

# p16<sup>INK4A</sup> as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep<sup>TM</sup> smears

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**Aim:** To examine the potential of p16<sup>INK4A</sup> as a biomarker for dysplastic squamous and glandular cells of the cervix in tissue sections and ThinPrep<sup>TM</sup> smears.

**Methods:** Immunocytochemical analysis of p16<sup>INK4A</sup> expression was performed on 22 normal cervical tissue samples, five cervical glandular intraepithelial neoplasia (cGIN), 38 cervical intraepithelial neoplasia 1 (CIN1), 33 CIN2, 46 CIN3, and 10 invasive cancer cases (eight squamous and two adenocarcinomas). All samples were formalin fixed and paraffin wax embedded, and immunohistochemical analysis was carried out using a mouse monoclonal anti-p16<sup>INK4A</sup> antibody after antigen unmasking. The staining intensity was assessed using a 0 to 3 scoring system. In addition, the expression status of p16<sup>INK4A</sup> was examined in 12 normal ThinPrep smears, one smear exhibiting cGIN, and a total of 20 smears exhibiting mild, moderate, and severe dyskaryosis. Human papillomavirus (HPV) detection was carried out using a modified SYBR green assay system. Fluorogenic polymerase chain reaction (PCR) and solution phase PCR were used for specific HPV typing.

**Results:** p16<sup>INK4A</sup> immunoreactivity was absent in all normal cervical tissues examined. Dysplastic squamous and glandular cells were positive for p16<sup>INK4A</sup> expression in all cases included in this study, except for one CIN3 case. p16<sup>INK4A</sup> expression was mainly nuclear in CIN1 cases, and both nuclear and cytoplasmic in CIN2, CIN3, cGIN, and invasive cases. All cases positive for HPV expressed the p16<sup>INK4A</sup> protein, although not all cases found positive for p16<sup>INK4A</sup> were HPV positive. In general, the p16<sup>INK4A</sup> staining intensity was lower in cases negative for HPV or those containing a low risk HPV type.

**Conclusion:** This pattern of overexpression demonstrates the potential use of p16<sup>INK4A</sup> as a diagnostic marker for cervical squamous and also glandular neoplastic lesions. In addition, the technique can be used to identify individual dyskaryotic cells in ThinPrep smears. Thus, p16<sup>INK4A</sup> is a useful marker of cervical dyskaryosis.

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Cervical cancer is one of the most common forms of cancer in women worldwide. In developing countries, cancer of the uterine cervix is ranked second, with a relative frequency of 15% of all cancers in women, whereas in developed countries cervical cancer is ranked fifth, with a relative frequency of 4.4%.<sup>1</sup> The Papanicolaou (Pap) test, as described by G Papanicolaou, is a cytological staining technique, which allows the identification of asymptomatic women who have preneoplastic lesions or early cancer of the uterine cervix. Although the introduction of mass screening programmes in developed countries has been effective in reducing cervical cancer mortality and morbidity rates, the success of the Pap smear test is limited with respect to sensitivity and specificity. False negative rates for cervical premalignant lesions and cervical cancer lie between 15% and 50% and false positive rates of approximately 30% have been reported.<sup>2</sup> This failure may reflect the subjectivity of cytological diagnosis. In addition, histological analysis of biopsy samples taken from women with abnormal smears can (as with cytology) be affected by interobserver discrepancies.<sup>3</sup> The failure of the Pap test to eradicate this potentially preventable disease outlines the limitations of current screening programmes and emphasises the need for the identification of specific biomarkers for dysplastic epithelial cells of the cervix to aid in primary screening and lesion diagnosis. The use of specific markers of dysplasia of the cervical epithelium in conjunction with current cytological or histological procedures could greatly improve the accuracy, precision, and sensitivity of cervical cancer screening programmes.

"The failure of the Pap test to eradicate this potentially preventable disease outlines the limitations of current screening programmes and emphasises the need for the identification of specific biomarkers for dysplastic epithelial cells of the cervix"

A wide array of immunohistochemical markers have been tested to evaluate their specificity in staining dysplastic cells in either biopsies or cytological smears.<sup>4</sup> Two proteins involved in the regulation of DNA replication, Cdc6 and Mcm5, are specific markers for active replication. Cdc6 and Mcm5 have been shown to mark dysplastic cells, although their clinical usefulness is limited because they are unable to differentiate precisely between proliferating dysplastic cells and normal proliferating cells.<sup>5</sup> The MN antigen, a transmembrane glycoprotein containing a carbo-anhydrase domain, has also been described as a potential diagnostic marker for dysplastic and cancerous cervical epithelial cells. However, immunoreactivity of MN has been seen in normal columnar and squamous cells in immature metaplasia, and several cases with dysplastic lesions were negative for MN immunoreactivity.<sup>6</sup> Thus, the identification of a definitive marker for dysplastic cervical epithelial cells is still required to aid in the future diagnosis,

**Abbreviations:** BSA, bovine serum albumin; cdk, cyclin dependent kinase; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; Pap, Papanicolaou test; PBST, phosphate buffered saline/Tween 20; PCR, polymerase chain reaction; pRb, retinoblastoma protein; TBS, Tris buffered saline

