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Title: Triage of LSIL/ASCUS with p16/Ki-67 dual staining and Human Papillomavirus testing: a two year prospective study

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Running title: HPV and p16/Ki-67 triage of minor cervical abnormalities

Conflicts of interest

This study was performed under CERVIVA, the Irish Cervical Screening Research Consortium, funded by the Health Research Board Ireland and supported by the Irish Cancer Society. Roche Diagnostics and Qiagen are commercial partners in CERVIVA. All HPV testing and immunocytochemistry kits and associated reagents for Hybrid Capture 2 and CINtec® PLUS were purchased for this study. This was an investigator led study and all opinions detailed in the manuscript are those of the investigators.

Abstract

Objective: To investigate the utility of HPV DNA testing and p16/Ki-67 dual staining for detecting CIN2+ and CIN3 in women referred to colposcopy with minor abnormal cervical cytology LSIL and AS-CUS. The clinical performance of both tests was evaluated as standalone tests and combined tests, for detection CIN2+ and CIN3 over 2 years.

Methods: ThinPrep® liquid based cytology specimens were collected from 1349 women with repeat LSIL or ASC-US. HPV DNA was performed using Hybrid Capture. Where adequate material remained (n=471), p16/Ki-67 over expression was assessed. The clinical performance for detection of histologically diagnosed CIN2+ and CIN3, was calculated for both tests.

Results: Overall 62.2% of the population were positive for HPV DNA, and 30.4% were positive for p16/Ki-67. p16/Ki-67 showed no significant difference in positivity between LSIL and ASC-US referred patients (34.3% vs 28.6%; $p=0.189$). Women under 30 years had a higher rate of p16/Ki-67 positivity compared to those over 30 years (36.0% vs 26.6%; $p=0.03$). Overall HPV DNA testing produced a high sensitivity for detection of CIN3 of 95.8% compared to 79.2% for p16/Ki-67. In contrast, p16/Ki-67 expression offered a higher specificity, 75.2% versus 40.4% for detection of CIN3. Combining p16/Ki-67 with HPV DNA testing improved accuracy in distinguishing between CIN3 and <CIN3. The absolute risk of CIN3 increased from 15.6% in women who were HPV DNA positive to 27% in women positive for HPV DNA and p16/Ki-67. Those negative for HPV DNA and p16/Ki-67 had a low risk of 1.2%, of CIN3.

Conclusion: The addition p16/Ki-67 to HPV DNA testing lead to a more accurate stratification of CIN in women presenting with minor cytological abnormalities.

Key words: Human Papillomavirus, p16/Ki-67, Triage, Low grade squamous intraepithelial lesion, Atypical squamous cells of undetermined significance

Introduction

Based on the known causal relationship between high risk human papillomavirus (HR HPV) and cervical cancer (1), HPV testing has become an important tool in developing strategies for cervical cancer screening. It was initially approved as a triage test for minor cytological abnormalities, providing improved detection of cervical intraepithelial grade 2 or worse (CIN2+) compared with repeat cytology (2-4). However, despite the utility of HPV testing in triage, concern remains over the reported suboptimal specificity of HPV DNA based tests (5). This is due to the fact that HPV DNA testing cannot discriminate transforming infections from transient infections of minor clinical relevance. Knowledge of HPV pathophysiology has enabled the identification of a number of biomarkers with potential to distinguish those at risk of disease progression. Several host cell biomarkers have been evaluated for their potential to improve the diagnostic specificity of cervical screening (6). One of the most promising cellular protein markers to be identified is the cyclin dependent kinase inhibitor p16^{INK4A} (referred to as p16 hereafter) (7-9). However, p16 can be over expressed in some non-dyskaryotic cells (10) and, as a consequence, morphological criteria are needed (11). It is known that increased expression of p16 signals functional activation of E2F mediated by HPV E7. Combining p16 with the proliferation marker Ki-67 signals HPV transformed

cells undergoing proliferation. Studies have reported on the clinical performance of dual staining for p16 and Ki-67 for detection of CIN2+ and CIN3 (13-19). However, longitudinal data is limited on its utility as a triage tool for minor cytological abnormalities LSIL (low grade squamous intraepithelial lesion) and ASC-US (atypical cells of undetermined significance).

The purpose of this two year prospective study was to examine two testing modalities, HPV DNA and p16-Ki-67, with an aim to identify an approach to best manage women with LSIL and ASCUS on cytology.

