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RESEARCH

p16 and Ki-67 immunohistochemical staining reduces inter- and intra-observer variability in the grading of cervical squamous intraepithelial lesions of South African women

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Background: Cervical carcinoma was the second leading malignancy in South African women (following breast carcinoma) in 2010. This study aimed to correlate histopathological criteria and immunohistochemical stains in terms of the grading of cervical intraepithelial precursor lesions and evaluate intra- and inter-observer variability with only histology and with additional immunohistochemical stains.
Methods: Archival tissue from large-loop excision of the transformation zone (LLETZ) was graded on two separate occasions by an independent observer in terms of lesional severity. The section with the highest grade precursor lesion was selected and submitted for immunohistochemical stains that included p16 and Ki-67. These stains were also evaluated on two separate occasions by an independent observer.
Results: This study showed kappa values of 0.47 and 0.46 respectively for the separate histological evaluations of the observer and the original pathology report. The kappa value for the two evaluations of the observer was 0.57. Thus inter- and intra-observer variability is fair with the use of routinely stained histological slides. The two Ki-67 assessments had a kappa value of 0.85 and the p16 had a value of 0.80. Intra-observer agreement was markedly higher when using immunohistochemistry.
Conclusion: Although in most cases of precursor lesions of the cervix the grading can be made on routinely stained sections, intra- and inter-observer variability remains high. Immunohistochemical markers reduce this variability and aid in deciding in which group to place ambiguous lesions.

Keywords: CIN, HPV, LLETZ, observer variability,South Africa

Introduction

Cervical carcinoma is the second most common cancer in South African females of all races following breast carcinoma. It is, however, the leading female cancer and leading cause of cancer deaths in females aged 15 to 44 years in South Africa.[1]

The human papillomavirus (HPV), notably the high-risk genotypes, has been identified as the main causative factor in cervical neoplasia and invasive carcinoma.[2,3] This virus is transmitted mainly via sexual contact.[2] The division between high- and low-risk categories is partly based on the affinity in which the HPV-type specific oncoproteins bind cellular regulatory proteins. Carcinogenic or high-risk types include HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82.[2,4] Low-risk types are HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108.[4] These oncoproteins, namely E6 and E7, inactivate the p53 and retinoblastoma (Rb) tumour suppressor proteins respectively, disrupting the cell cycle with an increase in proliferative activity. HPV DNA integration into host DNA is needed, this being performed by disrupting the E1/E2 open reading frame with subsequent loss of E6 and E7 regulation by E2.[5]

The cell cycle is finely controlled by the activity of cyclin-dependent kinases (CDK), CDK1, CDK2, CDK4 and CDK6, their activating co-enzyme cyclins A, B, D and E as well as cyclin-dependent kinase inhibitors. These inhibitors are divided into two families, the CIP/KIP family with p21, p27 and p57 and the INK4 family with p15, p16, p18 and p19. INK4 inhibits cyclin D by interacting with CDK4 and CDK6. The KIP family affects cyclin E, cyclin A/CDK2 and cyclin B/CDK1.

The cyclin-dependent kinase inhibitor, p16, regulates CDK4 and CDK6 by interacting with cyclin D1. Inactivation of this protein by means of hypermethylation or genetic deletion is an event in several malignancies. In HPV-associated tumours, the E7 oncoprotein inactivates Rb and this results in a marked increase in p16.[5]

Precursors to invasive carcinoma can be detected by means of screening; however, implementation has proved problematic in low-resource areas.[6,7] To complicate matters further, histological grading (which largely guides the clinical management of the patient) of these precursor lesions can be subjective.[8,9]

Cervical carcinoma may take up to three decades to develop, allowing time to screen for its precursor lesions by means of cervical cytology. These precursors can be divided into two groups: high-grade squamous intraepithelial lesions (HSIL), which encompasses the old cervical intraepithelial neoplasia (CIN II and III), and low-grade squamous intraepithelial lesions (LSIL), which includes the old terminology of CIN I. These lesions can be identified by Pap smear and on histological evaluation. The majority of low-grade lesions will regress; however, with

high-grade lesions only 50% will regress. An increase in grade corresponds to an increase in risk of progression to invasive carcinoma. The grading of these precursors is important as the treatment differs.[10,11]

The mortality rate has dramatically reduced in some countries with the introduction of screening and early treatment. In 2000, the South African Department of Health introduced a national cervical screening policy; however, implementation has proved problematic, particularly in the areas where resources are limited.[12]

The precursor lesions are treated be means of either cold knife biopsy, laser evaporisation, large-loop excision of the transformation zone (LLETZ), also known as loop excision by electrosurgical procedure (LEEP) and cryotherapy of the transformation zone. Of these, the LLETZ procedure allows tissue to be submitted for histopathological examination, including grading of precursor lesions.[13]