**Table 1** Primers and probes used in human papillomavirus (HPV) detection and typing

HPV	Primer or probe	Sequence
DgHPV	Primer 1 Primer 2	TTT GTT ACT GTG GTA GAT AC GAA AAA TAA ACT GTA AAT CAG C
HPV-16 L1A	U primer 6564 L primer 7012 U probe 6862	CCT TAT TGG TTA CAA CGA GCA C GCG TCC TAA AGG AAA CTG ATA TA CCC CAG GAG GCA CAC TAG AAG AT
HPV-18 L1A	U primer 6548 L primer 6993 U probe 6902	GTT ACA TAA GGC ACA GGG TCA T CGT CCA AGG GGA TAT TGA TC AAA GGA TGC TGC ACC GGC T
HPV-31 L1A	U primer 6490 L primer 6930 U probe 6852	GAT GCA ACG TGC TCA GGG A GCG ACC CAG TGG AAA CTG ATC TA CCC AAA AGC CCA AGG AAG ATC
HPV-33 L1A	U primer 6490 L primer 6964 L probe 6787	GGT TAC TTC CGA ATC TCA GTT ATT T TCC CAA AGG AAA CTG ATC TAA A TGT TAA ACC AAA TTG CCA ATC TTC T
HPV-6B	Primer 1 Primer 2	CCT GTT TCG AGG CGG CTA TCC ATA GTA CAA TTT AGC TTT ATG AAC CGC GCC TTG GTT
HPV-11B	Primer 1 Primer 2	TGT GTG GCG AGA CAA CTT TCC CTT TGG TTA TTT AGT TTT ATG AAG CGT GCC TTT CCC

A primer pairs were designed by Swan *et al.*<sup>18</sup> B primer pairs were designed based on the following GenBank accession numbers: HPV-6, AF12648, X00203.1; HPV-11, NC 001525.1, M14119.1.

prognosis, and treatment of cervical intraepithelial lesions and cervical cancer.

The important role of human papillomaviruses (HPV) infection in cervical carcinogenesis is now well established. Indeed, HPV infection has been detected in almost all preneoplastic and neoplastic lesions of the cervix.<sup>7</sup> HPV is the most diverse group of DNA viruses involved in human disease, with more than 80 different types identified, approximately 30 of which can infect the cervical epithelium and give rise to various lesions of the cervix. HPV subtypes are subdivided into three categories according to risk: high, intermediate, and low. High risk HPV types, in particular HPV types 16 and 18, have been identified in more than 99% of cervical cancers.<sup>8</sup> HPV contributes to neoplastic progression predominantly through the action of two viral oncoproteins, namely E6 and E7, which interact with various cell cycle regulatory proteins.<sup>9</sup>

One such regulatory protein is the retinoblastoma gene product (pRb). pRb is a tumour suppressor, which inhibits the progression of cells into S phase and is regulated via phosphorylation by cyclin D1, complexed with cyclin dependant kinases (cdks).<sup>10</sup> Progressive and prolonged phosphorylation of the Rb protein leads to its inactivation and reduction of its growth suppressive activity.<sup>11</sup> The HPV E7 protein specifically binds to and inactivates pRb. This inactivation is mediated by the release of E2F-like transcription factors from pRb, which allows the activation of cdk and transcriptional activation of target promoters.<sup>12 13</sup>

The CDKN2A gene product, the p16<sup>INK4A</sup> protein, is a tumour suppressor protein that inhibits cdk4 and cdk6, which phosphorylate the Rb protein. A reciprocal relation between p16<sup>INK4A</sup> and pRb expression has been seen, suggesting the presence of a negative feedback loop allowing pRb to limit the concentration of p16<sup>INK4A</sup>.<sup>14 15</sup> p16<sup>INK4A</sup> overexpression has been demonstrated in cervical cancers as a result of functional inactivation of pRb by the HPV E7 protein.<sup>16</sup> This overexpression highlights the possible potential of p16<sup>INK4A</sup> as a marker for cervical intraepithelial lesions and cervical cancer.