Materials and Methods

Study population

The study setting was Ireland. At commencement of the study guidelines from CervicalCheck, the National Cervical Screening Program, state the following for referral to colposcopy following minor cytological abnormalities on ThinPrep® liquid based cytology: 1.) three consecutive ASC-US; 2.) two consecutive LSIL; 3.) two consecutive cytology samples graded a combination of ASC-US and LSIL; or 4.) having any three ThinPrep® liquid based cytology test results that are not normal in the previous 10 years without referral to colposcopy (12). Women gave written informed consent to take part in the study at their first visit to colposcopy following referral for repeat LSIL and ASC-US cytology at the National Maternity Hospital, Holles Street, Dublin from October 2008 to July 2011. Women were excluded from the study if they were pregnant, under the age of 18 or had been treated for CIN in the previous 5 years. All women were assessed by BSCCP (British Society for Colposcopy and Cervical Pathology) trained colposcopists. Women were followed over the period of time they spend under surveillance where

they were managed according to the standard protocol of the clinic. This involved follow up by 6 month cytology following the initial colposcopy visit. If a high grade lesion was identified at the initial colposcopy or during follow up, women were brought back within a shorter period, for repeat colposcopy and treatment. Women with persistent minor abnormalities identified at their initial colposcopy (Time 0 months) and up to three or more subsequent follow up visits (time 18 months), were offered treatment. Women were followed over a two year period or until they reached defined study endpoints. Study endpoints included having two consecutive normal cytology results and discharge from the clinic without treatment, or alternatively, having a LLETZ (Large Loop Excision of the Transformation Zone). The majority of women, 90.7% (427/471), had a histologic diagnosis over the two year follow up period by punch biopsy and/or LLETZ. Histological diagnosis was based on a standard protocol outlined in the CervicalCheck guidelines. In this study CIN was diagnosed by a pathologist in daily routine practice to allow test performance to be evaluated in a routine population-based setting. All results were collected and recorded from the participating hospital. The study was approved by The Research Ethics Committee at the National Maternity Hospital, Holles Street, Dublin.

HPV DNA testing

A ThinPrep® liquid based cytology specimen was taken for HPV testing and immunocytochemistry (ICC) prior to first colposcopic examination. Detection of DNA from oncogenic HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 was performed using Hybrid Capture 2 (HC2) (Qiagen, GmbH, Hilden, Germany), as described by the manufacturer. The RLU/CO negative cut off value was 1.0. Specimens below this detection limit were considered negative.

p16/Ki-67 dual stain

Cytology slides from residual ThinPrep® material, from the same sample used for HPV testing, were prepared using a T2000 slide processor. The CINtec PLUS® kit (Roche mtm Laboratories AG, Mannheim, Germany) was used for ICC staining of p16 and Ki-67 in accordance with the manufacturer's instructions. A positive result was interpreted as brown cytoplasmic staining for p16 expression and red nuclear staining for Ki-67 expression. The presence of one or more stained epithelial cells, showing simultaneous expression, signified a positive result. All cases were subjected to a pathologist review, blinded to histology and HPV status. All testing on ThinPrep® Specimens was performed within three months of obtaining the specimen. Results from HPV testing and ICC were not disclosed to the participants or used for patient management.

Statistical Analysis

The final outcome was based on the histological grade taken as the worse of the histological findings on punch biopsy or LLETZ during a patient's time at colposcopy. The primary disease endpoint was histologically confirmed CIN2+ and CIN3, diagnosed within two years of the first colposcopy visit. While staining for p16 has been recommended to improve diagnosis of CIN2 (20) it was not possible for this study. We included CIN3 as a clinical endpoint as it is considered the true pre-cursor to cervical cancer. Women who had a normal colposcopy, without biopsy, at initial or follow up visits were classified as <CIN2/3. Data was analysed using Minitab statistical software version 16. Confidence intervals were calculated where appropriate. McNemar's test was used to compare differences in disease detection between HPV DNA and p16/Ki-67. A p value <0.05 was

considered statistically significant. The clinical performance of both tests, HPV DNA and p16/Ki-67, was assessed by calculating the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and the relative 95% confidence intervals (CI) for detecting CIN2+ and CIN3 over a two year follow up period in those with a histological diagnosis only. Additional sensitivity, specificity, PPV and NPV were calculated stratified by age and referral cytology.

Results

Among the 1346 women recruited in to the study, 1079 had a complete HPV DNA result. Of the 1079, 764 had residual material available to prepare a ThinPrep® slide. As additional tests, not included in this analysis, were performed on the same samples cellularity was low in some cases and resulted in exclusion of a further 293 cases. The final study population was 471. All subsequent results are based on this group of 471 women.

The median age of the population at enrolment was 31 years (interquartile range 27-38). LSIL referral was more common representing 56.3% (265/471) of referrals compared to ASC-US at 43.7% (206/471). Within a two year period, 29.3% (138/471) of women had a CIN2+ diagnosis on histology, 65.2% (90/138) of whom were classified as CIN2 and 34.8% (48/138) classified as CIN3. The majority, 83.3% (115/133), of CIN2+ was identified on histology at the first visit, generally following punch biopsy. The remaining 18 cases were identified at follow up visits (Figure 1A). Figure 1B illustrates the time at which treatment occurred for high grade CIN. Within the two year follow up period, a total of 33.1% (156/471) of women received a LLETZ treatment, 87.2% (136/156) to treat a suspected high grade lesion and 12.8% (20/156) for a persistent low grade lesion.

