P634A4 and TAp73 immunocytochemistry in liquid-based cervical cytology—potential biomarkers for diagnosis and progress prediction of cervical neoplasia

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P63 and p73 are two homologues of the important tumor suppressor gene p53. In this study, we investigated p63 and p73 expression by immunocytochemistry using antibodies for TAp73 and p634A4 isoforms in 91 high-grade and 107 low-grade squamous intraepithelial lesions, 212 atypical squamous cells of undetermined significance, 9 squamous cell carcinomas and 63 normal samples from an Asian screening population together with 47 hospital samples of carcinomas. There was significant correlation between the TAp73 and p634A4 indices ($P < 0.0001$). Significantly, higher TAp73 and p634A4 indices were found in high-grade lesions or carcinoma when compared with atypical squamous cells and low-grade lesions ($P < 0.0001$). Among atypical squamous cells, p634A4 indices of cases that subsequently progressed to low-grade ($P = 0.031$) or high-grade lesions ($P = 0.006$) were significantly higher than those that did not. For atypical squamous cells positive for high-risk human papillomavirus (HPV) as detected by Digene (61%), cases with high p634A4 index were still more likely to have subsequent high-grade lesions detected ($P = 0.016$). Among low-grade lesions, significantly higher TAp73 ($P = 0.038$) was found in cases that subsequently progressed to high-grade lesions. There was significant correlation between presence of high-risk HPV and p634A4 index ($P = 0.01$). In summary, p63 and p73 immunocytochemistry are potential good markers for detection of carcinoma and high-grade lesions in cervical cytology samples and for triage management of women with atypical squamous cells and low-grade lesions.

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Cervical cytology screening is effective in the prevention of cervical cancer by detecting asymptomatic cervical cancer and its precursors although there is limitation in its sensitivity and specificity.¹ Among cytological abnormalities detectable in a screening population, atypical squamous cells of undetermined significance (ASC-US) and low-grade squamous intraepithelial lesion (LSIL), especially

the former, are the most common diagnostic categories.^{2–4} Both were associated with a higher possibility of subsequent development of cervical cancer when compared with women with negative cytology.³ Colposcopic examination of all such cases poses significant demand on the community resources. To improve the efficiency of cervical cancer screening, human papillomaviruses (HPV) DNA testing has been introduced for the triage of women with ASC-US and for primary screening.^{5,6} Although HPV test shows high sensitivity, additional markers are necessary to improve the specificity of identifying cervical cancers or high-grade precursors.^{7–11} It is also necessary to discover adjunct markers for management of LSIL as HPV testing is not so effective in triage management of LSIL.

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p63 and *p73* belong to the family of *p53* proteins. *p53* is the most extensively studied tumor suppressor gene in human cancers. *p63* and *p73*, share both structural homology and functional similarities with *p53*. The latter includes activation of *p53*-downstream genes, regulation of cell cycle checkpoints and induction of apoptosis.^{12–14} However, unlike *p53*, inactivating mutations of *p63* and *p73* in cancers are uncommon.^{12–14} On the other hand, multiple isoforms of *p63*^{15–17} and *p73*^{16,18} exist.

The full-length wild-type isoforms of *p63* (*TAp63*) and *p73* (*TAp73*) contain an NH2-terminal transactivation domain (TA) that can activate downstream target genes and induce apoptosis. In contrast, the NH2-terminal truncated forms, $\Delta Np63$ and $\Delta Np73$, lack the TA and exert negative regulatory effect on *p53* and wild-type *TAp73* and execute anti-apoptotic function. As a result, *TAp63* and *TAp73* are *p53*-synergistic, anti-oncogenic and pro-apoptotic nature, whereas $\Delta Np73$ are oncogenic and anti-apoptotic^{15,16,18–21}

Aberrant expression of *p63* isoforms has been found in several human cancers^{22–26} in association with specific cell type differentiation and progress of cancers. Dysregulated expression of *p73* variants has been studied even more extensively. These studies, including our previous reports on cervical cancers, suggest that *p73* isoforms are involved in human carcinogenesis correlating with prognosis, radiosensitivity and chemosensitivity.^{27–31}

In this study, we evaluated the expression of *TAp73* and *p63A4* isoforms in liquid-based cervical cytology samples of different diagnostic categories and correlated with follow-up profile of such cases.

