ORIGINAL ARTICLE

p16^{INK4A}, CDC6, and MCM5: predictive biomarkers in cervical preinvasive neoplasia and cervical cancer

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J Clin Pathol 2005;**58**:525–534. doi: 10.1136/jcp.2004.018895

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Accepted for publication 12 November 2004

Aim: To analyse and compare expression patterns of three potential biomarkers—p16^{INK4A}, CDC6, and MCM5—and evaluate their use as predictive biomarkers in squamous and glandular cervical preinvasive neoplasia.

Methods: Immunocytochemical analysis of p16^{INK4A}, MCM5, and CDC6 expression was performed on 20 normal, 38 cervical intraepithelial neoplasia 1 (CIN1), 33 CIN2, 46 CIN3, 10 squamous cell carcinoma, 19 cervical glandular intraepithelial neoplasia (cGIN), and 10 adenocarcinoma samples. Staining intensity was assessed using a 0–3 scoring system. p16^{INK4A}, MCM5, and CDC6 expression was also examined in ThinPrep slides exhibiting mild, moderate, and severe dyskaryosis. Human papillomavirus (HPV) was detected using a modified SYBR green assay. Fluorogenic polymerase chain reaction (PCR) and solution phase PCR were used for specific HPV typing.

Results: All three markers showed a linear correlation between expression and grade of dysplasia. p16^{INK4A} and MCM5 protein expression was upregulated in all grades of squamous and glandular dysplasia. CDC6 protein was preferentially expressed in high grade lesions and in invasive squamous cell carcinoma.

Conclusion: p16^{INK4A} expression was closely associated with high risk HPV infection—all grades of squamous and glandular cervical lesions were immunohistochemically positive. MCM5 staining intensity was independent of high risk HPV infection, highlighting its potential as a biomarker in both HPV dependent and independent cervical dysplasia. CDC6 may be a biomarker of high grade and invasive lesions of the cervix, with limited use in low grade dysplasia. p16^{INK4A} was the most reliable marker of cervical dysplasia. Combinations of dysplastic biomarkers may be useful in difficult diagnostic cases.

The introduction of the Papanicolaou (PAP) cervical smear test by George Papanicolaou in the 1920s greatly reduced the morbidity and mortality rates of cervical cancer. However, limitations with respect to its sensitivity and specificity have prompted the search for specific biomarkers of dysplastic squamous and glandular cells of the cervix. It is hoped that these biomarkers can be used in conjunction with conventional screening programmes to enhance diagnostic consistency and reproducibility. Investigation of potential biomarkers will allow comparison of the efficacy of human papillomavirus (HPV) vaccine approaches and will help to unravel new pathways involved in the pathogenesis of cervical dyskaryosis.[1 2]

> ''Human papillomavirus contributes to neoplastic progression predominantly through the action of two viral oncoproteins—E6 and E7—and is manifested by changes in the expression of host cell cycle regulatory proteins''

It is well established that high risk human papillomavirus plays a pivotal role in the development and progression of cervical cancer.[3] In fact, HPV infection has been detected in almost all preneoplastic and neoplastic lesions of the cervix.[4 5] HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins—E6 and E7[6]—and is manifested by changes in the expression of host cell cycle regulatory proteins. Such differentially expressed host proteins and nucleic acids may have a role as ''biomarkers'' of dysplastic cells. To date, a wide array of molecular markers has been evaluated. Three markers that have shown the greatest potential are the cyclin dependant kinase inhibitor p16^{INK4}[7–9] and the DNA replication licensing proteins CDC6 (cell division cycle protein 6) and MCM5 (mini chromosome maintenance 5).[10–13]

The CDKN2A gene product, p16^{INK4A}, is a tumour suppressor protein that inhibits cyclin dependant kinases 4 and 6, which phosphorylate the retinoblastoma (Rb) protein. A reciprocal association between p16^{INK4A} and Rb expression has been found, suggesting the presence of a negative feedback loop that allows Rb to limit levels of p16^{INK4A}.[14–16] p16^{INK4A} overexpression has been demonstrated in cervical cancers, and is thought to be the result of inactivation of Rb by the HPV E7 protein.[17] p16^{INK4A} may also be directly induced by the transcription factor E2F, which is released from the Rb protein after the binding of HPV E7.[18] The expression pattern of p16^{INK4A} in dysplastic squamous and glandular cervical cells in tissue sections and in cervical smears has been investigated extensively.[4 7–9 19–23] It is now widely accepted that p16^{INK4A} is a sensitive and specific marker of squamous and glandular dysplastic cells of the cervix, and is a valuable adjunctive test in cervical cancer lesion diagnosis and cervical screening.[8]

In all eukaryotes, a conserved mechanism of DNA replication exists, which ensures that DNA replication occurs only once in a single cell cycle.[24] This mechanism is often termed the ''licensing'' of DNA replication.[25] DNA replication requires the regulated assembly of prereplicative complexes

Abbreviations: CDC6, cell division cycle protein 6; cGIN, cervical glandular intraepithelial neoplasia; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; MCM, mini chromosome maintenance; PCR, polymerase chain reaction; Rb, retinoblastoma protein

Table 1 Correlation between HPV status and p16^{INK4A} staining intensity

		p16^{INK4A} staining score				
Sample	**HPV status**	**0**	**0a**	**1**	**2**	**3**
Normal (n = 20)	HPV−	18/20 (90%)				
	HPV-6/11	2/20 (10%)				
	HPV-16					
	HPV-18					
	HPV unknown					
% Total		100%				
CIN1 (n = 38)	HPV−			2/38 (5%)	2/38 (5%)	
	HPV-6/11			3/38 (8%)	1/38 (3%)	
	HPV-16					21/38 (55%)
	HPV-18					
	HPV unknown				7/38 (19%)	2/38 (5%)
% Total				13%	27%	60%
CIN2 (n = 33)	HPV−			3/33 (9%)		
	HPV-6/11			2/33 (6%)		
	HPV-16				8/33 (24%)	14/33 (43%)
	HPV-18				1/33 (3%)	
	HPV unknown			2/33 (6%)		3/33 (9%)
% Total				21%	27%	52%
CIN3 (n = 46)	HPV +	1/46 (2%)		1/46 (2%)	1/46 (2%)	
	HPV-6/11			1/46 (2%)		
	HPV-16				8/46 (18%)	20/46 (44%)
	HPV-18				2/46 (4%)	2/46 (4%)
	HPV unknown			2/46 (4%)	3/46 (7%)	5/46 (11%)
% Total		2%		8%	31%	59%
SCC (n = 10)	HPV−					
	HPV-6/11					
	HPV-16					9/10 (90%)
	HPV-18					
	HPV unknown					1/10 (10%)
% Total						100%
cGIN (n = 7)	HPV unknown				1/17 (6%)	6/17 (35%)
hcGIN (n = 10)	HPV unknown				1/17 (6%)	9/17 (53%)
% Total					12%	88%
Adenocarcinoma (n = 10)	HPV unknown					10/10 (100%)
% Total						100%

