ORIGINAL ARTICLE

Immunocytochemistry of p16^{INK4a} in liquid-based cervicovaginal specimens with modified Papanicolaou counterstaining

G Negri, G Moretto, E Menia, F Vittadello, A Kasal, C Mian, E Egarter-Vigl

J Clin Pathol 2006;**59**:827–830. doi: 10.1136/jcp.2005.030726

See end of article for authors' affiliations

Correspondence to: G Negri, Department of Pathology, Central Hospital Bolzano, Via Boehler 5, 39100 Bolzano, Italy; ginegri@gmail.com

Accepted for publication 9 August 2005
Published Online First 7 February 2006

Aim: To evaluate the feasibility and value of a modified Papanicolaou counterstain for p16^{INK4a} immunostaining in liquid-based cervicovaginal samples.

Methods: Immunocytochemical analyses were carried out with p16^{INK4a} and modified Papanicolaou counterstain on 81 liquid-based samples, including 23 of within normal limits (WNL), 6 of low-grade squamous intraepithelial lesion (LSIL), 20 of high-grade squamous intraepithelial lesion (HSIL), 16 of atypical squamous cells of undetermined significance (ASC-US) and 16 of atypical squamous cells, high-grade lesion cannot be excluded (ASC-H). Results were compared with histological or cytological follow-up. For comparison, samples from 29 more cases (10 of LSIL, 10 of ASC-H and 9 of HSIL) were immunostained with p16^{INK4a} and conventionally counterstained with haematoxylin. The intensity of immunostaining in cases of squamous intraepithelial lesion (SIL) was assessed using a 0–3 scoring system. Interobserver agreement was calculated by κ statistics.

Results: Expression of p16^{INK4a} was detected in 3 of 23 cases of WNL, 4 of 6 cases of LSIL, all cases of HSIL, 5 of 16 cases of ASC-US and 13 of 16 cases of ASC-H. Excluding two cases with no residual dysplastic cells in the immunocytochemistry, all cases of cervical intraepithelial neoplasia (CIN)2 or CIN3 at follow-up expressed p16^{INK4a} and none of the p16^{INK4a}-negative cases showed a high-grade lesion at follow-up. No evident differences in pattern or intensity of p16^{INK4a} expression were observed between the specimens of the study and control groups. Interobserver agreement was significantly better in the study group than in the group with conventional immunostaining (combined κ 0.773 *v* 0.549; $p<0.05$), and still better, albeit statistically not significant, than with conventional immunostaining and cervical smear test together (combined κ 0.773 *v* 0.642).

Conclusion: Immunocytochemistry with p16^{INK4a} and modified Papanicolaou counterstain may add to the cervicovaginal cytology the full potentiality of p16^{INK4a} without the need of a further slide and the risk of loss of dysplastic cells, yet maintaining the typical morphological features of the smear test.

The value of p16^{INK4a} as a diagnostic marker for cervical dysplasia and carcinomas of the cervix uteri has already been shown. On histological specimens, a diffuse p16^{INK4a} staining is immunohistochemically detected in almost all high-grade precanceroses and carcinomas of squamous and glandular epithelia of the cervix.[1–5] Several studies have already highlighted the possibility of carrying out p16^{INK4a} staining also in liquid-based cytology samples.[6–12] These samples, however, are typically analysed for immunocytochemistry on a subsequent slide that, although prepared from the same sample, always shows cells different from the original slide. As some non-neoplastic epithelia may also be stained with p16^{INK4a},[6 7 12] the interpretation of immunocytochemical results requires a careful evaluation of the morphology of the stained cells. Unfortunately, the usual counterstain with haematoxylin produces a poor chromatic contrast, which may result in difficult evaluation of the cytological features. This may be particularly critical in cases of immature metaplasia or of a cytopathic effect induced by human papillomavirus (HPV), in which the chromatic features of the cytoplasm are often a key to distinguishing dysplastic changes from reactive ones. The interpretation of conventional immunostainings may be particularly troublesome for cytologists who are not used to the evaluation of immunohistochemical stains. This may lead to a poor acceptance of new immunocytochemical techniques. Thus, an alternative counterstain may be useful in improving the interpretation of immunostained specimens. However, immunocytochemical analysis with p16^{INK4a} is a sensitive technique in which pretreatment and post-treatment have critical roles with regard to the final result.

