)	26 (13.7)	3 (6.7)	5 (11.1)
Invasive adenocarcinoma	6 (3.1)	7 (3.7)	0 (0)	0 (0)
Total	190 (100)	190 (100)	45 (100)	45 (100)

[d]LSIL: low-grade squamous intraepithelial lesion; [e]HSIL: high-grade squamous intraepithelial lesion; [f]ISC: Invasive squamous carcinomas.

being considered good to very good with p < 0.0001, see Table 4.

The distribution of the cytomorphological interpretation of the gold standard that was the diagnosis by biopsy or cone with the corresponding staining. Because in some cases there was a discrepancy between cytology and biopsy, specifically cases in which cytology reported HSIL or invasive cancer and in biopsy reported cervicitis or LSIL, diagnostic cones were performed in some cases. Therefore, it was considered in these cases the final or conclusive diagnosis is the result of the cervical cone (Table 5).

For the Dual staining P16/KI67, it is observed that for both types of cytologies, conventional and double staining with P16/KI67, the sensitivity is similar 78.64 and 78.95% respectively. However, the specificity was different being 75.85 and 94.74% respectively, higher for double staining because there are fewer false positive cases (Table 6).

With respect to PPV, that is, the percentage of probability that the positive test is positive in the patient who is ill (diagnosis ≥ HSIL) was higher in cytology with double staining 95.74% with 95%CI 91.13-100.0. The PPV of conventional cytology and colposcopy was 79.41. The NPV, that is the percentage or probability that the test being negative, the patient is healthy, was similar in both types of cytologies with 75% in each one. The sensitivity, specificity, PPV and NPV of double staining in cytology were compared, according to two age groups, in < 30 and older > 30 years. The confirmatory test was the biopsy or cervical cone with P16 staining. He evidenced that the highest sensitivity and PPV of the test is in the group > 30-years-old, 78.43 and 95.24% respectively. Specificity and NPV were slightly higher in the < 30 years old group: 95.83 and 83.33% (Table 7).

Discussion

When comparing the interpretation of the two types of cytology, (conventional staining or pap and double staining P16/KI67), there were differences especially in the cytological diagnoses of ASCUS and LSIL.

In the cases less than HSIL, the results between cytology with conventional staining and double staining were different: ASCUS 27/190 (14.21%) and 4/190 (2.11%); LSIL 54/190 (28.42%) and 24/190 (12.63%) respectively, therefore the difference for ASCUS of 12% and LSIL of 16%. The use of this screening technique would have prevented a 25% extension of the study because it was low grade, with which initial follow-up, or eventually colposcopy, could be performed to determine the next step.

For lesions of HSIL there was a 4% overall difference between both types of stains, which could suggest that it detects fewer cases (less sensitivity) or as histological correlation seems to show, which has less false positives.

The interobserver reproducibility of good concordance, demonstrates a simplification and greater coherence and safety in the diagnosis. In this sense, the interobserver reproducibility of P16/KI67 staining in cervical smear cytology is reflected in the concordance index with weighted Kappa that was 0.66 to 0.83 (qualifiable as good); in a similar but liquid based study Wentzensen, et al. reported that it was from 0.65 to 0.81 [18], like what we found.

In histology, P16 staining was evaluated with respect to the interobserver concordance index or agreement between pathologists. In this study it was found that the weighted Kappa index of three pathologists for histomorphological interpretation of biopsies and cervical cones with P16 staining was between 0.83-0.89 and 0.76-0.90 respectively; this concordance is considered good to very good, with significance (p < 0.0001). In a similar study the Concordance Index was evaluated giving an average of 0.89 for biopsies and cervical cones [19].

Regarding the results of cones, with H&E and P16 staining, it is observed that in 3 cones with P16 they are reclassified from microinvasive to invasive. This may be relevant in influencing the surgical management of the patient for definitive treatment.

The present study reports a sensitivity to detect cytomorphological changes corresponding to lesion of HSIL with conventional staining and with double staining, 78.64 and 78.95% respectively. Therefore, the sensitivity value in both cytologies was similar.

In a meta-analysis which reviewed 24 articles, the

Table 6: Sensitivity, specificity, PPV and NPV of conventional cytology staining versus staining with P16/Ki67.

Test method	Sensitivity, 95%CI	Specificity, 95%CI	PPV, 95%CI	NPV, 95%CI
Conventional	78.64, 70.24-87.04	75.86, 66.30-85.43	79.41, 71.07-87.75	75.00, 65.38-84.62
P16/Ki67 staining	78.95, 71.03-86.87	94.74, 89.06-100.0	95.74, 91.13-100.0	75.00, 65.82-84.18

Table 7: Sensitivity, specificity, PPV, NPV of P16/Ki67 by age group.

	Sensitivity, (95%CI)	Specificity, (95%CI)	VPP, (95%CI)	NPV, (95%CI)
< 30 años	41.67, (9.61-73.73)	95.83, (85.76-100)	83.33, (45.18-100)	76.67, (59.87-93.47)
> 30 años	78.43, (69.96-86.90)	92.31, (69.96-86.90)	95.24, (90.09-100)	68.57, (56.98-80.16)

sensitivity of conventional cytologies was 65%. This suggests that in our environment the sensitivity is higher, however, we must consider that our study was conducted in patients referred to the Colposcopy Unit with cytology results greater than or equal to ASCUS [20]. Therefore, to detect lesions of HSIL (which is clinically relevant) the sensitivity of dual staining was similar to conventional, but dual staining P16/KI67 has a greater PVV with specificity of 94.74% versus 75.86% of the cytology with conventional staining.

Comparable studies, such as that of Murphy, et al. [21], report a similar specificity, having a value of 95.7%, and report that the false positive rate for conventional cytology is 30%. The latter is somewhat higher than ours, but again we must consider that our sample had more than 50% of women with a lesion of HSIL.

Two age groups were also analyzed, depending on whether they were under or over 30-years-old. Double staining in less than 30 years presented a sensitivity and specificity of 41.67% and 95.83% respectively, however, in the group above 30 years the sensitivity and specificity was 78.43% and 95.24% respectively.

These data are comparable with those of a study conducted by Jaume Ordi, et al. [13] which showed that in patients younger than 30 years the sensitivity and specificity was 78% and 91% respectively, and in over 30 years the sensitivity and specificity was 86.5 and 94.8% respectively. The specificity is similar in our study and sensitivity is likely to be more limited because the number of cases with lesions greater than HSIL was much lower among women who referred us at a younger age.

Conclusion

Dual staining P16/KI67 in women older than 30 years showed better sensitivity and PPV values, suggesting greater utility in this age group, although the sensitivity was like conventional cytology, but with greater specificity and PPV.

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Conflict of Interest

None to declare

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