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ORIGINAL ARTICLE

Correlation of p16^{INK4a} immunoexpression and human papillomavirus (HPV) detected by *in-situ* hybridization in cervical squamous neoplasia

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Abstract

Persistence and eventual integration of high-risk HPV (hrHPV) into the cervical cell is crucial to the progression of cervical neoplasia and it would be beneficial to morphologically identify this transformation in routine surgical pathology practice. Increased p16^{INK4a} (p16) expression is a downstream event following HPV E7 binding to pRB. A study was conducted to assess the correlation between hrHPV detection using a commercial *in-situ* hybridization assay (Ventana INFORM HPV ISH) and p16 immunoexpression (CINtec Histology Kit) in cervical squamous intraepithelial lesions and squamous carcinoma. 27 formalin-fixed, paraffin-embedded cervical low-grade squamous intraepithelial lesions (LSIL), 21 high-grade squamous intraepithelial lesions (HSIL) and 51 squamous carcinoma (SCC) were interrogated. hrHPV was significantly more frequent in HSIL (76.2%) and SCC (88.2%) compared to LSIL(37.0%). p16 expression was similarly more frequent in HSIL (95.2%) and SCC (90.2%) compared to LSIL(3.7%). That the rates of hrHPV when compared with p16 expression were almost equivalent in HSIL and SCC while p16 was expressed in only 1 of the 10 LSIL with hrHPV, are expected considering the likelihood that transformation has occurred in HSIL and SCC but does not occur in majority of LSIL.

Keywords: human papillomavirus, *in-situ* hybridization, p16^{INK4a} immunoexpression, viral integration

INTRODUCTION

Human papillomavirus (HPV) identification has become an important aspect in the management and prevention of cervical cancer.[1,2] More notably, it is now known that persistence and eventual integration of the HPV into the host genome, rather than mere presence of HPV infection, determines progression of disease.[3] Hence it would be beneficial and of practical importance if the transformation event can be more readily identified in routine surgical pathology practice. Immunohistochemical expression of p16^{INK4a} (p16) has been proposed as a surrogate marker of HPV-induced neoplastic transformation.[4-6] Normally, p16, a cyclin-dependent kinase inhibitor, downregulates cyclin-dependent kinase 4 and 6 (CDK4 and CDK6) and prevents phosphorylation of the retinoblastoma susceptible gene product (pRb) by CDK4 and CDK6. Hypophosphorylated pRb sequesters E2F transcription factors as ineffective pRb-E2F complexes and prevents E2F from driving the $G_{1-}S$ transition of the cell cycle.[7] In general this eventually leads to cell senescence.[5] When HPV E7 binds to pRB, E2F is released from sequestration. Accumulating E2F in cycling cells results in an autoregulatory pathway with reflex upregulation of p16[8] and implicit in this argument would be that p16 upregulation is associated with hrHPV driven transformation of the host cell. A study was conducted to assess p16 expression as a risk stratifier in cases of hrHPV detected by a commercial *in-situ* hybridization assay (Ventana INFORM HPV ISH).

MATERIALS AND METHODS

All cervical intraepithelial neoplasia grade 1 (CIN 1), grade 2 (CIN 2) and grade 3 (CIN 3) and squamous carcinoma (SCC) histologically-diagnosed for the first time between 1st January

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2006 to 31st December 2008, retrieved from the archives of the Department of Pathology, University of Malaya Medical Centre for an earlier study[4] were considered for the current study. The study was conducted with approval from the Institutional Review Board of the University of Malaya Medical Centre, Kuala Lumpur (MEC 751.1).