N refers to the number of cases tested. Not all cases included in our study were tested for HPV status. No case was found to be positive for HPV-31 or HPV-33. Staining score: 0, no positive staining of dysplastic cells; 0a, basal layer staining; 1, basal layer staining plus <10% positive staining of dysplastic cells; 2, >10% but <50% positive staining of dysplastic cells; 3, >50% positive staining of dysplastic cells.
cGIN, cervical glandular intraepithelial neoplasia; hcGIN, high grade cervical glandular neoplasia; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; SCC, squamous cell carcinoma.

on to DNA during the G1 phase of the cell cycle. Prereplicative complexes render the chromatin competent or ''licensed'' to replicate. Among the proteins known to assemble to form the prereplicative complexes are CDC6 and the mini chromosome maintenance (MCM) proteins.[26 27] Deregulation of DNA replication results in genomic instability and contributes to the malignant transformation of cells.[25 28] Not surprisingly then, increased expression of MCM5 and CDC6 is seen in dysplastic cells. In normal cells, MCM5 and CDC6 are present only during the cell cycle and are lost from the cell during quiescence and differentiation. As a consequence, MCM5 and CDC6 are specific biomarkers of proliferating cells. Several studies have highlighted their potential use in the identification and/or diagnosis of a variety of dysplastic and neoplastic conditions.[13 29–35] In 1998, Williams *et al* first identified CDC6 and MCM5 as potential molecular biomarkers of precursor malignant cells of the cervix.

The aim of our study was to compare and contrast the expression pattern of p16^{INK4A}, MCM5, and CDC6 and to assess their potential as markers of dysplastic squamous and glandular cells of the cervix. Immunohistochemical analysis of MCM5, CDC6, and p16^{INK4A} was carried out using mouse monoclonal antibodies on formalin fixed and paraffin wax embedded samples of normal cervix, cervical intraepithelial neoplasia 1 (CIN1), CIN2, CIN3, cervical glandular intraepithelial neoplasia (cGIN), invasive cervical squamous carcinoma, and adenocarcinoma. The expression of p16^{INK4A}, MCM5, and CDC6 was also analysed on a series of ThinPrep smears exhibiting mild, moderate, and severe dyskaryosis. HPV detection was carried out by means of a modified SYBR green assay system, which uses a ''degenerate'' primer set that detects sequences within the L1 reading frame of at least nine HPV types. Fluorogenic polymerase chain reaction (PCR) and solution phase PCR were used for type specific HPV typing.

MATERIALS AND METHODS

Tissues and cytology samples

Formalin fixed and paraffin wax embedded cervical biopsy samples were selected from the pathology files of the Coombe Women's Hospital, Dublin, Ireland. Haematoxylin and eosin stained slides of all biopsy samples were reviewed by a pathologist and classified according to criteria outlined by the World Health Organisation. Ethical approval for use of all specimens was obtained from the research ethics committee of the Coombe Women's Hospital. Overall, 20 normal cervical biopsies were selected, in addition to 38 CIN1, 33 CIN2, 46 CIN3, 10 invasive squamous cell carcinomas, 19 cGIN, and 10 adenocarcinomas. Because some biopsies could not be tested for all antigens, the numbers in some comparisons are less than the total available biopsies. Cells from the C33A (HPV negative), HeLa (HPV-18 positive), and CaSki (HPV-16 positive) cervical carcinoma cell lines were collected in

Table 2 Correlation between HPV status and MCM5 staining intensity

		MCM5 staining score				
Sample	**HPV status**	**0**	**0a**	**1**	**2**	**3**
Normal (n = 20)	HPV−		18/20 (90%)			
	HPV-6/11		2/20 (10%)			
	HPV-16					
	HPV-18					
	HPV unknown					
% Total			100%			
CIN1 (n = 31)	HPV−	1/31 (3%)		1/31 (3%)	1/31 (3%)	1/31 (3%)
	HPV-6/11				1/31 (3%)	2/23 (6.5%)
	HPV-16			5/31 (16%)	6/31 (19%)	5/31 (17%)
	HPV-18					
	HPV unknown	1/31 (3%)		4/31 (13%)	1/31 (3%)	2/31 (6.5%)
% Total		6%		32%	28%	33%
CIN2 (n = 25)	HPV−			1/25 (4%)	1/25 (4%)	1/25 (4%)
	HPV-6/11				2/25 (8%))	
	HPV-16	1/25 (4%)		8/25 (32%)	5/25 (20%)	3/25 (12%)
	HPV-18					
	HPV unknown			2/25 (8%)	1/25 (4%)	
% Total		4%		44%	36%	16%
CIN3 (n = 38)	HPV−	1/38 (3%)		1/38 (3%)		1/38 (3%)
	HPV-6/11				1/38 (3%)	
	HPV-16			7/38 (17%)	9/38 (24%)	6/38 (15%)
	HPV-18			1/38 (3%)	1/38 (3%)	
	HPV unknown	2/38(5%)		5/38 (13%)	1/38 (3%)	2/38 (5%)
% Total		8%		36%	33%	23%
SCC (n = 8)	HPV−					
	HPV-6/11					
	HPV-16				2/8 (25%)	6/8 (75%)
	HPV-18					
	HPV unknown					
% Total					25%	75%
cGIN (n = 5)	HPV unknown	1/17 (6%)		2/17 (12%)	2/17(12%)	
hcGIN (n = 12)	HPV unknown	2/17 (12%)			2/17 (12%)	8/17 (46%)
% Total		18%		12%	24%	46%
Adenocarcinoma (n = 10)	HPV unknown			1/10 (10%)	4/10 (40%)	5/10 (50%)
% Total				10%	40%	50%

N refers to the number of cases tested. Not all cases included in our study were tested for HPV status. No case was found to be positive for HPV-31 or HPV-33. Staining score: 0, no positive staining of dysplastic cells; 0a, basal layer staining; 1, basal layer staining plus <10% positive staining of dysplastic cells; 2, >10% but <50% positive staining of dysplastic cells; 3, >50% positive staining of dysplastic cells.
cGIN, cervical glandular intraepithelial neoplasia; hcGIN, high grade cervical glandular neoplasia; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; SCC, squamous cell carcinoma.

PreserveCyst liquid preservative and used to prepare ThinPrep slides (Cytyc Corporation, Boxborough, Massachusetts, USA).

