Cervical cancer screening by enhanced cytology: application of novel markers

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KEY MESSAGES

1. p634A4, TAp73, and DJ-1 immunocytochemistry as well as in situ hybridisation for the *OSMR1, PAPD7/POLS, PRAKK1*, and *TRIO* genes helped to identify cervical carcinoma and high-grade precursor cells.
2. p634A4 immunoreactivity is a potential marker for triage of atypical squamous cells of undetermined significance.
3. TAp73 immunoreactivity is a potential marker for triage of low-grade squamous intraepithelial lesions.

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Introduction

Cervical cytological examination is the most widely applied screening method for cervical cancer and its precursors. Liquid-based cytology produces good-quality smears[1] and enables the use of ancillary laboratory techniques to distinguish neoplastic from benign cells.[1]

Immunohistochemical and molecular markers have been tested to identify dysplastic cells in cervical smears. When testing for human papillomavirus (HPV) or chromosome aneusomy, *MIB1* (Ki67) and p16 immunocytochemistry helps to highlight dysplastic or cancer cells.[2] Such markers may be useful for triage of patients with borderline smears of atypical squamous cells of undetermined significance (ASCUS), which is the most common abnormal cytological finding and is associated with a significantly higher risk of developing cervical cancer and its high-grade precursors.

In this study, immunocytochemical markers (*p63, p73, Eif-5A2*, and *DJ-1* genes) were tested in cervical cancer screening using immunocytochemistry and chromogenic in situ hybridisation. Differential expression of these genes in cervical cytology is useful in diagnosing cervical cancer.

p63 and *p73* genes are members of the *p53* family and play important roles in carcinogenesis. Different isoforms of p63 and p73 can enhance or suppress neoplastic cell growth. Differential expression of p63 and p73 isoforms was reported in normal and neoplastic cervical epithelium. *p73* expression is associated with radiosensitivity of cervical cancer.[3] In a study of frequently amplified regions at 3q26.2, *Eif-5A2* was found to be important in ovarian carcinogenesis.[4] Similar regions at chromosome 3q have also been identified in cervical cancer. *Eif-5A2* is a potential marker for cervical cancer. *DJ-1* has been identified as a suppressor of *PTEN*, which is a tumour suppressor gene. *DJ-1* overexpression has been noted in human malignancies compared with healthy tissue.

Novel genes can be identified in specific chromosome regions by array comparative genomic hybridisation. In our previous studies on cervical cancer samples and cell lines, the frequently amplified regions were 1q, 3p, 3q, and 5p.[5] Besides *Eif-5A2*, eight gene loci can be identified and the amplification status can be detected by chromogenic in situ hybridisation. This study aimed to assess the application value of adjunct markers in liquid-based cervical cytology for detection of carcinoma cells and precursors.

Methods

This study was conducted from March 2007 to February 2009. Residues of liquid-based ThinPrep Pap Test (Hologic, Bedford, MA, US) cervical cytology samples of high-grade squamous intraepithelial lesions (HSIL) [n=80], low-grade SIL (LSIL) [n=100], ASCUS (n=200), invasive cervical carcinoma (n=40), and healthy tissue (n=60) findings were retrieved from the Cervical Cytology Laboratory, Queen Mary Hospital, University of Hong Kong for immunocytochemistry and in situ hybridisation.[1] Cervical cancer cell lines HeLa, SiHa, ME180, and C4-1P3 as well as the ovarian cancer cell line UACC1895 were used as controls. Expression of *p63, p73, Eif-5A2*, and *DJ-1* genes was studied immunocytochemically.

To identify the eight target genes for chromogenic in situ hybridisation experiments, quantitative real-time polymerase chain reaction (PCR) using DNA extracted from healthy tissue (n=6), HSIL (n=14), and squamous cell carcinoma (SCC) [n=12] cytology samples was performed. The DNA probes for *Eif-5A2* and the eight other selected gene loci on 1q, 3p, 3q, and 5p were labelled with biotin, and chromogenic in situ hybridisation was performed. The subsequent cytology and colposcopic histology findings of the cases were traced. The HPV molecular test results, if available, were also retrieved for correlation.