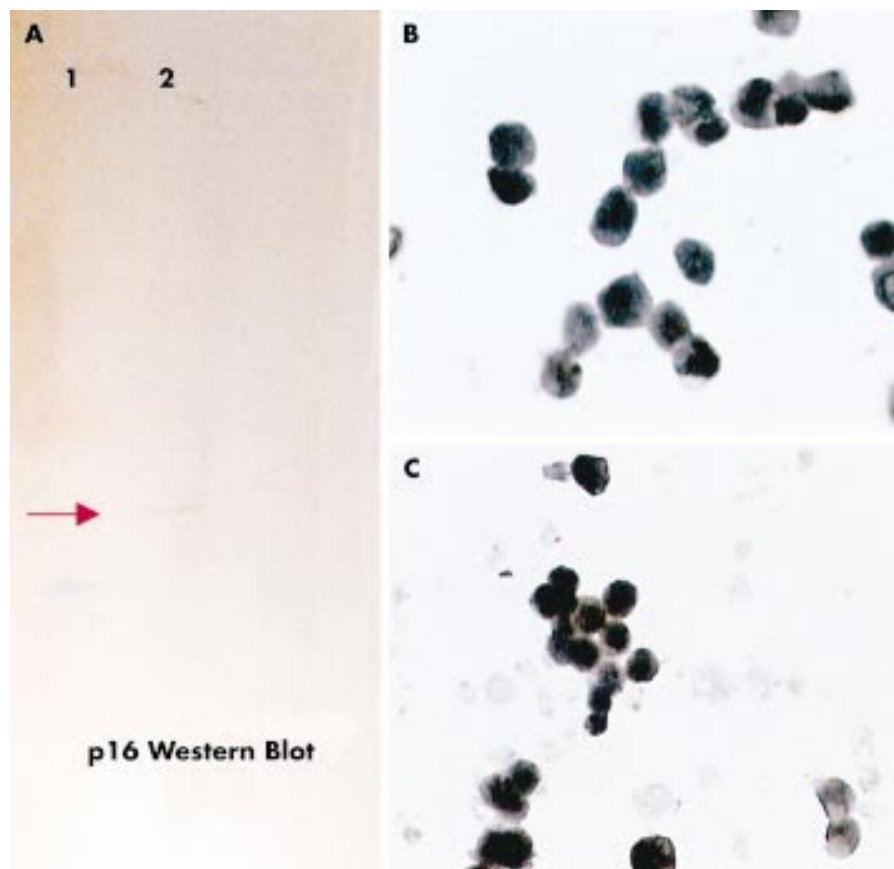
In our study, we examined the potential of p16<sup>INK4A</sup> as a biomarker for dysplastic squamous and glandular cells of the cervix. Immunocytochemical analysis of p16<sup>INK4A</sup> expression was carried out using an anti-p16<sup>INK4A</sup> mouse monoclonal anti-

body on a large number of formalin fixed and paraffin wax embedded samples of normal, cervical intraepithelial neoplasia 1 (CIN1), CIN2, CIN3, cervical glandular intraepithelial neoplasia (cGIN), and invasive cancer cases. The expression status of p16<sup>INK4A</sup> was also examined on several ThinPrep<sup>™</sup> smears exhibiting mild, moderate, and severe dyskaryosis. p16<sup>INK4A</sup> immunoreactivity was absent in all normal cervical tissues examined. Neoplastic squamous and glandular cells were positive for p16<sup>INK4A</sup> expression in all cases included in our study, except for one CIN3 case. This expression was nuclear in a small number of CIN1 cases, although the remaining CIN2, CIN3, cGIN, and invasive cases showed a combination of nuclear and cytoplasmic staining. As previously reported by Klaes *et al.*,<sup>17</sup> our results demonstrate that p16<sup>INK4A</sup> is a reliable and sensitive marker of dysplastic squamous cells of the cervix. Our results also indicate that p16<sup>INK4A</sup> is a sensitive and specific marker of glandular intraepithelial neoplasia and adenocarcinoma of the cervix. In addition, we found that the immunocytochemical assay for p16<sup>INK4A</sup> can be performed successfully on ThinPrep slides. In our study, p16<sup>INK4A</sup> identified exfoliated cells exhibiting CIN and cGIN in ThinPrep smears. No non-specific staining of normal squamous cells was seen in the normal smears examined; however, sporadic staining of inflammatory and metaplastic cells was identified.

## MATERIALS AND METHODS

### Tissues and cytology samples

Cervical biopsy samples were selected from the pathology files of the Coombe Women's Hospital, Dublin. All samples were fixed in formalin and embedded in paraffin wax by conventional techniques. Haematoxylin and eosin stained slides of all samples were reviewed and classified by a certified pathologist. A total of 22 normal cases were selected, in addition to five cGIN, 38 CIN1, 33 CIN2, and 46 CIN3 cases. Eight invasive squamous and two adenocarcinomas were also selected. A total of 12 normal ThinPrep smears, in addition to one smear exhibiting cGIN and a total of 20 smears exhibiting mild, moderate, and severe dyskaryosis, were also included in our study. Cervical samples were collected using the ThinPrep cytology collection system (Cytoc Corporation, Boxborough, Massachusetts, USA), according to the manufacturer's protocol.



**Figure 1** Western blot analysis of p16<sup>INK4A</sup> protein extracted from CaSki cells using mouse monoclonal anti-p16<sup>INK4A</sup> antibody (clone G175-405). (A) Lane 1, molecular weight standards; lane 2, anti-p16<sup>INK4A</sup> clone G175-407. (B,C) Immunocytochemical analysis of cell line cytopins using anti-p16<sup>INK4A</sup> antibody (clone G175-405). Strong immunoreactivity was seen both in (B) CaSki and (C) C33A cervical carcinoma cell lines.

### HPV detection and typing

After dewaxing of paraffin wax embedded sections, DNA extraction was carried out using a Gentra DNA isolation kit (Puregene, Minneapolis, Minnesota, USA), according to the manufacturer's protocol. Absolute measurement of extracted DNA was carried out using a Taqman real time quantitative polymerase chain reaction (PCR) assay for  $\beta$  actin (Applied Biosystems, Foster City, California, USA). HPV detection was then carried out using a modified SYBR green assay system (Applied Biosystems). A general HPV "degenerate" primer set that 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 (table 1). Each 25  $\mu$ l PCR reaction contained 1 $\times$  SYBR buffer, 4.5mM MgCl<sub>2</sub>, 200nM deoxynucleoside triphosphates, 150nM each primer (GAP1 and 2), 1.25 U TaqGold polymerase, and 100 ng template DNA. After denaturation for 10 minutes at 95°C, amplification conditions were as follows: 40 cycles (each) of 15 seconds at 95°C, 30 seconds at 42°C for primer annealing, and 30 seconds extension at 72°C. Amplification was carried out in a 7700 sequence detection system (Applied Biosystems). Interpretation of results was performed using Applied Biosystems' sequence detector software.