Materials and methods

Clinical Samples

The residues of 529 ThinPrep-processed (Cytoc, Boxborough, MA, USA) cervical cytology samples were collected from the Department of Pathology, the University of Hong Kong, Queen Mary Hospital. The samples include 91 high-grade squamous intraepithelial lesions (HSIL), 107 LSILs, 212 ASC-US, 63 normal samples and 56 invasive cervical squamous cell carcinomas (Table 1). All except 47 carcinomas were archived samples from the Cervical Cytology Laboratory of the University of Hong Kong and they were collected from a screening population in Hong Kong.² The remaining cancer cases were collected from the laboratory and clinics of Queen Mary Hospital. The use of cytology residues was approved by the Institutional Ethics Review Board.

All the cases of ASC-US have been tested for high-risk HPV by the Digene Hybrid Capture Test II Test (Qiagen, USA). The cytology and histology follow-up data of the patients within 2-year after initial cytology diagnosis were retrieved. Such follow-up

Table 1 Age and cytological diagnosis distribution of women whose samples were included in the study

Age	Number of samples				
	Negative	ASC-US	LSIL	HSIL	CA
<20	3	2	4	1	0
20–29	14	41	20	12	0
30–39	17	80	19	27	6
40–49	16	75	52	35	5
50–59	12	13	13	11	25
>60	1	1	4	5	20
	63	212	112	91	56

CA, squamous cell carcinoma.

data were available in 193/212 (91%) of ASCUS and 106/107 (100%) of LSIL cases.

Cell Lines

The human cervical squamous cell carcinoma cell lines C4-1 and ME180 were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in MEM (Life Technologies Inc., Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin.³⁰

Preparation of Slides

The cell pellet of the liquid-based cytology residues of the above clinical samples collected after centrifuge was resuspended in PreservCyt solution. C4-1 and ME180 cells were detached from culture flasks, washed with PBS and fixed in PreservCyt solution. Thin-layer cytology slides were made using the ThinPrep 2000 Processor and were fixed in 95% alcohol⁹ until use for immunocytochemistry.

Immunocytochemistry

Thin-layer cytology slides were dried overnight in an oven at 37°C. Antigen retrieval was performed by heating immersed slides in 10 mM sodium citrate buffer, pH 6.0, at 95°C in a microwave oven for 15 min. Endogenous peroxidases were blocked by treatment with 3% H₂O₂ in methanol. Mouse anti-human *p63*, Clone 4A4 (Dako, Glostrup, Denmark) and mouse anti-human *TAp73* (Zymed laboratories, CA, USA) were applied at the dilution of 1:1000 and 1:100, respectively. The anti-*p63* 4A4 antibody reacts with both *TAp63* and $\Delta Np63$ isoforms, whereas the anti-*TAp73* antibody detects the *TAp73 α* isoform. Immunocytochemistry was performed using EnVision + Dual Link System (Dako, Carpinteria, CA, USA).³² A light hematoxylin counterstain was used. The cervical carcinoma cell lines ME 180³³ and C4-1 were used as positive control for *p63* and *p73*, respectively.

Table 2 Scoring system for the assessment of *p63A4* and *TAp73* immunoreactivity (modified from Wentzensen, N *et al*)

Score	Staining pattern	Interpretation
0	<i>p63A4/TAp73</i> negative	Negative
1	<i>p63A4/TAp73</i> positive but not in atypical cells	Negative
2	<i>p63A4/TAp73</i> positive in atypical cells but scanty	Positive
3	<i>p63A4/TAp73</i> positive in moderate numbers of atypical cell	Positive
4	<i>p63A4/TAp73</i> positive in most atypical cells	Positive

Replacement of the primary antibody with preimmune IgG serum was used as a negative control. The experimental conditions were uniform for all samples.

Scoring for *p63* and *p73* Immunoreactivity

A modified Wentzensen scoring system³⁴ incorporating morphologic criteria was used for the assessment of the immunoreactivity of the cells for *TAp73* and *p63A4* (Table 2). The whole slide was screened in each case. More than 10 epithelial groups in each slide with the highest immunoreactivity were assessed. Cells with atypical cytological features were evaluated for immunoreactivity and an immunocytochemical index of 0–4 was obtained. The immunoreactivity for *TAp73* (Tsun and Cheung) and *p63A4* (Ng and Cheung) in the samples were evaluated by two authors. If indicated, cases with discrepancy in scoring were discussed under multi-head microscope. The specific cytological diagnosis of the samples was disclosed after the scoring was performed.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for Social Science 16 for Windows (SPSS Inc., Chicago, IL, USA). The significance of difference in *TAp73* and *p63A4* expression among all cervical cytology diagnostic categories was examined by non-parametric Kruskal–Wallis *H* rank test and Chi-square test. The significance of difference between any two diagnostic groups was analyzed by non-parametric Mann–Whitney *U* test. Correlation coefficient was calculated by Pearson correlation test. All statistical tests were calculated in two-sided and *P*-value of <0.05 was considered statistically significant.