Apart from *Eif-5A2*, potential genes located on 1q, 3p, 3q, and 5p for in situ hybridisation were explored through literature review followed by screening by quantitative PCR (qPCR).[5] The genomic sequences of potential target genes were retrieved from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). Primers were designed using NCBI/Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). qPCR was performed on DNA extracted from pilot samples with ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA).

After screening by real time PCR, eight genes with demonstrable amplification status in the cervical cancer samples were selected for chromogenic in situ hybridisation. These included *MUC1* (on 1q), *MST1R* (on 3p), *DVL3* and *FGF12* (on 3q), and *OSMR1, PAPD7/POLS, PRAKK1*, and *TRIO* (on 5p). The probes were designed and manufactured using the assay services of Empire Genomics (New York, NY, USA). Bacterial artificial chromosome clones for these eight genes and non-repetitive sequences of *MAN2A1* on chromosome 5, which acted as control for the genes on chromosome 5, were prepared. The location and clone percent coverage are listed in the Table. Plasmids containing pericentromeric regions for chromosomes 1 and 3 were used as controls for the genes on these chromosomes. After culture, the plasmid DNA was isolated by the alkaline lysis method followed by biotin labelling using BioPrime DNA Labeling System (Invitrogen; Life Technologies, Paisley, UK).

Chromogenic in situ hybridisation was performed following established protocols.[2] The slides were first incubated with 0.1% Triton X-100 and proteinase K. The labelled probes were added to the hybridisation mix, followed by overnight hybridisation. Immunocytochemistry was performed to visualise in situ hybridisation signals by peroxidase activity. Known positive and negative control slides were included in each batch of experiments. The morphology of the nuclei was taken into consideration during evaluation of in situ hybridisation signals.

Regarding immunocytochemistry, mouse anti-human *p63* (Clone 4A4; Dako, Glostrup, Denmark), mouse anti-human *TAp73* (Zymed Laboratories, San Francisco, CA, USA), DJ-1 (a gift from Prof Tak W Mak, Ontario Cancer Institute, Toronto, Canada), and mouse monoclonal antibody to *EIF-5A2* (ab57421; Abcam, Cambridge, MA, USA) were applied to the ThinPrep thin-layer cytology slides. Immunocytochemical studies were performed using the EnVision+ Dual Link System (Dako, Carpinteria, CA, USA). A light haematoxylin counterstain was used. Appropriate negative and positive controls were included. A modified Wentzensen scoring system incorporating morphologic criteria for the assessment of the immunoreactivity of the cells was used.

Chi-squared test (2-tailed) was used to compare the immunocytochemical and in situ hybridisation signals among the healthy tissue, ASCUS, LSIL, HSIL, and cervical carcinoma groups. A P value of <0.05 was considered statistically significant.

Results

Immunocytochemistry

p634A4 protein expression was found mainly at the nuclei, although weak cytoplasmic expression

TABLE. Location and clone percent coverage of in situ hybridisation probes

Gene	Chromosome	Clone	Clone percent coverage (%)	Chromosome start and stop location
DVL3	3q	RP11-814O14	100	185355977-185374008
Eif-5A2	3q	RP11-115J24	93.5	172090216-172251852
FGF12	3q	RP11-767C10	73.2	193362761-193557602
MST1R	3p	RP11-915H6	100	49782629-49932767
MUC1	1q	RP11-263K19	100	153361602-153541655
OSMR	5p	RP11-113D9	100	38808720-38981485
PAPD7	5p	RP11-681G24	100	6761120-6934896
PRKAA1	5p	RP11-357L11	100	40786785-40949438
TRIO	5p	RP11-481M12	53.7	14308043-14504474
MAN2A1	5q	RP11-259F13	95.3	109057383-109227354

was detected focally (Fig 1a). Significantly higher p634A4 expression was found in HSIL or carcinoma when compared with ASCUS and LSIL (P<0.05). Among ASCUS, p634A4 expression in cases that subsequently progressed to LSIL (P<0.05) or HSIL (P<0.05) was significantly higher than those that did not. For ASCUS positive for high-risk HPV detected by hybrid capture II, cases with a high p634A4 index were more likely to have subsequent HSIL detected (P<0.05).

TAp73 protein expression was found mainly at the nuclei, although weak cytoplasmic expression was detected focally (Fig 1b). Significantly higher TAp73 expression was found in HSIL or carcinoma when compared with ASCUS and LSIL (P<0.05). Among LSIL, significantly higher TAp73 (P<0.05) was found in cases that subsequently progressed to HSIL. TAp73 correlated with the p634A4 indices (P<0.0001).