Fluorogenic PCR was used for specific HPV typing. Each 50  $\mu$ l PCR reaction contained 10mM Tris (pH 8.3), 50mM KCl, 4.5mM MgCl<sub>2</sub>, 200 $\mu$ M deoxynucleoside triphosphates, 0.3 $\mu$ M each primer, 50nM each fluorogenic probe (probe and primer pair sequences for HPV 16, 18, 31, and 33 are shown in table 1), 0.025 U Taq polymerase/ $\mu$ l, and 10  $\mu$ l of template DNA. After template denaturation for two minutes at 95°C, amplification conditions were as follows: 40 cycles (each) of 30 seconds at 94°C, 10 seconds at 60°C, and two minutes at 65°C.<sup>18</sup> Amplification was carried out in a 7700 sequence detection system (Applied Biosystems). Interpretation of results was performed using Applied Biosystems' sequence detector software.

Solution phase PCR was used for HPV-6 and HPV-11 typing. Each 50  $\mu$ l PCR reaction contained 10mM Tris/HCl (pH 8.3), 50mM KCl, 1.5mM MgCl<sub>2</sub>, 0.01% gelatin, 200 $\mu$ M of each dNTP, 1.0 $\mu$ M of each primer, 2.5 U Taq polymerase, and 5  $\mu$ l of template DNA. Samples were subjected to 40 cycles of PCR in a Perkin Elmer Gene Amp PCR system 2400. Each PCR cycle consisted of one minute at 94°C, two minutes at 55°C, and three minutes at 72°C (extended to 10 minutes in the final cycle).

### Antibodies

Several commercial p16<sup>INK4A</sup> specific monoclonal antibodies are currently available. These include clone DCS-50 (Oncogene, Research Products, Cambridge, Massachusetts, USA), clone ZJ11, and clone JC8 (Neomarkers, Fremont, California, USA). Clone G175-405 (PharMingin, San Diego, California, USA) was chosen for our study based on performance results published by Geradts *et al.*<sup>19</sup>

### Characterisation of p16<sup>INK4A</sup> antibody by western blot analysis

CaSki cells were homogenised in RIPA buffer (Santa Cruz Biotechnology, San Diego, California, USA). The protein extract was then separated by electrophoresis on a 15% sodium dodecyl sulfate polyacrylamide electrophoresis gel and then 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, 0.5 NaCl, and 0.05% vol/vol Tween 20). The blot was then incubated with purified mouse anti-human p16<sup>INK4A</sup> antibody (Clone G175-405, PharMingin). After three 10 minute washes in phosphate buffered saline/Tween 20 (PBST; 136mM NaCl, 26mM KCl, 15mM KH<sub>2</sub>PO<sub>4</sub>, 82mM Na<sub>2</sub>HPO<sub>4</sub>, and 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 diaminobezaminidine (Vector Laboratories) (fig 1A).

### Immunocytochemistry on paraffin wax embedded tissues

Sections (4 µm thick) were cut from formalin fixed, paraffin wax embedded biopsy samples and mounted on saline coated glass slides. Sections were dewaxed by passage through xylene and then rehydrated in graded alcohol. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H<sub>2</sub>O<sub>2</sub>/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 (TBS; pH 7.4) non-specific antibody binding was reduced by incubating the sections in 0.1% bovine serum albumin (BSA) for 30 minutes. After decanting excess serum, sections were incubated for one hour at room temperature with a purified mouse antihuman p16<sup>INK4A</sup> monoclonal antibody (PharMingen, Becton Dickinson) at a 1/75 dilution with 0.1% BSA in TBS). After washing thoroughly with TBS, 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 diaminobezaminidine (Vector Laboratories) for approximately one minute and counterstained lightly with haematoxylin.

### Immunocytochemistry on ThinPrep smears

The procedure for p16<sup>INK4A</sup> immunocytochemical analysis on ThinPreps was identical to the above described procedure for immunohistochemical analysis except that the dewaxing step in xylene was omitted and a 1/100 dilution of purified mouse antihuman p16<sup>INK4A</sup> monoclonal antibody in 0.1% BSA in TBS was used for all ThinPrep samples. ThinPreps of CaSki and C33A cells (ECACC number 87020501) were used as positive controls to evaluate the specificity of each staining run.

### Interpretation of p16<sup>INK4A</sup> expression in biopsy tissues

All formalin fixed, 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), 1 (< 10% positive staining), 2 (> 10% but < 50% positive staining), and 3 (> 50% positive staining).

## RESULTS

### HPV detection and typing

SYBR green HPV analysis found the CaSki cell line positive for HPV. Further specific HPV typing by fluorogenic PCR found the cell line to contain HPV-16 DNA sequences. The C33A cervical cancer cell line was found to be negative for HPV DNA by SYBR green HPV analysis.