HPV detection and typing

After dewaxing, DNA extraction was carried out using a Gentra DNA isolation kit (Puregene, Minneapolis, Minnesota, USA), according to the manufacturer's specified protocol. Absolute quantitation of extracted DNA was carried out using a Taqman real time quantitative PCR assay for β actin (Applied Biosystems, Foster City, California, USA). Broad spectrum HPV detection was then carried out using a modified SYBR green assay system (Applied Biosystems). A general HPV ''degenerate'' primer set (GAP 1 & 2), which detects sequences within the L1 open reading frame of at least HPV types 6, 11, 16, 18, 30, 31, 32, 33, and 39 was used. Fluorogenic PCR was then used for HPV type 16, 18, 31 and 33 detection and typing. Solution phase PCR was used for HPV-6 and HPV-11 typing. Reaction conditions and primer pair and probe sequences have been described previously.[7]

Antibodies

Commercially available mouse monoclonal antibodies for $p16^{INK4A}$ (PharMingen, San Diego, California, USA), MCM5 (Novacastra, Newcastle upon Tyne, UK), and CDC6 (Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA) were used.

Characterisation of $p16^{INK4A}$/MCM/CDC6 antibodies by western blot analysis

CaSki cells were homogenised in RIPA buffer (Santa Cruz Biotechnology). The protein extract was then separated by electrophoresis on a 15% sodium dodecyl sulfate polyacrylamide electrophoresis gel and transferred to a nitrocellulose membrane using transfer buffer containing 25mM Tris/HCl, pH 8.3, 192mM glycine, and 20% methanol. The blot was preblocked with 3% Marvel in TTBS (20mM Tris/HCl, pH 7.5, 0.5M NaCl, 0.05% vol/vol Tween 20). The blot was then incubated with purified mouse antihuman $p16^{INK4A}$, MCM5, or CDC6 monoclonal antibody. After three 10 minute washes in PBST (136mM NaCl, 26mM KCl, 15mM KH_2PO_4, 82mM Na_2HPO_4, 0.05% vol/vol Tween 20) the blot was then incubated in biotinylated universal secondary antibody (Vectastain ABC kit; Vector Laboratories, Burlingame, California, USA) for 30 minutes. This was followed by three washes in PBST and incubation in an avidin–biotin complex (Vectastain ABC kit; Vector Laboratories) for 30 minutes. Immunoreactive bands were detected with diaminobenzidine (Vector Laboratories).

Immunocytochemistry on biopsy samples

Sections (4 μm thick) were cut from formalin fixed and paraffin wax embedded biopsy samples and mounted on 3-aminoproplytriethoxysilane coated glass slides. Sections were dewaxed by passage through xylene and then rehydrated in graded alcohol. Endogenous peroxidase activity

Table 3 Correlation between HPV status and CDC6 staining intensity

		CDC6 staining score				
Sample	**HPV status**	**0**	**0a**	**1**	**2**	**3**
Normal (n = 20)	HPV−	16/20 (80%)	2/20 (10%)			
	HPV-6/11	2/20 (10%)				
	HPV-16					
	HPV-18					
	HPV unknown					
% Total		90%	10%			
CIN1 (n = 32)	HPV−	1/32 (3%)		3/32 (9%)		
	HPV-6/11	2/32 (6%)		2/32 (6%)		
	HPV-16	5/32 (16%)		9/32 (28%)	1/32 (3%)	
	HPV-18					
	HPV unknown	4/32 (13%)		4/32 (13%)	1/32 (3%)	
% Total		38%		56%	6%	
CIN2 (n = 27)	HPV−	2/27 (7%)		1/27 (4%)		
	HPV-6/11			2/27 (7%)		
	HPV-16	7/27 (26%)		8/27 (30%)	3/27 (11%)	
	HPV-18			1/27 (4%)		
	HPV unknown	1/27 (4%)		2/27 (7%)		
% Total		37%		52%	11%	
CIN3 (n = 33)	HPV−	1/33 (3%)				
	HPV-6/11					1/33 (3%)
	HPV-16	3/33 (9%)		16/33 (49%)	1/33 (3%)	1/33 (3%)
	HPV-18			1/33 (3%)		1/33 (3%)
	HPV unknown	4/33 (12%)		4/33 (12%)		
% Total		24%		64%	3%	9%
SCC (n-10)	HPV−					
	HPV-6/11					
	HPV-16				4/8 (50%)	4/8 (50%)
	HPV-18					
	HPV unknown					
% Total					50%	50%
cGIN (n = 6)	HPV unknown	2/14 (14%)		1/14 (7%)	3/14 (215%)	
hcGIN (n = 8)	HPV unknown	1/14 (7%)			3/14 (21.5%	4/14 (29%)
% Total		21%		7%	43%	29%
Adenocarcinoma (n = 10)	HPV unknown	3/10 (30%)		1/10 (10%)	1/10	3/10
% Total		30%		10%	10%	30%

N refers to the number of cases tested. Not all cases included in our study were tested for HPV status. No case was found to be positive for HPV-31 or HPV-33. Staining score: 0, no positive staining of dysplastic cells; 0a, basal layer staining; 1, basal layer staining plus <10% positive staining of dysplastic cells; 2, >10% but <50% positive staining of dysplastic cells; 3, >50% positive staining of dysplastic cells.
cGIN, cervical glandular intraepithelial neoplasia; hcGIN, high grade cervical glandular neoplasia; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; SCC, squamous cell carcinoma.

was blocked by incubating the sections in 0.3% H_2O_2/methanol for 30 minutes. Antigen retrieval was performed in 0.01M citrate buffer (pH 6) using a pressure cooker method. After rinsing sections in Tris buffered saline (pH 7.4) non-specific antibody binding was reduced by incubating the sections in 0.1% bovine serum albumin for 30 minutes. After decanting excess serum, sections were incubated for one hour at room temperature with purified mouse antihuman $p16^{INK4A}$ (1/75 dilution; PharMingen), MCM5 (1/40 dilution; Novacastra), or CDC6 (1/40 dilution; Santa Cruz Biotechnology) monoclonal antibody. After washing thoroughly with Tris buffered saline, the sections were incubated with biotinylated universal secondary antibody (Vectastain ABC kit; Vector Laboratories) for 30 minutes. This was followed by incubation with the avidin–biotin complex (Vectastain; Vector Laboratories) for 30 minutes. Slides were developed with diaminobenzidine (Vector Laboratories) for approximately one minute and counterstained lightly with haematoxylin.

Immunocytochemistry on ThinPrep slides

The procedure for immunocytochemical analysis on ThinPreps™ was identical to the above described procedure for immunohistochemical analysis, except that the dewaxing step in xylene and the antigen retrieval steps were omitted. ThinPreps of CaSki, HeLa, and C33A cells were used as positive controls to evaluate the specificity of each staining run.