Results

The immunoreactivity for both *p63A4* and *TAp73* was predominantly found at the nuclei though weak cytoplasmic expression was focally and occasion-

ally found (Figure 1). There was no apparent effect of storage on the immunoreactivity. A comparison of the *p63A4* and *TAp73* indices in different categories of cervical cytology diagnosis was summarized in Figure 2 and Table 3. Although significant correlation between *TAp73* and *p63A4* indices ($P < 0.0001$, Pearson test) was found, statistically significant difference in both indices among the different diagnostic categories ($P < 0.0001$, Kruskal–Wallis test) was observed.

When compared with negative smears, ASC-US showed significant higher *TAp73* index ($P < 0.0001$, Mann–Whitney test) although no significant difference in *p63A4* index was noted between the two categories. There was also no significant difference in *TAp73* and *p63A4* indices between ASC-US and LSIL. HSIL, however, showed significant higher *TAp73* and *p63A4* indices than LSIL ($P < 0.0001$, $P < 0.0001$, Mann–Whitney test). The two antibodies could also detect isolated small HSIL cells. There was no significant difference in *TAp73* and *p63A4* indices between HSIL and carcinomas.

We were able to retrieve the follow-up cytology and/or histology data in 193/212 (91%) of ASC-US and 106/107 (99.6%) of LSIL cases. Correlation between *TAp73* and *p63A4* indices in ASC-US samples and subsequent development of LSIL/HSIL were shown in Table 4. Among cases of ASC-US, significantly higher *p63A4* index was found in cases that subsequently progressed to LSIL ($P = 0.031$) or HSIL ($P = 0.006$) or above than those that did not. On the other hand, there was no statistically significant correlation with *TAp73* index and subsequently development of LSIL ($P = 0.952$) or HSIL ($P = 0.365$).

Among the 212 ASC-US, 129 (61%) were positive for high-risk HPV as detected by Digene test. There was significant correlation between presence of high-risk HPV and *p63A4* index ($P = 0.01$) but not the *TAp73* index ($P = 0.891$). Among ASC-US cases positive for HPV, cases with high *p63A4* index were more likely to have subsequent HSIL detected ($P = 0.016$) whereas no predictive significance was found for subsequent detection of LSIL ($P = 0.124$). There was no significant correlation in *TAp73* index between HPV-positive ASC-US cases that have LSIL ($P = 1$) or HSIL ($P = 0.42$) or above subsequently detected.

Among LSIL, significantly higher *TAp73* index ($P = 0.038$) was also found in those cases that subsequently progressed to HSIL or above than those that did not (negative, ASC-US or LSIL) but no significant difference was observed for *p63A4* ($P = 0.689$).

Discussion

Novel Markers for Cervical Cytology

Cervical cancer screening by cytomorphological evaluation is one of the most successful cancer

Figure 1 *p634A4* and *TAp73* expression was assessed in liquid-based cytology by immunocytochemistry (magnification: 10×40): (a) Normal squamous cells are negative for both *p634A4* and *TAp73*. Focal weak to moderate immunoreactivity of *p634A4* and *TAp73* was noted in ASC-US (b) and LSIL (c). HSIL (d) and squamous cell carcinoma (e) cells were strongly positive for both *p634A4* and *TAp73*.

Figure 2 *p634A4* (a) and *TAp73* (b) protein expression in normal cervical cells, ASC-US, LSIL, HSIL and squamous cell carcinoma (CA) were analyzed by Mann–Whitney test.

Table 3 Comparison of *TAp73* and *p634A4* indices comparing different categories of cervical cytology diagnosis

	P-value (<i>TAp73</i> index)	P-value (<i>p634A4</i> index)
Negative vs ASC-US	<u><0.001</u>	0.524
ASC-US vs LSIL	0.606	0.540
ASC-US vs HSIL	<u><0.001</u>	<u><0.001</u>
LSIL vs HSIL	<u><0.001</u>	<u><0.001</u>
HSIL vs CA	0.355	0.084

CA, squamous cell carcinoma.