Histological assessment remains the current basis for treatment and serves as the follow-up of abnormalities identified by screening. Precursor lesions have well-recognised histological features, including the degree of maturation and mitotic activity level. However, application of these morphological criteria is variable and due to the presence of mimics (atrophy, immature metaplasia, reactive/inflammatory atypia) can result in significant lack of intra- and inter-observer reproducibility.[14]

More specific biomarkers might improve the situation, the p16 immunohistochemical stain being the most extensively studied and found to be specifically overexpressed in dysplastic and malignant cells.[3,15] Previous studies have shown that the degree of p16 expression as well as the Ki-67 expression correlated well with the degree of cervical neoplasia.[3,16–20]

Overexpression of p16 is characteristic of dysplastic and neoplastic cervical epithelium.[3,21] It has thus been used as a biomarker for dysplasia. The overexpression of p16 has a close association with high-risk HPV infection and correlates well with the degree of cervical neoplasia.[3,16,17,22] Klaes *et al*. demonstrated diffuse and strong overexpression of p16-specific antibody E6H4 in high-grade precursors, whilst normal cervical epithelium, inflammatory and metaplastic lesions did not show positive staining.[15]

p16 is overexpressed in almost all cases of high-grade lesions and squamous cell carcinomas but also in adenocarcinoma in situ as well as adenocarcinomas. According to Samarawardana *et al*., benign cervical lesions occasionally express p16, including the normal columnar cells of the cervix, tuboendometrial metaplasia, cervical endometriosis and squamous metaplasia. Thus the combination of p16 and Ki-67 on the same histological slide is advised.[18] Agoff *et al*. showed p16 to be more specific for neoplasia than Ki-67.[3]

Ki-67 (detected by the MIB-1 monoclonal antibody) is a nuclear protein associated with the transcription of RNA and progression of the cell cycle. It is overexpressed in high-grade precursor lesions of the cervix as well as in squamous cell carcinoma, adenocarcinoma and adenocarcinoma in situ, similar to the profile of p16. It is additionally expressed in the normal basal squamous mucosa and in benign proliferative lesions, but not in benign endocervical cells, squamous metaplastic cells or in foci of tuboendometrial metaplasia.

Ki-67 by itself does not aid differentiation between reactive changes and high-grade dysplasia. Furthermore, quantitative scoring can be complicated by proportional differences in glands seen in the field of the microscope.[18] Ki-67 expression is correlated with the grade of epithelial dysplasia, increasing from LSIL to infiltrating squamous cell carcinoma.[23] Ki-67 immunoquantative features are correlated with the presence of oncogenic HPV DNA.[20] Kruse *et al*. found Ki-67 to be useful to distinguish between different grades of cervical intraepithelial neoplastic lesions, as a potential method of quality control and possible indicator of progression in low-grade lesions.[20]

Samawardana *et al*. have shown that p16 and Ki-67 co-expression is present in almost all high-grade squamous and glandular lesions and rarely in benign conditions and that the markers in combination are more sensitive and specific than either of them used in isolation.[18] Keating *et al*. proved a strong relationship between Ki-67, cyclin E and p16 in the recognition of HPV-associated precursors as well as in distinguishing normal squamous mucosa from lesional tissue.[21]

Materials and methods

Histology

A total of 114 LLETZ biopsies performed between 2010 and 2011 were retrieved from the archives of the Department of Anatomical Pathology of the University of Pretoria and the National Health Laboratory Services (Tshwane Academic Division). All of these cases had conventional cytological smears that showed a high-grade or persistent low-grade squamous intraepithelial lesion. Dry swabs were also collected for HPV DNA typing.

Tissue submitted from the LLETZ biopsies, fixed in formalin, embedded in paraffin wax and stained with haematoxylin and eosin was graded by two primary observers in terms of lesional severity. The first observer was the pathologist initially allocated to the case and the second an independent observer without knowledge of the history or previous diagnoses.

In cases of discrepancy, a third unbiased observer was consulted. The second observer graded the lesions on two separate occasions in order to evaluate both inter- and intra-observer variability. The section with the highest-grade precursor lesion was then selected from each patient and submitted for immunohistochemical stains, the latter also being scored blindly on two separate occasions to assess intra-observer variability on the interpretation of the stains.

The criteria used for the grading of the histological slides were as follows:

CIN I indicating dysplasia involving the lower third of the cervical epithelium, CIN II having dysplasia involving the lower two-thirds of the epithelium with maturation and flattening of the surface epithelium still being present and CIN III showing full-thickness dysplasia with no evidence of maturation.