Interpretation of $p16^{INK4A}$/CDC6/MCM5 expression in biopsy tissues

All formalin fixed and paraffin wax embedded sections that showed either strong nuclear or cytoplasmic staining were considered positive. A certified pathologist then graded all sections qualitatively according to the following arbitrary scale: 0 (no positive staining of dysplastic cells), 0a (basal layer staining), 1 (basal layer staining plus < 10% positive staining of dysplastic cells), 2 (> 10% but < 50% positive staining of dysplastic cells), and 3 (> 50% positive staining of dysplastic cells).[7]

RESULTS

HPV detection and typing

SYBR green HPV analysis found the CaSki and HeLa cell lines positive for HPV DNA. Further specific HPV typing by fluorogenic PCR found that the CaSki cell line contained HPV-16 DNA sequences, whereas the HeLa cell line contained HPV-18 DNA sequences. The C33A cervical cancer cell line was found to be negative for HPV DNA by SYBR green HPV analysis. All cases positive for HPV using SYBR green PCR were also HPV positive in type specific PCR. Tables 1–3 outline the correlation between HPV status and $p16^{INK4A}$, MCM5, and CDC6 staining intensity.

Characterisation of monoclonal antibodies

Characterisation of mouse monoclonal anti-$p16^{INK4A}$, anti-MCM5, and anti-CDC6 antibodies was carried out by western

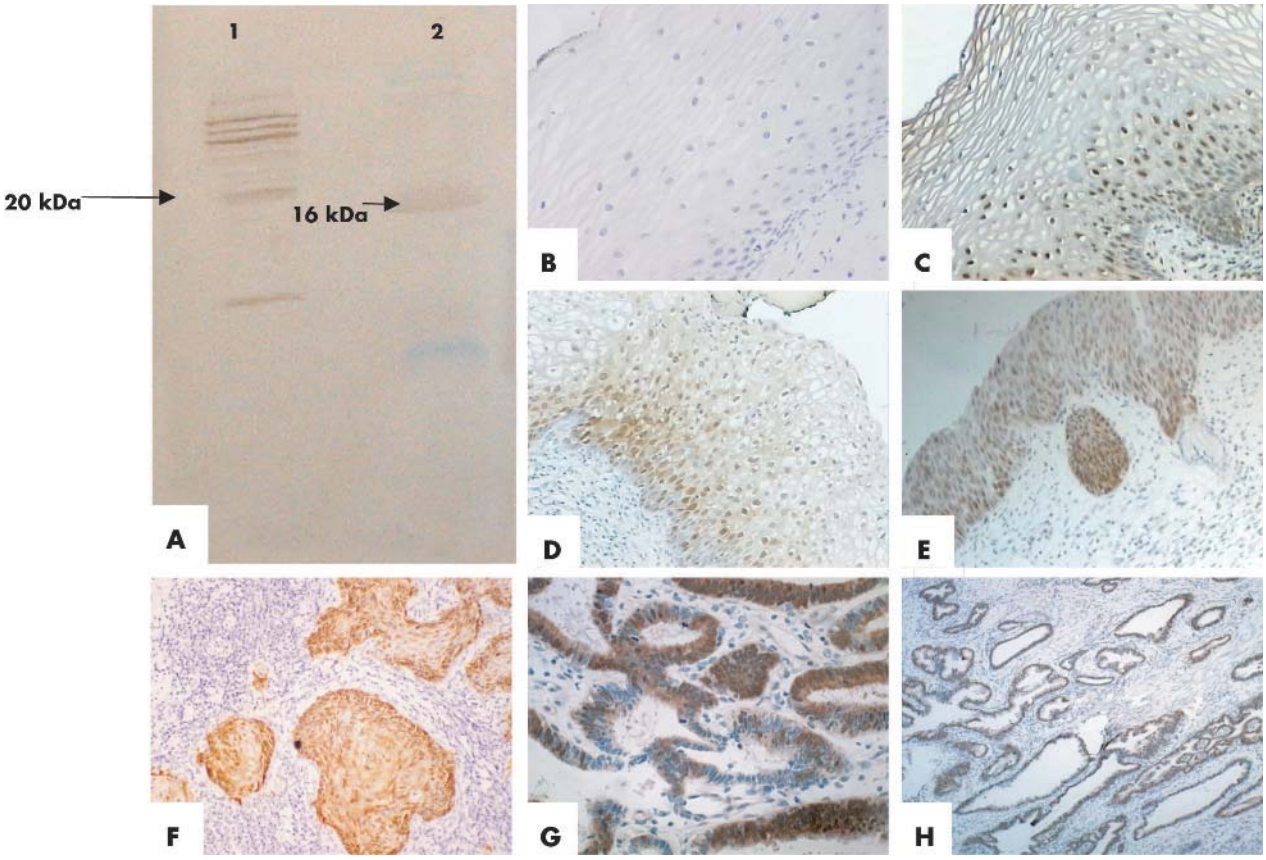

Figure 1 (A) Western blot analysis of $p16^{INK4A}$ protein extracted from CaSki cells using mouse monoclonal anti-$p16^{INK4A}$ antibody: lane 1, molecular weight standards; lane 2, anti-$p16^{INK4A}$. Immunohistochemical staining for $p16^{INK4A}$ in (B) normal cervical epithelium, (C) cervical intraepithelial neoplasia 1 (CIN1), (D) CIN1–2, (E) CIN3, (F) invasive squamous cell carcinoma, (G) cervical glandular intraepithelial neoplasia, and (H) adenocarcinoma.

blot analysis of whole protein extracted from CaSki cells. Western blot analysis revealed that all three antibodies were specific with no non-specific bands present: lane 2 in figs 1A–3A show bands of 16 kDa, 82 kDa, and 62.7 kDa, corresponding to the $p16^{INK4A}$, MCM5, and CDC6 proteins, respectively.

Immunocytochemical staining for $p16^{INK4A}$/MCM5/CDC6 in histologically normal tissue samples

Immunocytochemical analysis using mouse monoclonal anti-$p16^{INK4A}$ antibody was performed on 20 histologically normal cervical biopsies (fig 1B). In all cases normal epithelial, stromal, metaplastic, reactive, and inflammatory cells were not stained. Negative immunostaining with anti-$p16^{INK4A}$ antibody was also seen in glandular endocervical epithelium. In addition, normal areas adjacent to CIN lesions did not express $p16^{INK4A}$. Immunostaining using monoclonal anti-MCM5 antibody produced strong nuclear staining in the basal proliferating layers of the cervical squamous epithelium in all 20 normal biopsies examined. This type of staining of the basal proliferating layer was scored as 0a. Staining of the superficial differentiating layers was absent in all cases examined (fig 2B). A small number of normal biopsies showed occasional staining of inflammatory cells in the cervical stroma and intermittent staining in endocervical glands. Immunostaining using monoclonal anti-CDC6 antibody was negative in 18 of 20 normal cases, with two showing faint nuclear staining of the proliferating basal layer (staining intensity of 0a) (fig 3B). Normal areas adjacent to CIN and cGIN lesions, in addition to metaplastic, stromal, reactive, and inflammatory cells were negative for CDC6 expression.