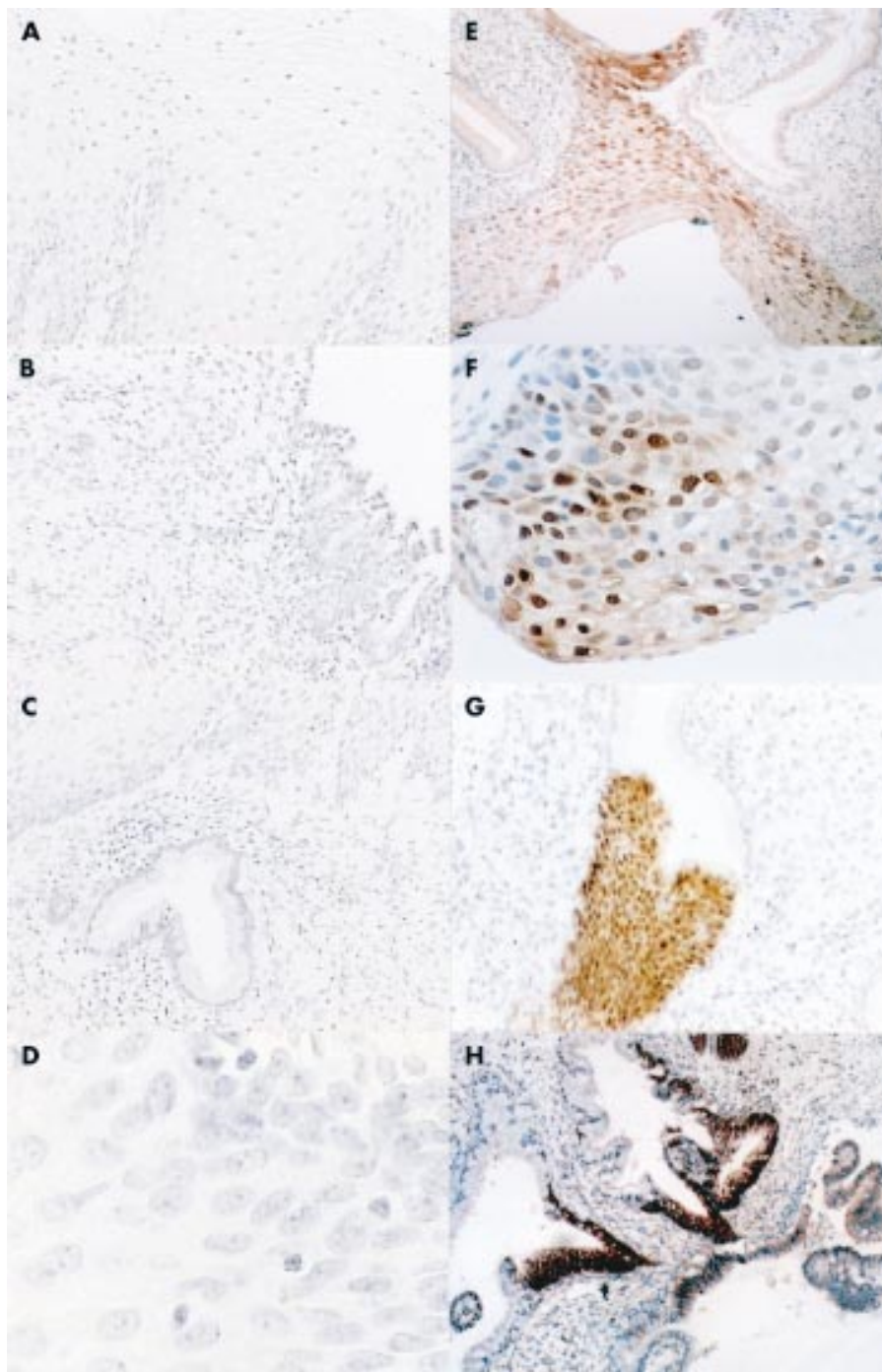
All cases positive for HPV using SYBR green PCR were also HPV positive for type specific PCR. No case included in our study was found to be positive for HPV-31 or HPV-33. This is not unusual in an Irish population. Table 2 shows the results of HPV typing.

**Table 2** Comparison between p16<sup>INK4A</sup> staining intensity and human papillomavirus (HPV) status

	N	P16 <sup>INK4A</sup> staining intensity	HPV -ive	HPV-6/11	HPV-16	HPV-18	HPV-31	HPV-33
Normal	20	0	18/20 (90%)	2/20 (10%)	–	–	–	–
		1	–	–	–	–	–	–
		2	–	–	–	–	–	–
		3	–	–	–	–	–	–
Total			90%	10%				
cGIN	3	0	–	–	–	–	–	–
		1	–	–	–	–	–	–
		2	–	–	–	–	–	–
		3	–	–	3/3 (100%)	–	–	–
Total					100%			
CIN 1	29	0	–	–	–	–	–	–
		1	2/29 (7%)	3/29 (10%)	–	–	–	–
		2	2/29 (7%)	1/29 (4%)	–	–	–	–
		3	–	–	21/29 (72%)	–	–	–
Total			14%	14%	72%			
CIN2	28	0	–	–	–	–	–	–
		1	3/28 (11%)	2/28 (7%)	–	–	–	–
		2	–	–	8/28 (28%)	1/28 (4%)	–	–
		3	–	–	14/28 (50%)	–	–	–
Total			11%	7%	78%	4%		
CIN3	36	0	1/36 (3%)	–	–	–	–	–
		1	1/36 (3%)	1/36 (3%)	–	–	–	–
		2	1/36 (3%)	–	8/36 (22%)	2/36 (5%)	–	–
		3	–	–	20/36 (56%)	2/36 (5%)	–	–
Total			9%	3%	78%	10%		
Invasive	9	0	–	–	–	–	–	–
		1	–	–	–	–	–	–
		2	–	–	–	–	–	–
		3	–	–	9/9 (100%)	–	–	–
Total					100%			

