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ORIGINAL ARTICLE

Implications of continued upregulation of p16^{INK4a} through the evolution from high-grade squamous intraepithelial lesion to invasive squamous carcinoma of the cervix

Phaik-Leng CHEAH *FRCPath, MD*, Lai-Meng LOOI *FRCPath, MD*, Kein-Seong MUN *MBBS MPath*, NAZARINA Abdul Rahman *MBBS, MPath* and Kean-Hooi TEOH *MBChB, MPath*

Department of Pathology, Faculty of Medicine, University of Malaya

Abstract

On integration into the host cervical keratinocyte genome, human papillomavirus (HPV) E7 protein binds pRB, releasing E2F from normally incompetent pRB-E2F complexes and allowing propagation of G1-S transition by the E2F. p16 ^{INK4a}, a tumour suppressor protein, increases in reflex response to counter this. 29 histologically re-confirmed low-grade squamous intraepithelial lesions (LSIL), 27 high-grade squamous intraepithelial lesions (HSIL) and 30 invasive cervical squamous carcinoma (SCC) were immunohistochemically stained for p16^{INK4a} expression using the CINtec Histology Kit (REF 9511, mtm laboratories AG, Heidelberg, Germany) to re-affirm the notion that integration of HPV occurs predominantly in SCC and possibly HSIL and less in LSIL and normal squamous epithelium (NSqE). Implicit was also the attempt to understand the role of E2F, as indicated by p16^{INK4a}, in evolution of SCC from HSIL. No ethnic predilection was noted for LSIL, HSIL or SCC. Patients with SCC were significantly older by about 14-years compared with HSIL ($p<0.05$) while there was no significant age difference between HSIL and LSIL. p16^{INK4a} expression was significantly increased ($p<0.05$) in both HSIL (88.9%) and SCC (83.3%) compared with LSIL (3.4%) and NSqE (0%); the NSqE being normal squamous epithelium noted in 17 of the LSIL, 19 HSIL and 5 SCC. From these findings there is suggestion that fundamental upstream events viz HPV integration, E7 upregulation followed by E2F activation occurs at point of transformation to HSIL and continues unrelentingly for another one to two decades before hitherto unclear factors convert a non-invasive lesion into an overtly invasive malignant counterpart. Interestingly, the occurrence of HSIL and LSIL in almost the same age group could mean that alteration from episomal to integrated form of HPV may not incur a prolonged incubation period, unlike from HSIL to SCC.

Keywords: p16^{INK4a}, pathogenesis, low-grade intraepithelial lesions (LSIL), high-grade intraepithelial lesions (HSIL), invasive cervical squamous carcinoma (SCC)

INTRODUCTION

Although vaccination against human papillomavirus (HPV) offers promise of eventual eradication of cervical carcinoma in the future, the current reality is that cervical carcinoma is still the second most common cancer in Malaysian females.[1] Although HPV has been identified as the aetiological agent and shown to be prevalent in about 80% of cases of cervical carcinoma from this institution,[2] there continues to be many unanswered questions regarding the pathogenesis of cervical carcinoma. Normally p16^{INK4a}, a tumour suppressor protein, prevents phosphorylation of the retinoblastoma susceptible gene product pRb by cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) via downregulation of the activity of the mentioned kinases. The E2F family of transcription factors, which drive G1-S transition of the cell cycle, are sequestered as incompetent pRb-E2F complexes with the hypophosphorylated pRb. Viral integration, in particular of high-risk HPV types, into the host cervical keratinocyte DNA results in overexpression of HPV E7 protein. HPV E7 selectively binds the pRB of the pRb-E2F complex thus releasing E2F and allowing it to initiate G_{1}-S transition. In turn, p16^{INK4a} is increased as

Address for correspondence and reprint requests: Dr P-L Cheah, Department of Pathology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. E-mail address: cheahpl@ummc.edu.my

a reflex attempt to damage containment.[3,4] In short, increased $p16^{INK4a}$ expression would be an indirect indicator of increased E2F in HPV associated cervical carcinogenesis.

We were interested to (1) confirm the impression that integration of HPV into the cervical keratinocyte and the subsequent activation of E2F with resultant increased $p16^{INK4a}$ occurs predominantly in invasive cervical squamous cell carcinoma (SCC) and possibly high-grade squamous intraepithelial lesions (HSIL) but less in low-grade intraepithelial lesions (LSIL) and normal squamous epithelium (NSqE). Inherent to the study was also (2) an attempt to further understand the role of E2F, as indicated by $p16^{INK4a}$, in the evolution of HSIL to SCC considering that only 30-40% of HSIL eventually transform to invasive cervical squamous carcinoma.[5] For purposes of this study, we adopted the two-tier division of pre-invasive squamous intraepithelial neoplasia incorporating cervical intraepithelial neoplasia (CIN) grades 2 and 3 as HSIL with CIN grade 1 being synonymous with LSIL.