Immunocytochemical staining for $p16^{INK4A}$ in cervical lesions

Dysplastic epithelial cells showed strong $p16^{INK4A}$ staining in 38 of 38 CIN1, 33 of 33 CIN2, 45 of 46 CIN3, and 10 of 10 invasive squamous cell carcinoma samples. A clear distinction was seen between dysplastic cells and adjacent normal cells. Interestingly, a small number of CIN1 cases showed exclusive nuclear staining, although in the remaining CIN1, CIN2, and CIN3 lesions and in squamous cell carcinomas a combination of nuclear and cytoplasmic staining was seen (fig 1C–F). Simple linear regression analysis revealed a highly significant linear relation (estimated slope, 0.46; $R2 = 0.32$; $p < 0.0001$) between $p16^{INK4A}$ staining and increasing grade of squamous dysplasia. In all 19 cGIN cases examined, either cytoplasmic staining or a combination of cytoplasmic and nuclear staining was seen (fig 1G). None of the cGIN cases showed a nuclear signal only. All 10 adenocarcinomas showed strong nuclear and cytoplasmic staining (fig 1H). In our study, $p16^{INK4A}$ had a median staining grade of 3 in all squamous and glandular dysplastic lesions and in all squamous cell carcinomas and adenocarcinomas examined. A strong correlation between HPV positivity and $p16^{INK4A}$ staining intensity was seen (table 1). $p16^{INK4A}$ positive cases that were positive for high risk HPV types 16 or 18 had a higher grade of staining intensity than HPV negative cases or cases harbouring low risk HPV types 6/11.

Immunocytochemical staining for MCM5 in cervical lesions

Strong nuclear staining for MCM5 was seen in 29 of 31 CIN1, 24 of 25 CIN2, and 35 of 38 CIN3 lesions examined. One

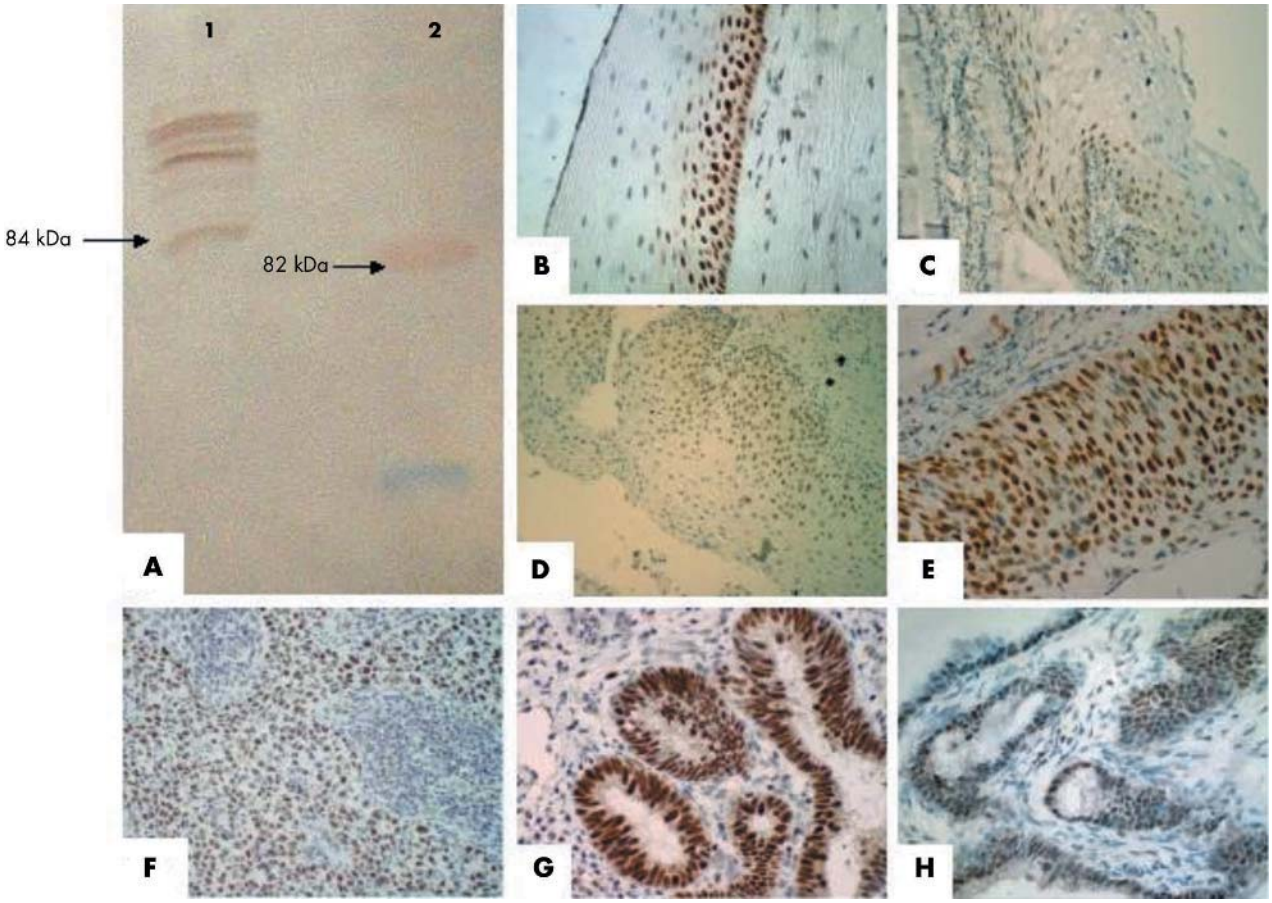

Figure 2 (A) Western blot analysis of MCM5 protein extracted from CaSki cells using mouse monoclonal anti-MCM5 antibody: lane 1, molecular weight standards; lane 2, anti-MCM5. Immunohistochemical staining for MCM5 in (B) normal cervical epithelium, (C) cervical intraepithelial neoplasia 1 (CIN1), (D) CIN2, (E) CIN3, (F) invasive squamous cell carcinoma, (G) cervical glandular intraepithelial neoplasia, and (H) adenocarcinoma.

isolated case showed intermittent staining of endocervical glandular cells. Intermittent staining of stromal cells was seen in a few isolated cases. Figure 2C–E illustrates MCM5 immunostaining in CIN lesions. All 10 squamous cell carcinomas showed intense nuclear staining for MCM5 (fig 2F). Simple linear regression analysis revealed a highly significant linear association between MCM5 staining and grade of squamous dysplasia (estimated slope, 0.59; $R2 = 0.26$; $p < 0.0001$). Strong immunopositivity for the MCM5 protein was also seen in 14 of 17 cGIN cases and all 10 adenocarcinomas. Glandular lesions and adenocarcinomas showed a dominant nuclear staining pattern (fig 2G, H). Cytoplasmic staining was not seen. MCM5 had a median staining grade of 2 in all grades of squamous dysplastic lesions and in glandular dysplastic lesions. In invasive squamous cell carcinoma and adenocarcinoma the median staining grade was 3. There was no apparent correlation between high risk HPV positivity and the grade of MCM5 staining intensity (table 2). Among the CIN1, CIN2, and CIN3 diagnostics groups the grade of staining intensity appeared to be independent of high risk HPV status.