Scoring was done as follows: 0 indicating CIN I, 1 CIN I–II, 2 CIN II, 3 CIN II–III and 4 CIN III.

Immunohistochemistry

Immunohistochemical stains were performed using the following primary monoclonal antibodies: (a) rabbit anti-human Ki-67 antigen and (b) mouse primary antibody against the p16INK4a protein (Ventana CINtec p16 histology).

The ready to use K8000 kit of Dako was used on the Dako AutostainerLink 48 (Denmark) for immunostaining. Formalin-fixed paraffin-embedded samples were cut into 3 µm thickness tissue sections. The sections were dewaxed and the antigens retrieved at high pH (9.4) with the EnVision Flex Target retrieval solution (Dako, Germany) at 96°C for 20 min followed by the EnVision Flex wash buffer rinse for 5 min. H_2O_2 blocking was then performed with EnVision Flex peroxidise blocking reagent for 10 min and then rinsed with the buffer solution for 5 min. The primary antibody was then incubated for 30 min at room temperature and again rinsed for 5 min with the buffer. The labelled polymer, EnVision Flex horseradish peroxidase, was applied for 30 min followed by 5 min of buffer. The substrate working solution followed, EnVision Flex DAB chromogen for 10 min. A 5 min buffer rinse was again applied. The sections were then counterstained with Mayer haematoxylin for 3 min and blued with tap water for 5 min. The sections were then dehydrated to xylene and mounted.

p16 has both nuclear and cytoplasmic expression, thus both of these staining patterns were considered to be positive and were scored as follows: 0 when no staining was observed, 1 when very occasional, single cells stained, 2 when patchy, strong staining (often not continuous with basement membrane) was seen and 3 when diffuse and strong staining (continuous from basement membrane, extending upward) was present. p16 was also correlated according to the HSIL present in terms of the proportion of dysplastic epithelium present.

The Ki-67 has nuclear staining only. A negative result was regarded as occasional positive basal or parabasal cells and a positive result as positive cells above the lower third of the epithelium.

Results

The concordance between the initial diagnosis and the review was 62.2% with a kappa value of 0.47. The cases were reviewed again by the observer without knowledge of the initial and second diagnosis and once again concordance was 60.7% with a kappa value of 0.46.

Thus inter-observer variability is fair with the use of routinely stained histological slides.

The concordance between the two diagnoses made by the observer was 67.9% with a kappa value of 0.55. Again, a fair intra-observer agreement is noted with routinely stained sections.

As the immunohistochemical stains were performed by the observer, two independent evaluations of these stains were made by the observer; again, the second evaluation without knowledge of the result of the first. The intra-observer agreement between the assessment of the p16 stain was 92% with a kappa value of 0.8. The intra-observer agreement for the Ki-67 stain was 96.4% with a kappa value of 0.85. Thus the intra-observer agreement is markedly higher when using immunohistochemistry.

For the initial diagnosis, 12 cases were regarded as CIN I (10.5%), 1 was CIN I–II (0.1%), 46 were CIN II (40.3%), 28 CIN II–III (24.6%) and 27 CIN III (23.7%).

When the first assessment of the observer was taken into account, 21 of the 114 cases were regarded as CIN I lesions (18.4%), 50 were CIN II (43.9%), 27 CIN II–III (23.7%) and 16 were CIN III (14%).

When the second assessment of the observer was taken into account, 19 of the 114 cases were regarded as CIN I lesions (16.7%), 42 were CIN II (36.9%), 37 CIN II–III (32.5%) and 16 were CIN III (14%).

For the initial diagnoses and comparison of the lesion present with the immunohistochemistry, 5 of the CIN I lesions (41.7%) showed negative staining with p16 and 5 (41.7%) showed diffuse and strong positivity. The one case that was assessed as a CIN I–II showed strong and diffuse p16 positivity. Of the CIN II lesions 4 (8.7%) showed no p16 staining and 31 (67.4%) strong and diffuse staining. The CIN II–III grouped had 2 (7.14%) which stained negative and 21 (75%) that showed strong positivity. Finally the CIN III group had 0 that had no p16 staining and 25 (92.6%) that had strong staining. The Fisher's exact test was 0.

For the Ki-67 stain in this group, 7 (58.3%) and 5 (41.7%) of the CIN I lesions had negative and positive results respectively. The one case of CIN I–II showed positive staining. The CIN II group had 5 (10.9%) that were negative and 41 (89.1%) that were positive. The CIN II–III group showed 2 (7.1%) negative cases and 26 (92.9%) positive cases. Only 1 (3.7%) of the CIN III cases was negative with Ki-67 and 26 (96.3%) were positive. The Fisher's exact test result was 0.005.