Two out of twenty women treated for a persistent low grade lesion had CIN2 on their LLETZ specimen. In total 22.7% (107/471) of women remained under surveillance after two years follow up. The majority of women who remained under surveillance, 85.0% (91/107), were under the age of 40 years. A further 44.2% (208/471) of the overall population exited the study having had two sequential normal cytology results and no treatment.

The prevalence of p16/Ki-67 and HPV DNA at recruitment indicated p16/Ki-67 over-expression was present in 30.4% (95% CI 28.5-32.3) of women, compared with a HPV DNA prevalence rate of 62.2% (95% CI 60.1-64.3). p16/Ki-67 was positive in 34.3% of women referred with LSIL and 28.6% (59/206) referred with ASCUS ($p=0.189$). HPV DNA was positive in 71.7% (190/265) of LSIL referrals and 50.5% of ASCUS referrals ($p<0.001$). The prevalence across each grade of CIN is shown in table 1. HPV DNA testing detected a higher proportion of CIN2+ and CIN3, compared to p16/Ki-67 testing ($p<0.001$). However, a positive HPV DNA result identified over twice as many women (57.9% and 84.4%) as p16/Ki-67 (27.8% and 73.2%) who, in fact, had no CIN2+ or CIN3 including women with persistent low grade abnormalities and those discharged with two sequential normal cytology results (table 1).

Table 2 shows, separately, the clinical performance characteristics of p16/Ki-67 and HPV DNA testing for detection of CIN2+ and CIN3. The calculations are based on those with a histological diagnosis over two years ($n=427$). HPV DNA detection demonstrated the highest sensitivity, 92.8% and 95.8%, but with limited specificity 48.9% and 40.4% for CIN2+ and CIN3 respectively. In comparison p16/Ki-67 had a lower sensitivity of 75.4% and 79.2%, and higher specificity of

88.3% and 75.2% for CIN2+ and CIN3. p16/Ki-67 demonstrated a significantly higher PPV of 26.6%, for detection of CIN3, compared to 15.4% for the HPV DNA test ($p < 0.001$). Whereas HPV DNA testing had a significantly higher NPV of 98.8%, compared to p16/Ki-67 at 97.0% ($p = 0.01$). p16/Ki-67 testing showed comparable sensitivity and specificity in both LSIL and ASC-US referrals, whereas HPV DNA testing had significantly higher sensitivity and specificity in ASC-US compared to LSIL. Table 3 contains the clinical performance, stratified by age. p16/Ki-67 was positive in 36.0% of women under 30 years and 26.6% of women over 30 years ($p = 0.029$). HPV DNA was positive in 70.9% of women under 30 years and 58.2% of women over 30 years ($p = 0.006$). Both tests demonstrated a similar sensitivity across both age groups but specificity increased in women aged 30 years and older for each test.

From the overall population 62.2% were positive for HPV DNA, 41.6% of whom had CIN2+ and 15.6% had CIN3 over the study period. There were 30.4% with a positive p16/Ki-67 result, 72.0% had a CIN2+ and 27.3% had a CIN3 diagnosis. Clinical outcome following combined testing of p16/Ki-67 and HPV DNA, is shown in figure 2. From the general population of 471 women, 29.5% had a double positive test result; 74.1% of these women had CIN2+ and 27.3% had CIN3 diagnosed. Over two years, the absolute risk of CIN2+ and CIN3 was 15% and 6.3% in women with a positive HPV result and a negative p16/Ki-67 result. The absolute risk of CIN2+ was 5.4% and CIN3 was 1.2% in women with a negative HPV DNA and negative p16/Ki-67 result.

Discussion

Minor abnormalities, LSIL and ASC-US, represent a large burden at colposcopy. A

large proportion of these will not lead to a diagnosis of CIN2+ or CIN3 yet still remain under extensive follow up. Efforts have been made to manage this by introducing HPV DNA triage of minor abnormal cytology. However, due to the low specificity, HPV DNA testing can still lead to over-referral to colposcopy. In this study we investigated potential options for triaging women attending colposcopy following repeat minor abnormal cytology. We found from a population of women attending colposcopy with LSIL and ASCUS on cytology only 29.3% and 10.2% had underlying or subsequent CIN2+ and CIN3 lesions over two years. We have shown that a combined HPV DNA and p16/Ki-67 testing approach could be a potential tool for predicting diagnosis of CIN2+ and CIN3 in these women.