Immunocytochemical staining for CDC6 in cervical lesions

Staining for CDC6 was seen in 20 of 32 CIN1, 17 of 27 CIN2, 25 of 33 CIN3, and eight of eight squamous cell carcinoma cases. A nuclear staining pattern was seen in all dysplastic lesions, with some higher grade lesions showing a faint cytoplasmic blush (fig 3C–E). All eight invasive squamous cell carcinomas examined were positive for CDC6 protein expression. A nuclear staining pattern was seen in squamous cell carcinomas, with some cases exhibiting cytoplasmic staining (fig 3F). Simple linear regression analysis revealed a highly significant linear relation (estimated slope, 0.7; $R2 = 0.2$; $p < 0.0001$) between CDC6 staining and increasing severity of cervical dysplasia. A median staining grade of 1 was seen for all grades of squamous dysplastic lesions, whereas the median staining for invasive squamous carcinomas was 3. Eleven of 14 cGINs showed staining of dysplastic nuclei, with a median staining grade of 2 (fig 3G). Strong nuclear and cytoplasmic staining was seen in seven of 10 adenocarcinoma cases, with a median staining grade of 2 (fig 3H). As previously described by Bonds *et al*, the proportion of cells positive for CDC6 protein expression increased with increasing grade of dysplasia.[10] Within areas of dysplasia, a strong correlation between CDC6 positivity and histological evidence of HPV was noted. Overall, a strong correlation between high risk HPV positivity and CDC6 positivity was seen (table 3). However, as for MCM5, there was no apparent correlation between high risk HPV positivity and the CDC6 staining grade.

Immunocytochemical staining for $p16^{INK4A}$/MCM/CDC6 in cell lines

To check the specificity of the purified mouse monoclonal antihuman $p16^{INK4A}$, MCM5, and CDC6 antibodies, immunostaining was performed in asynchronous CaSki (HPV-16 positive), HeLa (HPV-18 positive), and C33A (HPV negative) cells. The HPV-16 positive CaSki cells and HPV-18 positive HeLa cells showed strong immunopositivity for all three

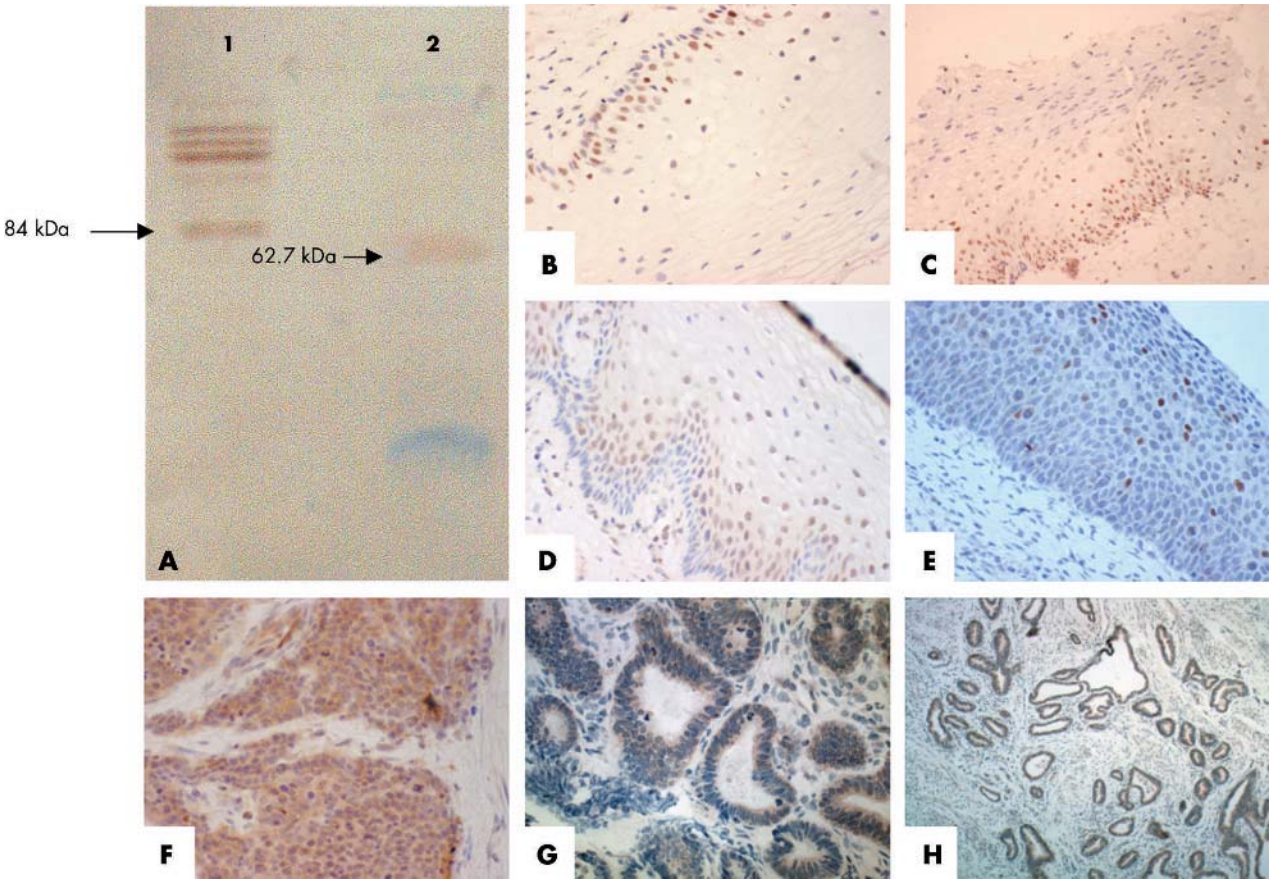

Figure 3 (A) Western blot analysis of CDC6 protein extracted from CaSki cells using mouse monoclonal anti-CDC6 antibody: lane 1, molecular weight standards; lane 2, anti-CDC6. Immunohistochemical staining for CDC6 in (B) normal cervical epithelium, (C) cervical intraepithelial neoplasia 1 (CIN1), (D) CIN1–2, (E) CIN3, (F) invasive squamous cell carcinoma, (G) cervical glandular intraepithelial neoplasia, and (H) adenocarcinoma.

antigens. The HPV negative C33A cell line (ATCC) was also strongly positive for $p16^{INK4A}$, MCM5, and CDC6 protein expression. MCM5 showed an intense nuclear staining pattern in all cell lines, whereas $p16^{INK4A}$ and CDC6 showed a combination of nuclear and cytoplasmic staining. This type of CDC6 nuclear and cytoplasmic expression in cervical carcinoma cell lines is in keeping with previous studies.[11]

Immunocytochemical staining for $p16^{INK4A}$ in ThinPrep smears

Immunocytochemical analysis using monoclonal anti-$p16^{INK4A}$ was carried out on a series of ThinPrep slides. The $p16^{INK4A}$ antibody assay was positive in all five smears showing mild dyskaryosis, six of seven smears showing moderate dyskaryosis and all eight slides showing severe dyskaryosis. The $p16^{INK4A}$ antibody assay was negative in all 12 normal smears examined.