MATERIALS AND METHODS

The archives of the Department of Pathology, University of Malaya Medical Centre were searched for the first thirty cases of LSIL, HSIL and SCC, histologically-diagnosed for the first time at the Department of Pathology, University of Malaya Medical Center between 1st January 2006 to 31st December 2008. The hysterectomy or large loop excision of the transformation zone specimen was preferably used for immunohistochemical staining, if either of these and a diagnostic biopsy specimen were available. All slides of the cases, including the diagnostic biopsy if available, were histologically-reviewed and only re-confirmed cases were considered. One 10% neutral buffered formalin-fixed, paraffin-embedded tissue block of each case was selected during the histological review for immunohistochemical study. 4 µm sections were cut from the selected paraffin block for immunohistochemical staining with $p16^{INK4a}$. Only cases where sufficient tissue was (1) available for immunohistochemical staining and (2) would also be left in the paraffin block for subsequent review of the case if necessary were finally entered into the study. Except for squamous carcinoma, all other histological types of invasive cervical carcinoma, were excluded. This study was conducted with approval from the Institutional Review Board.

Immunohistochemical staining for $p16^{INK4a}$ was carried out using the CINtec Histology Kit (REF 9511, mtm laboratories AG, Heidelberg, Germany) in accordance with the manufacturer's instructions. 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. After cooling the tissue sections for 20 min at room temperature, endogenous peroxidase blocking was followed by incubation with monoclonal $p16^{INK4a}$ antibody (clone E6H4) 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 SCC, previously proven to be $p16^{INK4a}$ 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 $p16^{INK4a}$ antibody in the staining of the positive control. Unequivocal brown staining of the cytoplasm and or nucleus was considered significant. Immunopositivity of $p16^{INK4a}$ 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). Furthermore, the staining must involve the basal and parabasal layers of the intraepithelial lesions.[6] Statistical analysis was by Student's t-test and chi-square with statistical significance at $p<0.05$.

RESULTS

Following histological re-confirmation and satisfaction of the inclusion criteria, 29 LSIL, 27 HSIL and 30 SCC were finally enrolled into the study. Normal squamous epithelium was observed in the vicinity of 17 of the LSIL, 19 HSIL and 5 SCC. Demographic profile of the cases in the three categories studied is shown in Table 1. Age of patients ranged between 18-68 years (mean=46.3 years) in LSIL, 24-66 years (mean=44.4 years) in HSIL and 33-80 years (mean=58.5 years) in SCC. Ages of patients with HSIL and LSIL did not differ significantly ($p>0.05$) but SCC was seen in significantly older patients ($p<0.05$) compared with both HSIL and LSIL. Ethnically, there was no statistically significant difference noted in the distribution of HSIL, SCC and LSIL between the Chinese, Malays and Indians.

TABLE 1: Demographic profile of cases of low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and invasive cervical squamous carcinoma (SCC)

		LSIL (n=29)	HSIL (n=27)	SCC (n=30)
Age range in years (mean)		18-68 (46.3)	24-66 (44.4)	33-80 (58.5)
Ethnicity	Malay	12 (41.4%)	4 (14.8%)	9 (30.0%)
	Chinese	13 (44.8%)	15 (55.6%)	13 (43.3%)
	Indian	4 (13.8%)	7 (25.9%)	7 (23.3%)
	Others	0	1 (3.7%)	1 (3.3%)

Table 2 shows the $p16^{INK4a}$ expression in NSqE, LSIL, HSIL and SCC. Diffuse continuous staining with $p16^{INK4a}$ was noted in 1 (3.4%) LSIL, 24 (88.9%) HSIL (Figure 1A) and 25 (83.3%) SCC (Figure 1B). All NSqE (41 in total) did not express $p16^{INK4}$. Both HSIL and SCC demonstrated a significantly increased $p16^{INK4a}$ expression when compared with LSIL and NSqE ($p<0.05$). Nevertheless HSIL and SCC demonstrated no difference in $p16^{INK4a}$ expression.

DISCUSSION

This study shows that $p16^{INK4a}$ expression was significantly increased in both HSIL (88.9%) and SCC (83.3%) compared with LSIL (3.4%) and NSqE (0%), findings which are comparable to those reported in other studies.[7-12] Although the prevalence of $p16^{INK4a}$ detection in HSIL and SCC are similar to that observed by other workers,[7-9] the finding of $p16^{INK4a}$ in 3.4% of LSIL is at variance with several studies e.g. Missaoui et al's 77.8% [8] and Lesnikova et al's 72.3%.[9] This variation is very likely due to the still undetermined and differing definitions of "immunopositivity" of $p16^{INK4a}$ adopted by various authors. In this study, we used a modified Van Niekerk et al's classification[13] and set the cut-off for immunopositivity for $p16^{INK4a}$ at a stringent expression in >75% of the squamous epithelium of intraepithelial lesions or > 75% of the tumour cells in SCC. In addition, a further caveat was that staining must be present in the basal and parabasal layers of the intraepithelial lesions to be considered positive.[6] Based on the current study's criteria of immunopositivity, both Missaoui *et al* and Lesnikova *et al* may have shown similar low $p16^{INK4a}$ expression in LSIL as the former group reported on weak focal $p16^{INK4a}$ staining, while the latter defined immunopositivity as moderate or strong staining in more than 10% of epithelial cells. The findings of this study seem to re-affirm the notion that HPV DNA integration into the cervical keratinocyte occurs at the HSIL stage rather than LSIL as indirectly evidenced by the increase in $p16^{INK4a}$ expression in HSIL and not LSIL.