DJ-1 protein expression was found at both the cytoplasm and the nucleus of SCC cells, whereas the healthy squamous cells were negative for DJ-1 protein. There was a significant difference in DJ-1 expression among the different diagnostic categories of healthy tissue, ASCUS, LSIL, HSIL, and SCC (P<0.05). Significantly higher DJ-1 expression was found in HSIL and SCC than in ASCUS and LSIL (P<0.05).

No significant Eif-5A2 protein expression was detected in SCC, HSIL, LSIL, and ASCUS cells. Only a few HSIL, LSIL, and ASCUS cells in a small number of samples showed weak Eif-5A2 expression. In contrast, strong expression of Eif-5A2 was demonstrated in the control ovarian cancer cell line UACC1895.

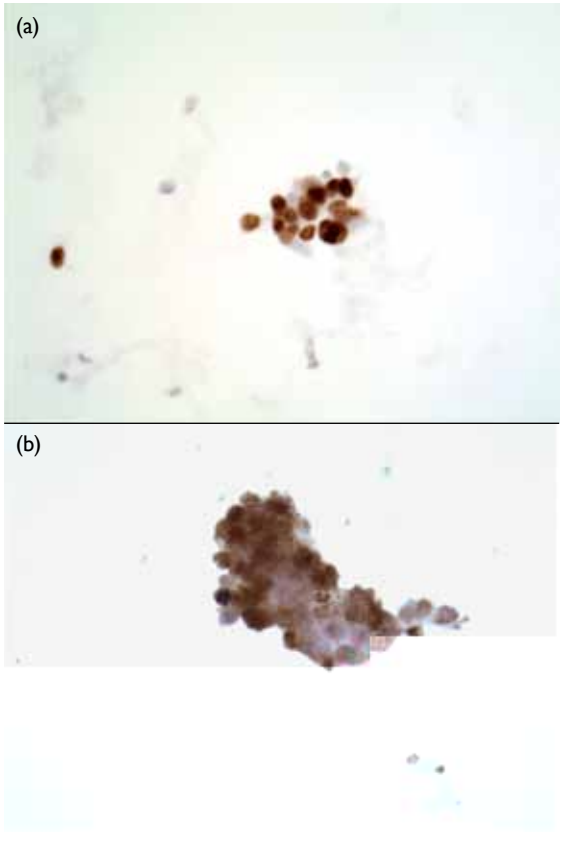

FIG 1. Overexpression of (a) p634A4 and (b) TAp73 in squamous cell carcinoma cells

Chromogenic in situ hybridisation

There were two to 14 copy signals for *DVL3* and three to 10 copy signals for *FGF12*, both on chromosome 3q, and two to 10 copy signals for *MST1R* on chromosome 3p detected in SCC and HSIL samples. Aneusomy of chromosome 3 was suggested by the multiple (2-8) copy signals for chromosome 3 centromere. There was no significant increase in the ratio of *DVL3, FGF12*, and *MST1R* to centromere 3 in SCC and HSIL samples (P>0.05), indicating no significant amplification of these genes in SCC and HSIL.

No increase in in situ hybridisation signals for *Eif-5A2* (on chromosome 3q) could be detected in carcinoma, HSIL, LSIL, and ASCUS cells. In contrast, multiple copy signals were found in the control ovarian cancer cell line UACC1895.

There were two to 10 copy signals for *MUC1* on chromosome 1q detected in SCC and HSIL samples. Aneusomy of chromosome 1 was suggested by the multiple (2-7) copy signals for chromosome 1 centromere. There was no significant increase in the *MUC1* to centromere 1 ratio in SCC and HSIL samples (P>0.05) suggesting absence of amplification of the *MUC1* gene in these lesions.

Overall, three to eight copy signals for *OSMR* and *PRAD7/POLS* each, and two to 10 copy signals for *PRKAA1* and *RIO* each on chromosome 5p were detected in SCC and HSIL samples, whereas two copy signals for the non-replicating *MAN2A-1* gene on chromosome 5 were detected (Fig 2). There was a significant increase in the ratio of *OSMR, PRAD7/POLS, PRKAA1*, and *TRIO* to centromere 5 in SCC and HSIL samples (P<0.05) suggesting amplification of these genes in SCC and HSIL.