Not all cases included in our study were evaluated for HPV status. Staining intensity: 0, no positive p16<sup>INK4A</sup> staining; 1, <10% positive p16<sup>INK4A</sup> staining; 2, >10% but <50% positive p16<sup>INK4A</sup> staining; 3, >50% positive p16<sup>INK4A</sup> staining.  
CIN, cervical intraepithelial neoplasia; cGIN, cervical glandular intraepithelial neoplasia; N, number of cases tested for HPV.





**Figure 2** Immunohistochemical staining for p16<sup>INK4A</sup> in histologically normal tissue samples. (A) Normal epithelium, (B) squamous metaplasia, (C) normal endocervical gland, and (D) reactive epithelium showing chronic inflammation. Immunohistochemical staining for p16<sup>INK4A</sup> in cervical genital lesions. (E) CIN1, (F) CIN2, (G) CIN3 in an endocervical gland, and (H) cGIN.

#### Immunocytochemical staining for p16<sup>INK4A</sup> in cell lines

To check the specificity of clone G175–405 (PharMingin) we performed immunostaining for p16<sup>INK4A</sup> in CaSki (HPV-16 positive) and C33A (HPV negative) cytopspins. The HPV-16 positive CaSki cells exhibited strong immunopositivity for p16<sup>INK4A</sup> protein. Unexpectedly, the HPV negative C33A cell line (ATCC) was also strongly positive for p16<sup>INK4A</sup> protein expression. Staining was predominantly cytoplasmic in both cell lines (fig 1B,C).

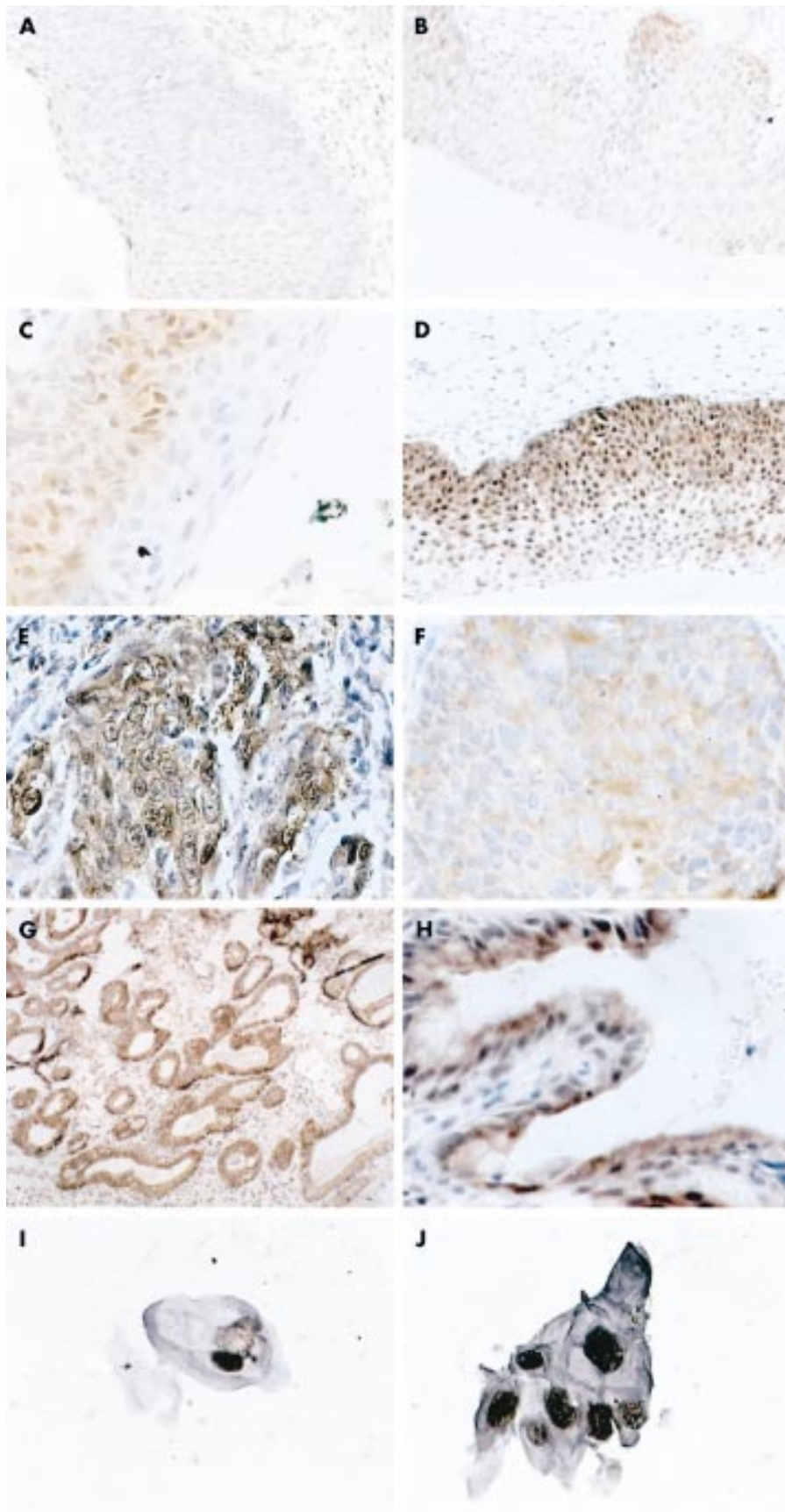
#### Immunocytochemical staining for p16<sup>INK4A</sup> in histologically normal tissue samples

