

## RESEARCH COMMUNICATION

# **p16<sup>INK4a</sup> is a Useful Marker of Human Papillomavirus Integration Allowing Risk Stratification for Cervical Malignancies**

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### **Abstract**

The present study was conducted to assess utility of p16<sup>INK4a</sup> immunopositivity as a surrogate marker for genomic integration of high-risk human papillomavirus infection (hrHPV). A total of 29 formalin-fixed, paraffin-embedded cervical low-grade squamous intraepithelial lesions (LSILs), 27 high-grade squamous intraepithelial lesions (HSILs) and 53 invasive squamous cell carcinomas (SCCs), histologically-diagnosed between 1st January 2006 to 31st December 2008 at the University of Malaya Medical Centre were stained for p16<sup>INK4a</sup> (CINtec Histology Kit (REF 9511, mtm laboratories AG, Heidelberg, Germany)). Immunopositivity was defined as diffuse staining of the squamous cell cytoplasm and or nucleus (involving > 75% of the intraepithelial lesions or SCCs). Staining of basal and parabasal layers of intraepithelial lesions was pre-requisite. One (3.4%) LSIL, 24 (88.9%) HSIL and 46 (86.8%) SCC were p16<sup>INK4a</sup> immunopositive. All normal squamous epithelium did not express p16<sup>INK4a</sup>. p16<sup>INK4a</sup> expression was significantly lower (p<0.05) in LSIL compared with HSIL and SCC with no difference in expression between HSIL and SCC. The increased p16<sup>INK4a</sup> immunopositivity in HSIL and SCC appears in line with the integrated existence of the hrHPV and may provide more insightful information on risk of malignant transformation of cervical squamous intraepithelial lesions than mere hrHPV detection.

**Keywords:** p16<sup>INK4a</sup> immunohistochemistry - high risk human papillomavirus - cervix - invasive squamous carcinoma

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### **Introduction**

Uterine cervical carcinoma remains an important cancer worldwide and is the second most common cancer in Malaysian females (Lim et al., 2008) with human papillomavirus (HPV) shown to be prevalent in about 80% of biopsied or excised cervical carcinoma from this centre (Cheah et al., 2011). Although high-risk HPV (hrHPV) testing has been advocated to improve risk stratification of pre-invasive squamous intraepithelial lesions (Nishino et al., 2011), testing for hrHPV is not easily affordable in emerging economies. Besides, detection of hrHPV may also not provide accurate prediction in the light of current understanding that new infections seem to carry little risk whereupon only persistent infections appear to determine whether invasive cancer eventually develops (Schiffman et al., 2011). The cut-off to definition of persistence is still being debated with 6 to 12 months as seemingly viable suggestions (Rodríguez et al., 2008; Syrjänen K, 2011). Although close follow-up with repeated testing for persistence of hrHPV in patients with pre-invasive lesions would seem ideal to guide further management, we were interested in assessing the use

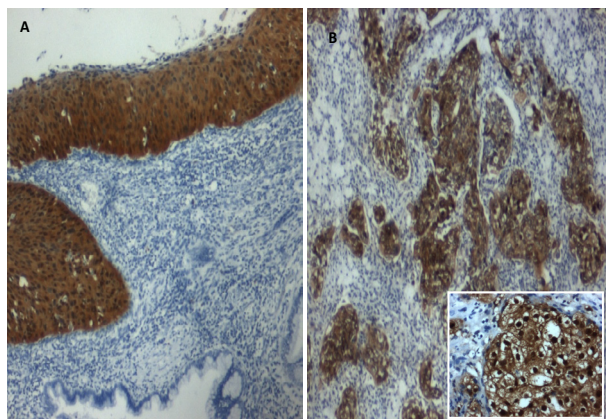
of p16<sup>INK4a</sup> immunohistochemical detection as a more readily available surrogate marker of hrHPV infection with genomic integration into the cervical squamous cell.

High-risk HPV integrates into the host genome and deregulates host tumour suppressor proteins, in particular p53 and pRB via the HPV's E6 and E7 proteins respectively (Doorbar, 2005). p16<sup>INK4a</sup> is a tumor suppressor protein that downregulates activity of cyclin-dependent kinase 4 and 6 (CDK4 and CDK6). By doing so, it prevents phosphorylation of the retinoblastoma susceptible gene product, pRb, by CDK4 and CDK6. Hypophosphorylated pRb in turn sequesters E2F transcription factors as incompetent pRb-E2F complexes and prevents E2F from driving the G1-S transition of the cell cycle (Sherr and Roberts, 2004). When E7 binds to pRB, E2F is released from sequestration with reflex upregulation of p16<sup>INK4a</sup>. Thus, it would appear that detection of p16<sup>INK4a</sup> over-expression may in fact be more revealing as a surrogate marker of integration of hrHPV and transition to malignant transformation. In contrast, E6 inactivates and destroys p53, a phenomenon not readily tracked by simple and readily available immunohistochemical testing (Doorbar, 2005).