Noteworthy in this study is the observation that SCC in our patients occurs about 14-years later than HSIL and LSIL. This seemingly long interval has also been observed in longitudinal studies which have shown an approximately 10-20 years interval for HSIL to progress to invasive carcinoma of the cervix. [14] Taking into consideration that $p16^{INK4a}$ is expressed as frequently in HSIL (88.9%) and SCC (83.3%) it would appear that fundamental upstream events viz HPV integration, E7 activation followed by

TABLE 2: $p16^{INK4a}$ immunohistochemical expression in normal squamous epithelium (NSqE), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL), invasive cervical squamous carcinoma (SCC)

	$p16^{INK4a}$ positive/ number tested (% positive)
NSqE	0/41 (0)
LSIL	1/29 (3.4)
HSIL	24/27 (88.9)
SCC	25/30 (83.3)

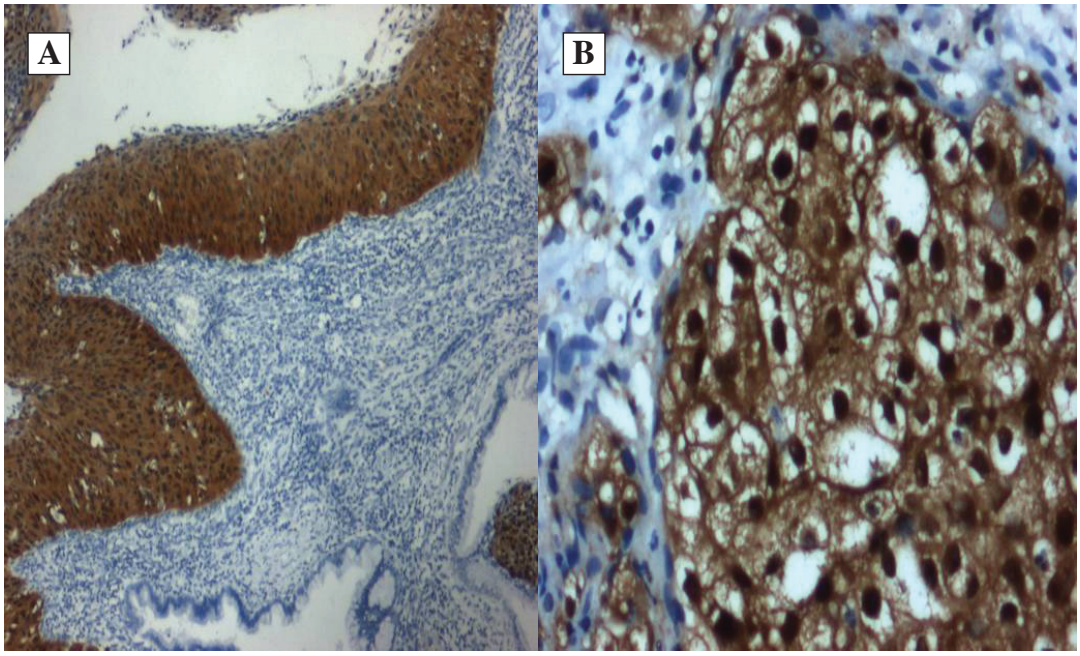

FIG. 1: (A) Unequivocal diffuse $p16^{INK4a}$ immunopositivity in high-grade squamous intraepithelial lesion (note that staining involves the basal and parabasal layers of the epithelium) and (B) nuclear and cytoplasmic $p16^{INK4a}$ immunopositivity in invasive cervical squamous carcinoma

E2F activation occurs at point of transformation to HSIL and continues unrelentingly for another one to two decades. It is still unclear at this stage, what are the critical steps which convert a non-invasive lesion into an overtly invasive malignant phenotype. These factors would however appear not to be universal in all HPV transformed squamous epithelium but occurring in only 30-40% of HSIL. Nonetheless, it is attractive to assume that E2F remaining active throughout this prolonged transformation process will create a propitious milieu for many genetic alterations which can lead to acquisition of invasive properties.

In contrast to SCC, LSIL appears to affect the same age-group as HSIL with mean age of patients in the former group at 46-years and the latter at 44-years. With the current understanding that most HPV remain in the episomal form in LSIL,[15] until and unless integration occurs, most LSIL should not progress into cervical carcinoma. An extrapolation to the observation of HSIL and LSIL occurring around the same age may mean that the process of alteration from episomal to integrated form of HPV, should it occur, does not incur a prolonged incubation period, unlike from HSIL to SCC.

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