The first assessment of the observer and the p16 stain showed 5 of the CIN I lesions (23.8%) with negative staining and 10 (47.6%) with diffuse and strong positivity. Of the CIN II lesions 4 (8%) showed no p16 staining and 38 (76%) strong and diffuse staining. The CIN II-III grouped had 1 (3.7%) which stained negative and 21 (77.8%) that showed strong positivity. The CIN III group had 1 (6.3%) that had no p16 staining and 14 (87.5%) that had strong staining. The Fisher's exact test was 0.004.

For the Ki-67 stain in this group, 8 (38.1%) and 13 (61.9%) of the CIN I lesions had negative and positive results respectively. The CIN II group had 2 (7.4%) that were negative and 45 (90%) that were positive. The CIN II–III group showed 2 (7.1%) negative cases and 25 (92.6%) positive cases. None of the CIN III cases were negative with Ki-67 and 16 (100%) were positive. The Fisher's exact test result was 0.005.

The initial dry swabs that were taken showed 5 cases (1.7%) that did not yield any HPV. HPV16 was identified in 38 (13.4%), HPV18 in 14 (4.9%), HPV31 in 23 (8.1%), HPV33 in 21 (7.4), HPV35 in 29 (10.2%), HPV39 in 12 (4.2%), HPV45 in 13 (4.6%), HPV51 in 33 (11.6%), HPV52 in 16 (5.6%), HPV54 in 1 (0.4%), HPV56 in 13 (4.6%), HPV58 in 28 (9.7%), HPV59 in 10 (3.5%), HPV68 in 10 (3.5%), HPV73 in 9 (3.1%) and HPV82 in 9 (3.1%).

Of the 109 cases that did yield HPV on dry swabs, 29 (26.6%) were infected with a single type, 27 (24.8%) with 2 types, 30 (27.5%) with 3 types, 13 (11.9%) with 4 types, 6 (5.5%) with 5 types and 4 (3.7%) had the maximum of 6 types of HPV identified in a lesion.

Discussion

South Africa is a unique country in terms of the diverse demographics of the population, distribution of the ethnic groups in the country and also the significant number of immunocompromised (HIV positive) individuals. The results of this study can therefore not be extrapolated to other countries or populations.

It is clear from the study that both inter- and intra-observer variability is only fair when using routine haematoxylin and eosin

(H&E) stained histological sections. The intra-observer agreement was significantly higher when interpreting the immunohistochemical stains. Though H&E sections remain the gold standard, the use of immunohistochemistry can augment and refine the initial diagnosis and, in cases where there is uncertainty, direct the diagnosis.

The Bethesda system, used in cytological Pap smears, has now been incorporated in the histological assessment of cervical precursor lesions. This two-tiered system, of low- and high-grade squamous intraepithelial lesions only, is less complicated than the previous three-tiered CIN system and will, to a certain degree, also decrease the inter- and intra-observer variability. For the purposes of this study (the cases were initially evaluated when the three-tiered CIN system was still in place) we used the CIN system, also to obtain a more detailed assessment. This can, however, readily be extrapolated to the current two-tiered classification by combining the CIN II and CIN III results to form the HSIL group.

It is still important, though, to make the distinction between the low- and high-grade lesions, as treatment differs dramatically and although theoretically it might seem like a clear distinction, cases where it is not apparent in which group a lesion belongs certainly exist. It is in these cases that immunohistochemistry will greatly aid the diagnostic process.

Interesting findings from this study include the high proportion (23.8–41.7%) of low-grade lesions that showed strong and diffuse p16 positivity as well as the 41.7–61.9% that showed positive Ki-67 staining. It is likely that these cases will progress to high-grade lesions as high-risk HPV types are present. If one can identify these cases where progression is more likely, more aggressive treatment (as for a high-grade lesion) might be considered.

There is a clear upward gradient for the CIN II–III group for both markers as the lesional severity increases.

Also demonstrated from the swabs that were taken is that HPV16 remains the most prevalent high-risk subtype, closely followed by HPV35 and 51. Nearly 80% of cases harboured between 1 and 3 HPV types, approximately 12% having 4 types and the remaining having 5 to 6 high-risk types. Co-infection by several high-risk HPV subtypes will also likely increase the chances of progression of the lesion.

Although in most cases of precursor lesions of the cervix the grading can be made on routinely stained sections, intra- and inter-observer variability remains high. Immunohistochemical markers reduce this variability and aid in deciding in which group to place ambiguous lesions. Also, the use of these markers may help in predicting progression of lesions that appear low grade.

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