Based on arguments that a two-tiered system is more robust than a three-tiered one in histologically classifying cervical intraepithelial neoplasia,[9,10] cervical intraepithelial neoplasia were re-classified using the CAP-ASCCP Lower Anogenital Squamous Terminology (LAST) standardization guidelines into low-grade squamous intraepithelial lesions (LSIL) equivalent to CIN 1 and high-grade squamous intraepithelial lesions (HSIL) which encompasses CIN 2 and CIN 3.[11,12] For all cases, hrHPV *in-situ* hybridization was carried out on the same formalin-fixed, paraffin-embedded tissue block as that selected for p16 immunohistochemical staining earlier. Only cases where sufficient tissue was still available for further *in-situ* hybridization examination and would be left in the paraffin block for any subsequent review of the case were entered into this study. A 4-μm section was cut from the selected paraffin block for *in-situ* hybridization. To prevent cross-contamination, microtome blades were changed with each case sectioned.

In-situ hybridisation for high-risk HPV

In-situ hybridization (ISH) for HPV was performed on the Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, Arizona) using the Ventana INFORM HPV ISH assay (Ventana Medical Systems Inc., Tucson, Arizona) with INFORM HPV III Family 16 probe according to the manufacturer's instructions. The assay targeted common high-risk HPV (hrHPV) types i.e. 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 66. In brief, the tissue sections were subjected to Ventana ISH Protease 2 digestion for 24 min before hybridization with the above-mentioned hrHPV probe cocktail labelled with Dinitro-Phenol (DNP). Visual detection of the hybridization reaction was via the Ventana ISH *i*VIEW Blue Plus Detection Kit. Briefly, the DNP labelled probe was detected by a rabbit anti-DNP antibody, amplified by adding mouse anti-rabbit antibody with subsequent binding of a biotin labelled goat anti-mouse antibody. The biotin then complexes with alkaline phosphatase conjugated streptavidin and finally reacts with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) to produce blue intra-nuclear diffuse or punctuate signals of hrHPV.[13-15] For the case to be entered, the lesion in question (LSIL, HSIL or SCC) should be preserved in the ISH- stained slide when compared with that noted in the earlier study. Signals, seen as intranuclear blueing (Fig. 1) were read using an Olympus BX51 microscope at 400x magnification. A case of cervical squamous carcinoma, known to be HPV 16 positive served as positive control and was included in each run, whilst the surrounding normal squamous epithelium served as an internal negative control.

p16 immunohistochemical staining

Immunohistochemical staining for p16 was carried out using the CINtec Histology Kit (REF 9511, mtm laboratories AG, Heidelberg, Germany).[4] Staining was according to the manufacturer's instructions whereby antigen retrieval was carried out by immersing the tissue sections in the Epitope Retrieval Solution at 95 - 99°C for 10 min. Endogenous peroxidase blocking was followed by incubation with monoclonal p16 antibody (clone E6H4) for 30 min. The Visualization Reagent and 3,3'-diaminobenzidine chromogen with haematoxylin counterstaining provided visualization of the reaction. A previously proven p16 immunoreactive invasive cervical squamous carcinoma served as positive control. The negative control was constituted by substituting Negative Reagent Control (monoclonal anti-Rat oxytocin-related neurophysin antibody) for p16 antibody in the staining of the positive control tissue. Both positive and negative controls were run with each batch stained. Staining of the cytoplasm or nucleus was considered and positive staining was defined as diffuse continuous staining i.e. involving >75% of the squamous epithelial cells in LSIL, HSIL or invasive carcinoma (Fig. 2). In addition, for the squamous intraepithelial lesions, the staining must involve the basal and parabasal layers of the squamous epithelium. Statistical analysis was carried out on the SPSS (IBM version 22) using Fisher exact test and chi-square with statistical significance at $p<0.05$.

RESULTS

Finally 27 LSIL, 21 HSIL and 51 SCC could be entered into the study based on the inclusion criteria of this study. The prevalence of hrHPV versus p16 expression of the squamous