The two triage modalities, p16/Ki-67 and HPV DNA, were initially explored as standalone tests. HPV DNA was over three times higher than p16/Ki-67 in women with persistent low grade lesions and those discharged with no CIN. Unlike HPV DNA testing, there was no significant difference in the proportion of women with p16/Ki-67 co-expression between LSIL and ASC-US referred patients, an important finding considering the reported limited use of HPV DNA triage in LSIL (2,3). Furthermore p16/Ki-67 showed only a modest difference in performance with respect to age of women compared to HPV testing, which showed a substantially reduced specificity in women under the age of 30 years. On the other hand, compared to p16/Ki-67, HPV DNA was positive in a significantly higher proportion of CIN2+ and CIN3 ($p < 0.001$). This highlights the lower sensitivity of p16/Ki-67 compared to HPV DNA testing. Although, sensitivity for p16/Ki-67 in the current study is similar to previous studies (13-19). Generally PPV appeared low, this is due to the low prevalence of CIN3 in this population, 10%, and is in line with other studies showing a similar prevalence of CIN3 (14, 15, 18). Overall

specificity and PPV demonstrated by p16/Ki-67 remained higher than that demonstrated by HPV DNA across all categories. However as HPV DNA testing outperformed with respect to sensitivity and NPV we investigated a combined testing approach in order to maintain the high sensitivity of HPV testing and improve specificity with p16/Ki-67.

When combined, 29.5% were found to be positive for both tests, from these 74.1% had CIN2+ and 27.3% CIN3 diagnosed. Combined testing presented the most efficient option for identifying CIN2+ and CIN3 in women with repeat minor abnormal cytology as it identified almost one third of the population as requiring immediate colposcopy, signified by a double positive result. A negative p16/Ki-67 and negative HPV DNA maintained a high level of reassurance against CIN3 (1.2%) similar to that of a negative HPV test alone. A risk of CIN3 <2% has been previously deemed safe to allow return to routine recall (20). An important consideration in the use of a combined testing approach is how to manage women with discordant results, i.e. who are HPV positive p16/Ki-67 negative. Due to the risk of CIN2+ and CIN3 in these individuals it would probably be best advised that they do not return to routine recall but have some form of follow-up or colposcopy referral. An approach of repeat HPV testing of women who are HPV positive cytology negative after one year has been previously recommended (21).

The strengths of this study are that enrolment was systematic through the Irish national screening program, CervicalCheck. Women were managed under a standard protocol outlined by CervicalCheck guidelines. These attributes allowed test performance to be evaluated in a routine population-based setting. The consequence of this real-world setting was, however, that some women were not

managed according to the protocol, but this probably reflects the day-to-day reality of colposcopy clinics. A limitation of this study is that it focused on a population of women attending colposcopy on the basis of repeat minor cytology rather than a single ASC-US or LSIL, which a secondary test would be applied. However, sensitivity in the current study remains consistent with previous studies (13-19). While a large number of samples were excluded based on low cellularity this is likely due to the fact two or more tests were performed on the samples prior to the ThinPrep® slide been made. In reality a triage test would be performed following only one HPV test, as such we do not anticipate cellularity to be an issue.

While this work has established that HPV DNA and p16/Ki-67 may play a role in cervical screening programmes, large studies with long term follow up are warranted in order to determine an optimal management algorithm. Management of HPV DNA positive p16/Ki-67 negative women will need to be addressed in order to predict the intervals for retesting and return to routine screening. Moreover, further studies investigating triage following an initial minor cytological abnormality and in the context of primary screening with HPV will be important. Currently triage of primary screened HPV positive women by cytology is recommended however a question still remains over how to manage HPV positive cytology negative women. Dual staining for p16/Ki-67 has been previously shown to help further stratify Pap negative/HPV positive women that at a highest risk of underlying high-grade disease (19). In addition, cost-effective analysis will be an important aspect to help provide guidelines on the delivery and implementation in of such models in cervical screening. While other studies have found p16/Ki-67 superior to HPV E6/E7 mRNA testing by the APTIMA HPV assay (18), further

studies on the role of the ATPIMA assay in combination with p16/Ki-67, in addition to number of positive cells and morphology should be explored.

In conclusion, we have shown that combining HPV DNA positive women with p16/Ki-67 testing could lead to more accurate stratification of CIN in women presenting with minor cytology, potentially reducing referrals to colposcopy. This strategy is worthy of further evaluation in terms of both clinical-effectiveness and cost-effectiveness. While adding an additional test to screening may be more costly, introducing further stratification of minor abnormalities, prior to colposcopy rather than at colposcopy, should reduce unnecessary work up and treatment. This will benefit women by reducing the psychosocial effects endured from having repeat abnormal smears and attending colposcopy clinics, in addition to reducing cost associated with colposcopy visits (22).

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