Immunocytochemical staining for MCM5 in ThinPrep smears

The MCM5 antibody assay was positive in one of five smears showing mild dyskaryosis, three of five smears showing moderate dyskaryosis, and four of five smears showing severe dyskaryosis. MCM5 staining was predominantly nuclear. The MCM5 antibody assay was also faintly positive in immature metaplastic cells in one of 10 normal smears examined.

Immunocytochemical staining for CDC6 in ThinPrep smears

CDC6 staining patterns and cellular localisation were examined in a series of ThinPrep slides. The CDC6 antibody assay showed faint nuclear positivity in one of five smears with mild dyskaryosis and in one of five smears with moderate dyskaryosis. In two of five smears showing severe dyskaryosis strong nuclear positivity was seen. One of 10 normal smears examined demonstrated faint staining of immature metaplastic cells.

DISCUSSION

A wide array of potential biomarkers has been evaluated for diagnostic usefulness in the evaluation of cervical cancer and its precursors. Three markers that have shown the greatest potential are the cyclin dependent kinase inhibitor $p16^{INK4A}$[4 7–9 21–23] and the DNA replication licensing proteins MCM5 and CDC6.[10 12 13 28] The aim of our study was to compare and contrast the expression patterns of these three proteins in normal epithelium, dysplastic squamous and glandular lesions, invasive squamous carcinoma, and adenocarcinoma of the cervix and to assess their diagnostic usefulness as biomarkers of cervical dysplasia. We found that all three markers showed a linear correlation between their presence or absence and the grade of dysplasia. However, differences were found between the diagnostic usefulness of all three markers.

Of the three markers assessed, $p16^{INK4A}$ showed the greatest diagnostic utility. Our findings clearly support numerous other reports confirming the hypothesis that $p16^{INK4A}$ is overexpressed in squamous and glandular dysplastic cells of the cervix and is a useful adjunctive test in lesion diagnosis and cervical screening.[4 8 9 21 36] We found a strong correlation between HPV positivity and $p16^{INK4A}$ positivity, although it should be noted that $p16^{INK4A}$

expression was also seen in a limited number of HPV negative cases. These lesions typically showed weaker $p16^{INK4A}$ expression. Milde-Langosch *et al* also reported $p16^{INK4A}$ overexpression in 41% of HPV negative adenocarcinomas.[37] These findings, coupled with the observed upregulated expression of $p16^{INK4A}$ in the HPV negative cell line C33A, could indicate that a non-HPV E7 mediated mechanism of $p16^{INK4A}$ upregulation may also exist.[37] Loss of transcriptional repression in the presence of inactivating mutations in the Rb gene is the most well defined non-HPV related mechanism of $p16^{INK4A}$ upregulation. Indeed, $p16^{INK4A}$ expression may in some cases be independent of pRb. Henshall *et al* suggested that continued proliferation, despite the growth inhibitory effects of increased $p16^{INK4A}$ expression, may be possible when cells also overexpress cyclin E or when $p27^{Kip}$ is unavailable.[38]

Of importance was the identification of an isolated HPV negative CIN3 lesion that was negative for $p16^{INK4A}$ protein expression but positive for both MCM5 and CDC6. One possible explanation for the absence of $p16^{INK4A}$ expression in this lesion could be methylation of the $p16^{INK4A}$ promoter, resulting in silencing of the $p16^{INK4A}$ gene.[39–45] Promoter hypermethylation of $p16^{INK4A}$ may be an alternative pathway resulting in disruption of the Rb–$p16^{INK4A}$ regulatory pathway in HPV independent cervical dyskaryosis. This case demonstrates that, although rare, $p16^{INK4A}$ negative cervical dyskaryosis does occur. This may have important implications for the use of $p16^{INKA}$ staining as a ''stand alone test'', and supports the use of combinations of markers of cervical dyskaryosis.

$p16^{INK4A}$ is strongly expressed in cervical glandular dysplastic cells. However, in addition to recognising cGIN, to be an effective biomarker, it is also essential that $p16^{INK4A}$ should distinguish these lesions from benign glandular mimic lesions, such as tubo-endometrioid metaplasia. The reported presence of $p16^{INK4A}$ expression in tubo-endometrioid metaplasia lesions may limit the use of $p16^{INK4A}$ as a stand alone biomarker for the diagnosis of cervical glandular dysplasia.[46] As was suggested by Cameron *et al*, $p16^{INK4A}$ should be used in combination with other biomarkers.[47]

''Of the three markers assessed, $p16^{INK4A}$ showed the greatest diagnostic utility''

MCM5 showed a strong correlation between its expression and the grade of dysplasia. We also saw very occasional intermittent staining of stromal cells and normal endocervical glands. Superficial differentiating cells were negative in all cases examined. Strong immunopositivity was seen in all invasive squamous carcinoma and in 88 of 94 CIN cases examined. In the glandular lesions examined, 14 of 17 cGIN cases were positive for MCM5 expression. All adenocarcinoma cases examined were positive for MCM5 protein expression. MCM5 also stains exfoliated dysplastic cells within ThinPrep slides and may have potential as a marker of exfoliated cells exhibiting moderate and severe dyskaryosis. Sporadic staining of morphologically normal metaplastic cells was identified. However, these cells can be easily identified by morphological criteria using the Papanicolaou stain. A large scale study of MCM5 staining in ThinPrep smears is currently under way.

Throughout our study, a striking increase in MCM5 protein expression was seen in cells showing histological HPV features. This may be caused by the release of Rb inhibition on E2F via binding of HPV E7 oncoproteins. The transcription factor E2F facilitates increased transcription of MCM5 by binding to MCM5 promoter sites.[48] A high rate of high risk HPV infection was present among all grades of dysplasia in our study. Because the Coombe Women's Hospital is located within a high risk area and is a tertiary referral centre for cervical disease this is not unexpected. Interestingly, however, there did not appear to be a significant relation between the grade of MCM5 staining intensity and HPV status among diagnostic categories. As was suggested by Davidson *et al*, this lack of correlation between HPV positivity and staining intensity indicates that MCM5 protein expression is a measure of proliferation and is independent of the presence of HPV.[29] Indeed, MCM5 upregulation is described in a variety of non HPV related neoplasms, indicating that although in the cervix MCM5 upregulation may be a consequence of HPV infection, it is not solely dependent on it. This highlights the potential of MCM as a biomarker in both HPV dependent and HPV independent cervical dysplasia. This may be particularly relevant in the case of glandular cervical lesions, the pathogenesis of which appears to be less associated with HPV infection.[37]