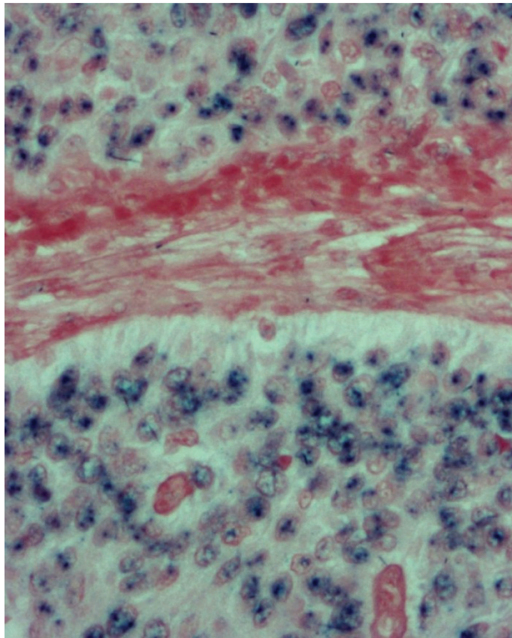

FIG. 1: High-risk HPV signals detected as blue intra-nuclear signals by in-situ hybridization in a case of cervical squamous carcinoma x 400

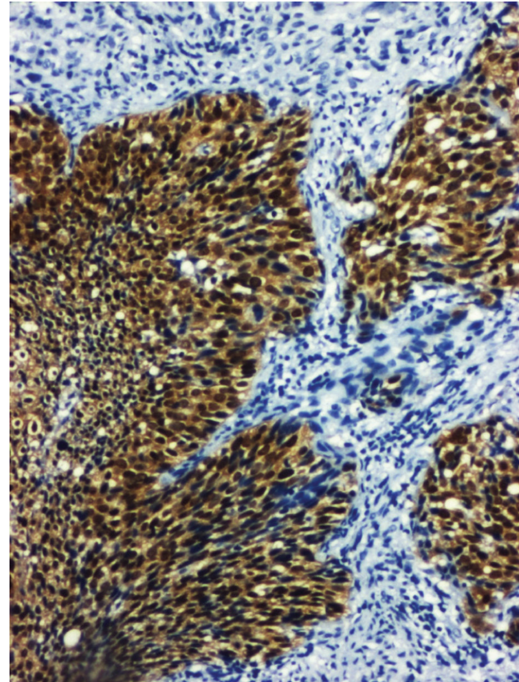

FIG. 2: p16^{INK4a} immunohistochemical expression in cervical squamous carcinoma, seen as nuclear and cytoplasmic staining x100

intraepithelial lesions and invasive squamous carcinoma is shown in Table 1. High-risk HPV was detected in 10 (37.0%) LSIL, 16 (76.2%) HSIL and 45 (88.2%) SCC with hrHPV being detected significantly more frequently in both HSIL and SCC compared with LSIL ($p<0.05$). Prevalence of hrHPV did not differ significantly between HSIL and SCC ($p=0.482$). p16 which was detected in 1 (3.7%) LSIL, 20 (95.2%) HSIL and 46 (90.2%) SCC was also significantly more frequent in both HSIL and SCC compared with LSIL ($p<0.05$). As with hrHPV, there was also no significant difference in p16 immunopositivity between HSIL and SCC ($p=0.197$). Of the 10 LSIL with hrHPV, only 1 showed p16 immunoexpression. All 16 HSIL with hrHPV expressed p16. However, 4 cases of HSIL which were negative for hrHPV also expressed p16. In the SCC group, 42 (93.3%) of the 45 cases with hrHPV expressed p16, while 3 did not. In contrast, 4 SCC without hrHPV expressed p16.

TABLE 1: High-risk HPV (hrHPV) detected by in-situ hybridization versus p16^{INK4a} (p16) expression in LSIL (n=27), HSIL (n=21), and SCC (n=51)

			hrHPV		
			positive	negative	Total
LSIL	p16	positive	1	0	1
		negative	9	17	26
	Total		10	17	27
HSIL	p16	positive	16	4	20
		negative	0	1	1
	Total		16	5	21
SCC	p16	positive	42	4	46
		negative	3	2	5
	Total		45	6	51