Specificity of predicting cervical lesions

When compared with the high-risk HPV test, p634A4 immunocytochemistry increased the specificity and positive predictive value, although the sensitivity and negative predictive value were reduced.

Discussion

The selected gene loci have been found to occupy essential niches in cervical carcinogenesis. Among the six major hallmarks of cancer, these genes have been found to play a role in evading apoptosis (*p63,*

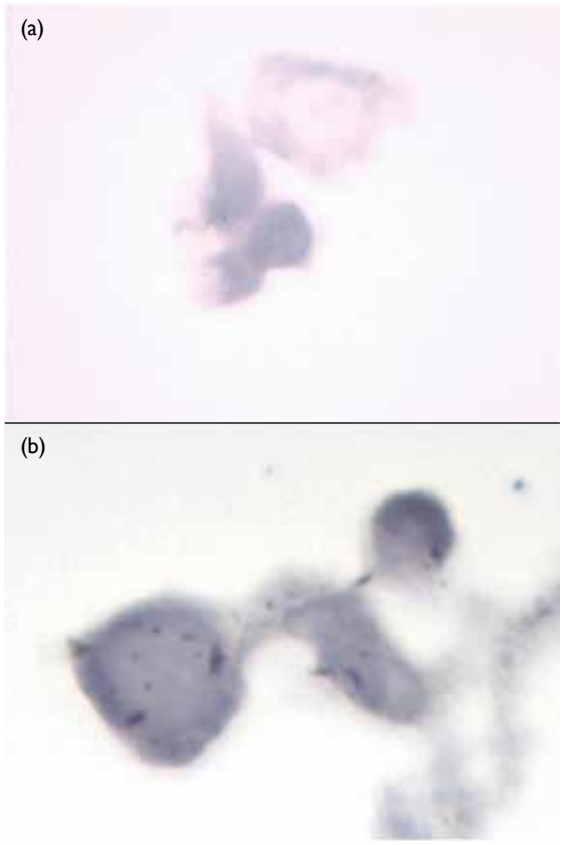

FIG 2. Amplification of *TRIO* is reflected by increases in in situ hybridisation signals for (a) *MAN2A1* and (b) *TRIO*

p73, DJ-1, TRIO, Eif-5A2), self-sufficiency in growth signals (*DVL3, FGF12, MST1R, OSMR, PAPD7/POLS*), insensitivity to anti-growth signals (*p63, p73, DJ-1, MUC1, PAPD7/POLS*), and sustained angiogenesis (*PRKAA1*).

In this study, the potential of p63 and p73 in highlighting SCC and HSIL, and in triaging ASCUS and LSIL (even among HPV-positive cases for ASCUS) was demonstrated. Nonetheless, the application of p63 and p73 immunocytochemistry, particularly p63, may have limitations for detecting glandular lesions in cervical cytology.

High expression of *DJ-1* rendered cells resistant to apoptosis and overexpression of *DJ-1* has been reported in various human cancers. In this study, high *DJ-1* expression was also found in SCC and HSIL, suggesting its role as cancer marker. However, *DJ-1* cannot be used to triage ASCUS or LSIL cases for subsequent development of HSIL or SCC.

Overexpression of *Eif-5A2* has been reported in several cancers, suggesting it is a potential oncogene. Almost all of the carcinomas with overexpression of *Eif-5A2* were adenocarcinomas. In our pilot study, *Eif-5A2* was found to be overexpressed in cervical adenocarcinoma, but to a much lesser extent in SCC. Overexpression or amplification of *Eif-5A2* was not detected in the cervical cytology samples. Tissue-specific functions of the *Eif-5A2* isoform may exist. Different gene expression profiles for adenocarcinoma and SCC have been demonstrated.

Dishevelled (*Dvl*) family gene products, cytoplasmic mediators of the Wnt/beta-catenin signalling pathway, are important in embryological development. *DVL3* was found to be overexpressed in cancers relative to metastatic ability. Nevertheless, amplification of *DVL3* was not detected in cervical cancers in this study.

In oesophageal SCC, *FGF12* was found to be associated with cellular migration, proliferation, and inhibition of apoptosis. Expression of *FGF12*, related to the MAPK signalling pathway, was validated by tissue microarray. Amplification of *FGF12* has not been reported in cervical cancers and was not detected in the cytology samples of cervical cancers in our study.