P-values were calculated by Mann–Whitney test. Those with significant P-values are underlined.

screening programs. Nevertheless, there still exist limitations in cervical cytology screening. New laboratory techniques are therefore being continuously explored to improve sensitivity and specificity of cervical cytology evaluation. The liquid-based cytologic preparation was popularly applied in the recent decade. It allows collection of exfoliated cervical epithelial cells in liquid buffer, reduces the interfering background of blood and inflammatory cells and improves the quality of the sample for screening of cancer cells.² Moreover, after the production of a thin layered cervical smear, the residual cells in the vial can conveniently be used for ancillary investigations.

Table 4 Correlation between *TAp73* and *p634A4* indices in ASC-US samples and subsequent development of LSIL/HSIL or above

	P-value (<i>TAp73</i> index)	P-value (<i>p634A4</i> index)
Among ASC-US		
Subsequent development of LSIL+	0.952	<u>0.031</u>
Subsequent development of HSIL+	0.365	<u>0.006</u>
Among HPV-positive ASC-US		
Subsequent development of LSIL+	1	0.124
Subsequent development of HSIL+	0.42	<u>0.016</u>
Among LSIL		
Subsequent development of HSIL+	<u>0.038</u>	0.689

LSIL+, LSIL or above; HSIL+, HSIL or above.

P-values were calculated by Mann–Whitney test. Those with significant P-values are underlined.

HPV molecular test performed on directly collected samples or from liquid-based cell residues is currently the most commonly applied ancillary test to cervical cytology.^{5,6,10} It enables triage of management of women with ASC-US, primary screening and follow-up of patients with cervical neoplasia. Besides HPV tests, cervical cytology samples can also be used for other evaluation including genetic and epigenetic studies.^{6,10} The ploidy of epithelial cells and the immunohistochemical detection of expression of genetic markers can be assessed with an intact morphological background by immunocytochemistry and *in situ* hybridization enhancing identification of dysplastic cells.^{8,9,35,36} For instance, *p16* immunohistochemical study on liquid-based cytology has been reported to be particularly hopeful in detection of cancer cells and their precursors although there still exist limitations in its application.^{6,36} Further exploration of novel biomarkers for identifying cervical cancers and their precursors is still an ongoing process.

We believe that combined cytopathologic evaluation and application of molecular markers in cervical cytology is a promising option to facilitate reliable detection of cervical cancer. Owing to the important roles of *p63* and *p73*, the two homologues of *p53*, in cervical epithelial differentiation and carcinogenesis, the significance of immunoreactivity for *p634A4* and *TAp73* in liquid-based cervical cytology was evaluated in this study. The anti-*p634A4* antibody recognizes both *TAp63* and $\Delta Np63$ isoforms, whereas the anti-*TAp73* antibody detects the *TAp73 α* isoform. They were selected for the study based on our previous experience on these two genes.^{25,26,29,37}

P63 and Cervical Epithelial Differentiation and Carcinogenesis

Upregulated expression of both *TAp63* and $\Delta Np63$ isoforms has been demonstrated in cervical intra-

epithelial neoplasia and invasive cancers.^{4,33} The expression of *p63* gene was found to be associated with poor survival and local recurrence of cervical squamous cell carcinoma.³⁸ In this study, p63A4 immunoreactivity was found to be significantly higher in HSIL and cancers. The antibody can also detect isolated small HSIL cells and can improve the sensitivity of detecting these 'litigation' cells that are potentially easy to be missed.

P63 has been suggested to be useful to distinguish squamous and glandular lesions in cervical samples³⁹ and the endocervical glandular cells in this study was found to be negative for p63A4. In the squamous mucosa, *p63* expression is often localized to the basal and parabasal cells of mature or metaplastic stratified squamous epithelium but may be present in all cell layers in atrophic epithelium. *p63* is indeed considered as a marker for reserve cells or stem cell like cells.^{25,40–42} *p63* expression by immunostaining alone may therefore highlight basal cells, which may become a diagnostic pitfall in atrophic samples. By combining *p63* immunoreactivity assessment and morphological evaluation, such false positive results may be minimized.