DISCUSSION

The prevalence rates of hrHPV in LSIL (37.0%), HSIL(76.2%) and SCC (88.2%) in this study as detected by the commercial Ventana INFORM HPV ISH system, parallels most recent studies from other centres which ranged between 33% to 80% in LSIL, 57% to 100% in HSIL and 78% to 96% in SCC.[16-21] The rate of HPV detection in SCC observed in this study appears comparable, if not marginally better, compared to the 70% detection rate in an earlier study carried out by the authors on formalin-fixed, paraffin-embedded cervical carcinoma using the polymerase chain reaction (PCR) as the method for detection.[22] As histology still remains the reference for confirmation and subsequent management of cervical neoplasia,[23] the current observation supports the possible use of a commercial *in-situ* hybridization system for detection of hrHPV in formalin-fixed, paraffin-embedded tissues. This is further attested to by the reasonably acceptable results observed by other workers using the same system.[24-26] At this juncture it is important to mention that results of HPV prevalence studies should always be interpreted in the light that varying HPV types may be embraced in the "high-risk" detection panels used by different workers and this is particularly so with borderline carcinogenic types which may or may not be included in different cocktail panels.[27]

p16 immunopositivity was significantly more common in HSIL (95.2%) and SCC (90.2%) compared with LSIL (3.7%) in this study, mirroring a trend which is generally observed by most workers.[21,28-32] Notwithstanding the above, the current lack of standardisation for interpreting p16 immunopositivity continues to make comparison of rates across studies difficult.[4,33] Illustrating this point further, it is noteworthy that p16 immunopositivity had been defined differently in all the various studies referenced above. While there appears to be general agreement in the prevalence rates of p16 immunopositivity in HSIL and SCC with most rates observed ranging from 80% to 100%, reported rates for LSIL appear to vary quite widely. Nishio *et al*[21] reported p16 in 21% of LSIL while Lesnikova *et al*[29] reported a rate of 72%. In this study, the authors had used a modified van Niekerk classification[34] with the cut-off for immunopositivity set at a stringent >75% of the squamous epithelium of the intraepithelial lesions or >75% of the tumour cells in SCC expressing p16, with an added caveat that positive staining must be present in the basal and parabasal layers of the intraepithelial lesions. The stringency of the cut-off for p16 immunopositivity in this study may explain for the lower prevalence observed in LSIL in this study, as even Nishio *et al*'s observed 21% immunopositivity was based on a cut-off set at 5% of cells with moderately intense p16 nuclear and/or cytoplamic staining. In a somewhat unrelated scenario, a p16 cut-off of >70% was proposed by Larsen *et al* to provide better correlation with HPV presence in oropharyngeal SCC.[35]

That the rates of hrHPV when compared with p16 expression were almost equivalent in HSIL and SCC is to be expected as it can be assumed that transformation would have occurred in these two categories of lesions. In contrast, p16 was expressed in only 1 of the 10 LSIL with hrHPV, implying that majority of hrHPV positive LSIL may not have transformed and the hrHPV infection will probably clear in due course. The observation that expression of p16 predicts for transformation of LSIL to HSIL was also noted by Solares *et al*.[36] It is nevertheless interesting that 4 HSIL and 4 SCC in this study demonstrated p16 immunoexpression in the absence of hrHPV. Apart from the possibility that the HPV type may not have been included in the hrHPV panel used for detection here and inherent problems of formalin fixation preventing detection of the HPV, the presence of alternative non-HPV associated pathways leading to p16 overexpression[37] may also have to be considered in these cases. The finding of p16 immunonegativity in 3 hrHPV-positive SCC is equally interesting. This phenomenon has also been observed by Perez *et al* in their study where a case of SCC tested HPV-positive by the SPF_{10}-$LIPA_{25}$ assay was p16 immunonegative.[38] In our study, it is possible that the stringent cut-off for interpretation of p16 immunopositivity adopted resulted in these observations.

These observations underscore the possible use of p16 expression to further subcategorise equivocal and early premalignant cervical squamous lesions in which hrHPV is detected on screening. This study also brings out the possibility of use of an *in-situ* system for hrHPV detection in a routine surgical pathology diagnostic laboratory.

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