CDC6 showed a strong correlation between its expression and grade of dysplasia present. In C33A, HeLa, and CaSki cervical carcinoma cell lines, CDC6 showed intense nuclear and cytoplasmic staining, which is in keeping with previous studies.[11] Using immunofluoresent staining, Fujita *et al* demonstrated nuclear and cytoplasmic staining of CDC6 in asynchronous HeLa cervical carcinoma cells. Fujita *et al* reported that approximately 60–70% of cells showed dominant nuclear staining, with some cytoplasmic positivity, whereas remaining interphase cells showed strong cytoplasmic staining.[11] This pattern of staining closely correlates with our own findings for CDC6 expression in HeLa, CaSki, and C33A cells. In our study, a dominant nuclear staining pattern was seen in all dysplastic lesions, with some high grade lesions and squamous cell carcinomas showing cytoplasmic staining. After phosphorylation by cyclin A–cyclin dependent kinase 2, the CDC6 protein is translocated from its chromatin sites to the cytoplasm during the replication phase (S phase) of the cell cycle.[49 50] CDC6 is then degraded by ubiquitin dependant proteolysis by the anaphase promoting complex/cyclosome.[51 52] Relocalisation of CDC6 to the cytoplasm prevents re-initiation of replication and is necessary for coupling S phase with the following mitosis.[53 54] The presence of CDC6 cytoplasmic staining in several high grade lesions and in squamous cell carcinomas is probably the result of the accumulation of CDC6 protein in the cytoplasm after repeated and prolonged S phases in dysplastic cells.

Although CDC6 showed a significant linear association between staining and dysplastic grade, the numbers of CIN1 and to a lesser extent CIN2/CIN3 lesions positive for CDC6 expression were significantly lower than previously described by Williams *et al*, who used an alternative CDC6 polyclonal antibody.[13] However, our results do correlate well with a more recent study published by Bonds *et al*, which used an alternative mouse monoclonal anti-CDC6 antibody (Neomarkers, Freemont, California, USA).[10] Similar to us, they found decreased CDC6 positivity in lower grade lesions. In addition, although the proportion of cells positive for CDC6 expression increased with increasing grade of glandular and squamous dysplasia, the proportion of positive dysplastic cells was lower when compared with $p16^{INK4A}$ and MCM5. However, all squamous cell carcinomas examined showed strong nuclear and some cytoplasmic staining. CDC6 expression was present in 11 of 14 cGIN and seven of 10 adenocarcinoma cases. This correlates closely with the study of Bonds *et al*, in which 11 of 14 adenocarcinoma in situ cases and eight of 10 adenocarcinomas were positive for CDC6 expression.[10] The low or absent expression of CDC6 in low grade lesions may be a function of its role as a G2/M phase checkpoint regulator. Degradation of CDC6 is necessary for entry into M phase of the cell cycle.[55] This may explain why

Take home messages

- We assessed the usefulness of three potential markers—p16^{INK4A} , MCM5, and CDC6—as predictive biomarkers in cervical dysplasia
- p16^{INK4A} expression was closely associated with high risk human papillomavirus (HPV) infection and was the most reliable marker of cervical dysplasia
- MCM5 staining intensity was independent of high risk HPV infection, highlighting its potential as a biomarker in both HPV dependent and independent cervical dysplasia
- CDC6 may be a biomarker of high grade and invasive lesions of the cervix, with limited use in low grade dysplasia
- Combinations of dysplastic biomarkers may be useful in difficult diagnostic cases

CDC6 is either undetectable or expressed at relatively low levels in early neoplastic cells. It is possible that as a consequence of neoplastic progression, higher grade neoplastic cells may find a mechanism to evade CDC6 G2/M phase regulation, and thus continue proliferating in the presence of increased amounts of CDC6 protein. CDC6 immunostaining in ThinPrep smears showed that CDC6 expression is preferentially upregulated in higher grade exfoliated dysplastic cells. These results are in keeping with findings at the histological level.

We found a correlation between CDC6 positivity and high risk HPV positivity. However, as for MCM5, no correlation was seen between high risk HPV positivity and the grade of staining intensity. Similar to Bonds *et al*, we found that CDC6 was preferentially expressed in areas showing histological HPV changes.[10] This may suggest activation of genomic DNA replication processes by HPV associated oncoproteins. Inactivation of Rb by HPV E7 releases the inhibition of E2F and may result in transcriptional upregulation of CDC6. A recent publication by Vaziri *et al* reported that overexpression of CDC6 in combination with Cdt1 promotes re-replication in human cancer cells with inactive p53 but not in cells with functional p53.[56] Re-replication results in genomic instability and DNA damage, which causes DNA damage checkpoint pathways to activate the tumour suppressor protein p53. p53 prevents re-replication by inducing G1 phase cell cycle arrest or alternatively by the induction of apoptosis. High risk HPV E6 oncoprotein targets p53 for proteolytic degradation. Thus, HPV infected cells have developed a strategy that permits continued and prolonged re-replication, despite the presence of DNA damage and overexpression of CDC6 protein. Interestingly, the expression pattern of CDC6 closely mirrors that of the high risk HPV E6 oncoprotein, which is typically strongly expressed in high grade lesions and invasive carcinomas. These results indicate that although CDC6 undoubtedly plays a crucial role in the malignant transformation of cells, its potential role as a biomarker of cervical glandular and squamous dysplastic lesions is limited with respect to lower grade lesions. CDC6 may function as a biomarker of high grade and invasive cervical lesions.

> ''The low or absent expression of CDC6 in low grade lesions may be a function of it role as a G2/M phase checkpoint regulator''

In summary, all three markers evaluated in our study showed a significant relation between antibody staining and grade of dysplasia. Of the three potential biomarkers evaluated, p16^{INK4A} proved to be the most reliable marker of cervical dysplasia. We found that p16^{INK4A} marked all grades of squamous and glandular lesions of the cervix, and its expression was closely associated with high risk HPV infection. However, the failure of p16^{INK4A} to mark an isolated CIN3 case, and its reported staining of glandular mimics such as TEM, may limit its use as a stand alone test of cervical dysplasia. These findings suggest the use of combinations of dysplastic markers in difficult diagnostic cases. We found that MCM5 marked all grades of squamous and glandular dysplastic cells of the cervix, and staining intensity appeared to be independent of high risk HPV infection, highlighting the potential of MCM as a biomarker of both HPV dependent and HPV independent cervical dysplasia. Overexpression of the CDC6 protein was seen in all grades of cervical dysplasia, but it was preferentially expressed in high grade and invasive lesions. Our results indicate that CDC6 might function as a biomarker of high grade and invasive lesions of the cervix. Although our study showed that p16^{INK4A}, MCM5, and CDC6 can identify exfoliated dysplastic cells in cytology samples a larger study will be necessary to identify precisely the role of each marker in primary cervical screening.

ACKNOWLEDGEMENTS

The authors greatly acknowledge the support of the Royal City of Dublin, Hospital Trust, the Irish Cancer Society, and the Health Research Board for funding this study.

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