P73 and Cervical Cancers

Dysregulated expression of *p73* isoforms in cervical intraepithelial neoplasia and cervical cancer has been reported.^{29,43,44} In our earlier studies, the expressions of $\Delta Np73$ and *TAp73* were found to be independently associated with prognosis of patients with cervical cancers. The two *p73* isoforms have been found to be potential markers for predicting the prognosis and sensitivity to radiotherapy in patients with cervical squamous cell carcinoma.²⁹ In this study, *TAp73* immunoreactivity was also found to be higher in HSIL and squamous cell carcinomas.

Correlation with Progress of Disease

CIN is actually a dynamic lesion capable of spontaneous regression or progression into more serious lesion including cancers. It is believed that biomarkers such as *p16* can help in evaluating the likelihood for progression and regression in a particular CIN.⁷

Such markers may be particularly useful in assisting triage of patients with borderline smears reported as ASC-US. Our study on an Asian screening population in Hong Kong showed that, similar to other screening populations, ASC-US is the most common abnormal cytologic finding² and is associated with a significantly higher chance to develop cervical cancer and its high-grade precursors.³ HPV DNA test has been used for triage of ASCUS. However, majority of the ASC-US positive for high-risk HPV does not progress to high-grade cervical intraepithelial lesion. Management problem in

women with LSIL also exists. Although more than 50% of LSIL will regress, HSIL or above can still be found in a significant portion of these women.⁴⁵ Women with LSIL may be referred for colposcopy or followed up with cytology with variation in management guidelines in different countries.

In this study, high p63A4 immunoreactivity correlated with higher likelihood of progressing to LSIL or HSIL lesions in subsequent cytology or histology follow-up among ASC-US cases independent of their HPV status. On the other hand, high *TAp73* index in LSIL correlated with higher chance of progressing to HSIL or above. p63A4 may thus be a promising marker to further triage management of women with HPV-positive ASC-US whereas *TAp73* is a good marker for deciding management of women with LSIL. Ancillary immunocytochemical studies for p63A4 and *TAp73* can be performed in ASC-US and LSIL samples, respectively. Those with high p63A4 and *TAp73* expression will be referred for colposcopy whereas those with low expression can be followed up with repeat cytology. This will reduce the burden of unnecessary colposcopy.

Reaction with HPV

HPV are recognized carcinogen for cervical cancers and their precursors. The E6 and E7 genes of high-risk HPV genotypes are essential in promoting degradation and/or inactivation of the *p53* and retinoblastoma proteins, respectively, resulting in disruption of the important tumor suppressor functions of these two genes.

It is controversial whether there is a direct interaction between *p63* and *p73* with HPV viral oncoproteins. Interaction among HPV, *p63* and *p73* has been reported. A strong circumstantial association between *p63* immunoreactivity and HPV 16 has been reported in earlier study because of their colocalization in squamous cell carcinomas.⁴⁶ Indeed, *p63* is considered as a HPV target cell.⁴¹ Recently, Teisser *et al*⁴⁷ reported that the *p63* target genes could be activated by suppressing the E6/E6AP pathway. In our study, significant correlation between presence of high-risk HPV and p63A4 index was found among cases of ASC-US.

Park *et al*⁴⁸ showed that *p73* could also be inactivated by HPV-E6, but not by ubiquitin/proteasome-dependent mechanisms as in the case of *p53*. *p73* promoter has also been shown to be affected, at least in cell lines and primary human keratinocytes, by E7 proteins from oncogenic HPV types.⁴³ Studies on *p73*-/- HPV38 E6/E7 transgenic mice keratinocytes also showed that loss of *p53* could stop $\Delta Np73$ expression and partially restore the UV-activated cell cycle checkpoints. On the other hand, loss of $\Delta Np73$ led to restoration of the *p53* pathways.⁴⁹ In this study, however, the $\Delta Np73$ immunohistochemistry did not correlate with the HPV status.

In summary, in this immunocytochemical study on a relatively large number of liquid-based cytology samples, we showed that *TAp73* and *p63A4* immunoreactivity together with morphological assessment could distinguish cervical cancer and the high-grade precursors. In particular, *p63A4* and *TAp73* immunoreactivity correlated with subsequent detection of HSIL or above in patients with ASC-US and LSIL, respectively. Cases of ASC-US positive for *p63A4* are also more likely to harbor high-risk HPV. *p63A4* and *TAp73* immunoreactivity may thus be useful potential markers for triage of borderline and low-grade cervical smears, respectively.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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