Using a monoclonal antibody to p16<sup>INK4A</sup> (clone G175–405; PharMingin), immunocytochemical analysis was performed in histologically normal tissue samples. In all cases, normal

epithelial, stromal, metaplastic reactive, and inflammatory cells were not stained (fig 2A,B,D). Negative immunostaining with anti-p16<sup>INK4A</sup> was also seen in surface and glandular endocervical epithelium (fig 2C). In addition, normal areas adjacent to CIN lesions showed no detectable p16<sup>INK4A</sup> expression.

#### Immunocytochemical staining for p16<sup>INK4A</sup> in cervical genital lesions

In 117 CIN lesions, five cGIN lesions, and 10 invasive cervical carcinomas (eight squamous and two adenocarcinoma), p16<sup>INK4A</sup> expression was generally very strong in dysplastic epithelial cells. A clear distinction was seen between dysplastic cells and adjacent normal cells. Interestingly, a small number of CIN1 lesions exhibited exclusively nuclear staining,



**Figure 3** Scoring system for p16<sup>INK4A</sup> immunostaining in CIN3 lesions. (A) 0, (B) 1, (C) 2, and (D) 3. Immunohistochemical staining of p16<sup>INK4A</sup> in invasive squamous cell carcinoma; (E) nuclear staining, (F) cytoplasmic staining, (G) adenocarcinoma, and (H) cGIN. Immunocytochemical staining of exfoliated dysplastic cells in ThinPrep smears using p16<sup>INK4A</sup> specific antibody; (I) p16<sup>INK4A</sup> expression in a mild dyskaryotic cell; (J) mild to moderately dyskaryotic cell cluster exhibiting p16<sup>INK4A</sup> immunopositivity.

**Table 3** Results of immunocytochemical analysis of p16<sup>INK4A</sup>

	N	Score			
		0	1	2	3
Normal	21	21 (100%)	0 (0%)	0 (0%)	0 (0%)
cGIN	5	0 (0%)	0 (0%)	1 (20%)	4 (80%)
CIN 1	38	0 (0%)	3 (8%)	11 (29%)	24 (63%)
CIN 2	33	0 (0%)	9 (27%)	9 (27%)	15 (46%)
CIN 3	46	1 (2%)	3 (7%)	14 (30%)	28 (61%)
Invasive	10	0 (0%)	0 (0%)	0 (0%)	10 (100%)

Score: 0, no positive staining; 1, <10% positive staining; 2, >10% but <50% positive staining; 3, >50% positive staining. Examples of 0, 1, 2, and 3 are shown in fig 3A–D.  
CIN, cervical intraepithelial neoplasia; cGIN, cervical glandular intraepithelial neoplasia.

whereas in the remaining cGIN, CIN1, CIN2, and CIN3 lesions a predominantly cytoplasmic pattern of staining was seen (fig 2E–H). All invasive squamous carcinomas and adenocarcinomas exhibited strong nuclear and cytoplasmic staining (fig 3E–G). All lesions included in our study were qualitatively graded according to the following criteria: 0 (no positive staining), 1 (< 10% positive staining), 2 (> 10% but < 50%), 3 (> 50% positive staining) (table 3; fig 3A–D).

#### Immunocytochemical staining for p16<sup>INK4A</sup> in ThinPrep slides

To evaluate the potential of p16<sup>INK4A</sup> as a biomarker for dysplastic exfoliated cells in cytological samples, immunocytochemical analysis using monoclonal anti-p16<sup>INK4A</sup> was carried out on a series of ThinPrep slides. The p16<sup>INK4A</sup> antibody assay was positive in all five smears exhibiting mild dyskaryosis, six of seven smears exhibiting moderate dyskaryosis, and all eight ThinPrep smears showing severe dyskaryosis. Exfoliated cells exhibiting cGIN were also positive for p16<sup>INK4A</sup> expression. The p16<sup>INK4A</sup> antibody assay was negative in all 12 normal smears examined. Figure 3I and J shows the immunocytochemical analysis of the p16<sup>INK4A</sup> antigen on ThinPrep slides. This example demonstrates the ability of p16<sup>INK4A</sup> to identify dysplastic exfoliated epithelial cells. In all ThinPrep slides analysed, no non-specific staining of normal squamous cells was seen, although sporadic staining of morphologically normal metaplastic cells and inflammatory cells was identified (in one case).