## Materials and Methods

All cervical low-grade squamous intraepithelial lesions (LSIL), synonymous with cervical intraepithelial neoplasia (CIN) grade 1, high-grade squamous epithelial lesions (encompassing CIN 2 and CIN 3) and invasive cervical squamous carcinoma (SCC) histologically-diagnosed for the first time between 1<sup>st</sup> January 2006 to 31<sup>st</sup> December 2008 at the Department of Pathology, University of Malaya Medical Center were retrieved from the archives of the department. For any case, either hysterectomy or large loop excision of the transformation zone (LLETZ) or diagnostic biopsy was used for study provided the lesion was present in the material. All slides of the cases were histologically-reviewed and only re-confirmed cases were considered. If more than one grade of lesion was present in any one case, the most severe was entered into the study. One 10% neutral buffered formalin-fixed, paraffin-embedded tissue block of the lesion, preferably with adjacent normal squamous epithelium, was selected during the review for immunohistochemical staining. Preference was to use hysterectomy or LLETZ specimens to spare diagnostic biopsy material from being exhausted. 4 µm sections were cut from the selected paraffin block for immunohistochemical staining with p16<sup>INK4a</sup>. Only cases whereby sufficient tissue (1) was available for immunohistochemical staining and (2) would also be left in the paraffin block for subsequent review of the case, if necessary, were finally enrolled into the study. Except for squamous carcinoma, all other histological types of invasive cervical carcinoma, were excluded from the study. This study was conducted with approval from the Institutional Review Board and is an extension of an earlier study by this group (Cheah et al., 2011).

Immunohistochemical staining for p16<sup>INK4a</sup> was carried out using the CINtec Histology Kit (REF 9511, mtm laboratories AG, Heidelberg, Germany) in accordance with the manufacturer's instructions. Briefly, antigen retrieval was carried out in a water bath with the tissue sections immersed in Epitope Retrieval Solution at 95 - 99°C for 10 min. The tissue sections were cooled for 20 min at room temperature. Endogenous peroxidase



**Figure 1. Diffuse Continuous p16<sup>INK4a</sup> Expression in (A) a High-grade Squamous Intraepithelial Lesion and (B) an Invasive Squamous Cell Carcinoma. Both Nuclear and Cytoplasmic p16<sup>INK4a</sup> Immunopositivity are Noted Here (inset)**

**Table 1. p16<sup>INK4a</sup> Immunohistochemical Expression in Low-grade Squamous Intraepithelial Lesions (LSIL), High-grade Squamous Intraepithelial Lesions (HSIL) and Invasive Cervical Squamous Carcinoma (SCC)**

	p16 <sup>INK4a</sup> positive (%)	p16 <sup>INK4a</sup> negative (%)
LSIL (n=29)	1 (3.4)	28 (96.6)
HSIL (n=27)	24 (88.9)	3 (11.1)
SCC (n=53)	46 (86.8)	7 (13.2)

blocking was followed by incubation with monoclonal p16<sup>INK4a</sup> antibody (clone E6H4) provided in the CINtec Histology Kit for 30 min. Visualisation of the reaction was via the Visualization Reagent and 3,3'-diaminobenzidine chromogen with haematoxylin counterstaining. A positive control, consisting of a case of invasive cervical squamous carcinoma, previously proven to be p16<sup>INK4a</sup> positive, was included in each batch of cases stained. The negative control included in each batch run, was constituted by substituting Negative Reagent Control (monoclonal anti-Rat oxytocin-related neurophysin antibody) for p16<sup>INK4a</sup> antibody in the staining of the positive control invasive cervical squamous carcinoma case. Unequivocal staining of the cytoplasm and or nucleus was considered significant but staining intensity was not further subcategorised. Positive expression of p16<sup>INK4a</sup> was defined as diffuse (continuous) unequivocal staining of the cytoplasm and or nucleus of the squamous cells (involving > 75% of the squamous epithelium of the intraepithelial lesions or > 75% of the tumour cells in SCC). In addition, the staining must involve the basal and parabasal layers of the intraepithelial lesions to be considered positive (Bergeron et al., 2010). Chi-square and Student's t-test were used for statistical analysis of results with significance at p<0.05.