MST1R, which maps at 3p21.3, encodes a tyrosine kinase receptor closely related to the *MET* gene, whose mutations and dysregulated expression are associated with cancers of the lung and breast in association with metastasis and death. *MST1R* forms a complex with the epidermal growth factor receptor and acts as a transcriptional regulator in response to stress signals imposed on cancer cells. Nevertheless, amplification of *MST1R* was not detected in cervical cancers in this study.

Frequent amplification of *MUC1* at 1q has been reported in cancers of the breast, ovary, and thyroid in association with aggressive clinical behaviour and resistance to chemotherapy. High *MUC1* expression has been reported in cervical cancers, predominantly in adenocarcinoma. In this study, amplification of *MUC1* was not demonstrated in cytology samples of SCC and HSIL.

Amplification of *OSMR* at 5p has been reported in SCC of the cervix, adversely influencing overall patient survival independently of tumour stage. *OSMR* could activate downstream signalling pathways, including STAT3 and MAPK, and induce transcription of the angiogenic factor vascular endothelial growth factor. Our study confirmed the amplification of *OSMR* in SCC and HSIL cytology samples.

The *PAPD7/Pols* gene, which encodes a DNA polymerase, is necessary for chromosome segregation and establishing sister chromatid cohesion after S-phase. These processes are important for regulation of the cell cycle. Amplification of *PAPD7/Pols* has been demonstrated in cervical cancers as well as soft-tissue sarcomas. Amplification of *PAPD7/Pols* was also detected in SCC and HSIL cytology samples in this study.

The *PRKAA1* gene codes for the catalytic alpha 1 subunit of the AMP-activated protein kinase,

which is an important cellular metabolic stress regulator. Amplification and overexpression of *PRKAA1* has been reported in our earlier studies of cervical cancers, supporting its potential in cervical carcinogenesis. The amplification was confirmed by chromogenic in situ hybridisation in this study.

Amplification of *TRIO* at 5p15.2 has been demonstrated in oral, oesophageal, and urinary bladder cancers, in association with aggressive tumour growth and proliferation and reduced apoptosis. *TRIO* amplification is also found in primary cervical cancers, adversely influencing patients' survival. Amplification of *TRIO* was confirmed in this study.

Study limitations

This study was limited to evaluation of gene expression and amplification in SCC and squamous lesions in cervical cytology. Adenocarcinoma and its precursors were not studied.

Conclusion

In liquid-based cytology samples, TAp73, p634A4, and DJ-1 immunocytochemistry and detection of amplified gene copies of *OSMR1, PAPD7/POLS, PRAKK1*, and *TRIO* by in situ hybridisation together with morphological assessment could distinguish cervical cancer from its high-grade precursors. In addition, p634A4 and TAp73 immunoreactivity correlated with subsequent detection of HSIL or SCC in patients with ASCUS and LSIL, respectively, with enhanced specificity. p634A4 and TAp73 immunoreactivity may thus be potential markers for triage of borderline and low-grade cervical smears. Such adjunct markers can identify at-risk patients and reduce unnecessary referral for colposcopy.

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References

1. Cheung AN, Szeto EF, Leung BS, Khoo US, Ng AW. Liquid-based cytology and conventional cervical smears: a comparison study in an Asian screening population. Cancer 2003;99:331-5.
2. Cheung AN, Chiu PM, Tsun KL, Khoo US, Leung BS, Ngan HY. Chromosome in situ hybridisation, Ki-67, and telomerase immunocytochemistry in liquid based cervical cytology. J Clin Pathol 2004;57:721-7.
3. Liu SS, Leung RC, Chan KY, et al. p73 expression is associated with the cellular radiosensitivity in cervical cancer after radiotherapy. Clin Cancer Res 2004;10:3309-16.
4. Guan XY, Fung JM, Ma NF, et al. Oncogenic role of eIF-5A2 in the development of ovarian cancer. Cancer Res 2004;64:4197-200.
5. Huang FY, Chiu PM, Tam KF, et al. Semi-quantitative fluorescent PCR analysis identifies PRKAA1 on chromosome 5 as a potential candidate cancer gene of cervical cancer. Gynecol Oncol 2006;103:219-25.