#### DISCUSSION

Despite the success of conventional screening programmes questions have arisen concerning the reliability of conventional cervical cytology and histology.<sup>20</sup> These concerns have highlighted the need for improved screening technologies and prompted investigation into the possible usefulness of tumour associated antigen markers as an adjunct to conventional Pap testing. One such potential biomarker is the p16<sup>INK4A</sup> tumour suppressor gene. In our study, we wished to examine the potential usefulness of p16<sup>INK4A</sup> as a diagnostic marker for dysplastic squamous and glandular cells of the cervix.

We have extensively analysed the immunocytochemical distribution of p16<sup>INK4A</sup> in normal and neoplastic tissue of the cervix. In all normal cervical tissues examined all epithelial, metaplastic, endocervical, reactive, and inflammatory regions were not stained with the monoclonal anti-p16<sup>INK4A</sup> antibody. In addition, all normal regions adjacent to CIN lesions showed no detectable p16<sup>INK4A</sup> expression. In 117 cases (including CIN1, CIN2, and CIN3, five cGIN, and 10 invasive cancer cases) all but one CIN3 lesion exhibited overexpression of the p16<sup>INK4A</sup> gene product. Failure of this isolated CIN3 case to express p16<sup>INK4A</sup> could not be explained because this case demonstrated

#### Take home messages

- p16<sup>INK4A</sup> marks dysplastic squamous and glandular cells of the cervix with a sensitivity of 99.9% and a specificity of 100%
- All cases positive for human papillomavirus (HPV) expressed the p16<sup>INK4A</sup> protein, although not all cases found positive for p16<sup>INK4A</sup> were HPV positive. In general, the p16<sup>INK4A</sup> staining intensity was lower in cases negative for HPV or those containing a low risk HPV type
- p16<sup>INK4A</sup> is a reliable marker for dysplastic squamous and glandular cervical cells both in tissue sections and in cervical ThinPreps
- p16<sup>INK4A</sup> immunohistochemical analysis would provide a useful adjunct to conventional screening programmes and would help reduce false positive and false negative results, which could reduce patient anxiety and the overall cost of cervical cancer screening programmes

immunoreactivity for common leucocyte antigen and epithelial membrane antigen. All squamous cell carcinomas and adenocarcinomas examined exhibited strong overexpression of the p16<sup>INK4A</sup> protein. Although a small number of CIN1 cases showed exclusive nuclear staining, interestingly, the remaining CIN1, CIN2, CIN3, and invasive cancer cases showed a combination of nuclear and cytoplasmic staining. p16<sup>INK4A</sup> protein localisation is thought to be nuclear. The presence of p16<sup>INK4A</sup> in the cytoplasm may result from a type of post transcriptional modification or, more simply, overproduction of the protein may force its transfer into the cytoplasm. These findings clearly support previous studies confirming the hypothesis that p16<sup>INK4A</sup> is overexpressed in dysplastic cells of the cervix.<sup>12 13 15</sup>

In our study we performed p16<sup>INK4A</sup> immunohistochemical analysis on a series of ThinPrep slides. Smears categorised as normal, in addition to those showing mild, moderate, and severe dyskaryosis were analysed. We found that the anti-p16<sup>INK4A</sup> monoclonal antibody specifically stains exfoliated dysplastic cells within ThinPrep smears. The p16<sup>INK4A</sup> antibody assay also identified exfoliated cells exhibiting cGIN. Non-specific staining of normal squamous cells was not seen, although sporadic staining of inflammatory cells and morphologically normal metaplastic cells was identified (in one case). We found ThinPrep prepared monolayer slides easily adaptable to the p16<sup>INK4A</sup> immunohistochemical assay. The methanol based fixative used by the ThinPrep system is capable of morphological preservation and maintaining the integrity of cellular protein.<sup>21</sup> ThinPrep smears also have reduced amounts of blood and mucous, which have in conventional smears been reported to reduce immunostaining efficiency.

“We found ThinPrep prepared monolayer slides easily adaptable to the p16<sup>INK4A</sup> immunohistochemical assay”

All cases positive for HPV expressed p16<sup>INK4A</sup> protein, although not all cases found positive for p16<sup>INK4A</sup> protein expression were HPV positive. Table 2 compares the p16<sup>INK4A</sup> staining intensity with HPV type. In general, the p16<sup>INK4A</sup> staining intensity was lower in cases containing a low risk HPV type or those negative for HPV. In addition, the HPV negative cell line C33A is p16<sup>INK4A</sup> positive; this clearly indicates that a non-HPV dependent p16<sup>INK4A</sup> expression pathway may also exist.

In conclusion, p16<sup>INK4A</sup> marks dysplastic squamous and glandular cells of the cervix with a sensitivity of 99.9% and a specificity of 100%. The results of our study clearly indicate that p16<sup>INK4A</sup> is a reliable marker for dysplastic squamous and glandular cervical cells in tissue sections and in cervical ThinPreps. The use of p16<sup>INK4A</sup> immunohistochemical analysis as a complement to conventional screening programmes will aid in



the reduction of false positive and false negative results. This will ultimately result in a reduction in patient anxiety and the overall cost of cervical cancer screening programmes.

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