## Results

Following histological re-confirmation and taking into account the criteria set for inclusion, 109 cases (29 LSIL, 27 HSIL and 53 SCC) could be entered into the study. Table 1 shows the p16<sup>INK4a</sup> expression in LSIL, HSIL and SCC. Diffuse continuous staining with p16<sup>INK4a</sup> involving >75% of LSIL or HSIL and SCC was noted in 1 (3.4%) LSIL, 24 (88.9%) HSIL, 46 (86.8%) SCC (Figure 1). All normal squamous epithelium, observed in the vicinity of 18 LSIL, 18 HSIL and 9 SCC did not express p16<sup>INK4a</sup>. An abrupt transition from immunopositivity, in cases with p16<sup>INK4a</sup> expression, to immunonegativity in the adjacent normal squamous epithelium was noted in all cases. p16<sup>INK4a</sup> expression was significantly lower (p<0.05) in LSIL compared with HSIL and SCC while there was no difference in expression between HSIL and SCC.

## Discussion

The finding of significantly increased p16<sup>INK4a</sup> expression in HSIL (88.9%) and SCC (86.8%) when compared with LSIL (3.4%) mirrors observations of other studies (Kurshumliu et al., 2009; Lesnikova et al., 2009; Missaoui et al., 2010). The lack of p16<sup>INK4a</sup> expression

in normal squamous epithelium in the vicinity of the squamous intraepithelial lesions and invasive squamous carcinoma is to be expected and has also been noted by other workers (Murphy et al., 2005; Benevolo et al., 2006). As is currently acknowledged, all invasive squamous carcinoma of cervix and squamous intraepithelial lesions, irrespective of grade are associated with HPV with predominance of hrHPV in all (Walboomers et al., 1999; Stanley, 2010). The difference in potential for malignant transformation between LSIL and HSIL hinges on the tendency for existence of hrHPV in episomal form with complete expression of viral genes and formation of complete virions which are eventually shed in LSIL. In contrast, integration of the viral DNA into the host cell occurs in HSIL resulting in disruption of viral gene expressions, whereby deregulation of E6 and E7 lead to deleterious downstream effects (Stanley, 2010).

The findings in this study fully support this known natural turn of events with lack of p16<sup>INK4a</sup> expression in LSIL. The significantly increased p16<sup>INK4a</sup> expression in HSIL probably indicates the integrated state of hrHPV in the lesion directing an E7 over-production and the subsequent reflex p16<sup>INK4a</sup> over-expression. The similar p16<sup>INK4a</sup> over-expression in SCC implies that hrHPV E7-pRB binding maintains its importance throughout the lifespan of the neoplastic cell. Thus p16<sup>INK4a</sup> immunohistochemical expression, a technologically less demanding and less expensive test, which can be readily available in most surgical pathology laboratories, may actually provide more insightful information on risk for eventual progression to SCC of squamous intraepithelial lesions than mere detection and demonstration of hrHPV and this has also been advocated in recent studies (Reuschenbach et al., 2011; Ukpo et al., 2011).

Notwithstanding the above, it has to be cautioned that interpretation of p16<sup>INK4a</sup> expression still remains a challenge for surgical pathologists as cut-off thresholds for immunopositivity range between studies, although majority agree that staining should be diffuse and there should be staining of the basal/parabasal layers of the squamous epithelium to be considered positive (Klaes et al., 2001; Eleutério et al., 2007; Kong et al., 2007; Van Niekerk et al., 2007; Bergeron et al., 2010). A modified Van Niekerk et al's classification (Van Niekerk et al., 2007) was utilized here and the cut-off for immunopositivity was set at a stringent >75% of the squamous epithelium of the intraepithelial lesions or > 75% of the tumour cells in SCC with inclusion of a further caveat that positive staining must be present in the basal and parabasal layers of the intraepithelial lesions. The stringent cut-off for immunopositivity was to reflect the authors' interpretation of "diffuse". As in all immunohistochemical techniques, other factors that may influence results should be considered, including the fixative used for the tissues, duration of fixation, size of tissue being fixed, antigen retrieval methods etc. In addition, the relevance of nuclear and cytoplasmic expression of p16<sup>INK4a</sup> was not differentiated in this study which accepted both or either as positive staining. On the molecular aspects, lack of p16<sup>INK4a</sup> expression in about 10% of HSIL and SCC need not necessarily indicate lack of hrHPV or suboptimal

immunohistochemical techniques. E7 transcription failure or failure of p16<sup>INK4a</sup> expression due to mutation or failure of upstream controls need also to be considered.

It is unfortunate that the case of LSIL exhibiting p16<sup>INK4a</sup> was lost to follow-up following the biopsy. It would have been interesting to follow the development of the lesion through long-term follow-up.

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