In this study, we evaluated the feasibility and usefulness of a modified Papanicolaou counterstain for p16^{INK4a} immunostaining on liquid-based cervicovaginal cytological samples.

MATERIALS AND METHODS

Immunocytochemical analysis with p16^{INK4a} and modified Papanicolaou counterstaining was carried out on 81 liquid-based cervicovaginal cytological samples, including 23 of within normal limits (WNL), 6 of low-grade squamous intraepithelial lesion (LSIL), 20 of high-grade squamous intraepithelial lesion (HSIL), 16 of atypical squamous cells of undetermined significance (ASC-US) and 16 of atypical squamous cells, high-grade lesion cannot be excluded (ASC-H). For comparison, 29 ThinPrep samples (10 of LSIL, 10 of ASC-H and 9 of HSIL) were stained with p16 and routinely counterstained with haematoxylin. Histological or cytological follow-up was available in all cases.

Abbreviations: ASC-H, atypical squamous cells, high-grade lesion cannot be excluded; ASC-US, atypical squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; SIL, squamous intraepithelial lesion; WNL, within normal limits

All ThinPrep slides for immunocytochemical analysis were processed with the TP 3000 processor (Cytic Corporation, Boxborough, Massachusetts, USA), according to the manufacturer's instructions.

For the immunocytochemical staining, slides were first fixed with Merckofix spray fixative (Merck, Darmstadt, Germany) and then rinsed in 50% ethanol for 30 min. Immunostaining with $p16^{INK4a}$ was carried out using the CINtec $p16^{INK4a}$ Cytology Kit (clone E6H4, Dako Cytomation, Glostrup, Denmark). Antigen retrieval was first carried out for 40 min at 95–99℃ in a water bath. After blocking the endogenous peroxidase activity, the slides were incubated with the primary antibody for 30 min. Secondary antibody and chromogen (diaminobenzidine) were used according to the instructions on the kit. After immunostaining, the slides from the study group were rinsed in distilled water for 90 s and then stained with Harris haematoxylin (Papanicolaou solution 1a, Merck) for 30 s. After further rinsing in distilled water (30 s), a watery solution of 0.05% hydrochloric acid (HCl; 30 s) and again in distilled water (30 s), the slides were dehydrated in ethanol at 80℃ and 90℃, and then stained with Orange II solution (Papanicolaou solution 2b, Merck) for 1 min. After further rinsing twice in ethanol at 95℃ for 30 s, slides were finally stained with polychromatic solution EA50 (Papanicolaou solution 3b, Merck) for 90 s, rinsed in ethanol and xylol, and mounted with entellan new (Merck).

Specimens from the control group were conventionally counterstained with haematoxylin (ChemMate Hematoxylin, Dako Cytomation) for 2 min.

The intensity of immunostaining for cases of SIL was assessed using a 0–3 score system: 0, no stain; 1, weak; 2, moderate; and 3, strong. For assessing interobserver agreement, three cytologists (GM, EM and CM) first received a set containing immunostainings with the modified Papanicolaou counterstain. Each of them independently reviewed and classified the samples according to the Bethesda system. Subsequently, they received the control immunostainings with the conventional haematoxylin counterstain. Finally, interobserver agreement in the control group was also analysed with the conventional immunostaining together with the respective liquid-based cytology. To avoid a statistical bias due to the different number of samples in the two groups, only 6 cases of LSIL, 10 cases of ASC-H and 9 cases of HSIL were randomly selected from each group. Interobserver agreement was assessed by combined κ (Fleiss–Nee–Landis test).[13] The null hypothesis of no difference between the combined κ values of the two groups was tested by a χ^2_1 statistic.[14]

RESULTS

In the study group, $p16^{INK4a}$ expression was observed in all cases of HSIL, 4 of 6 cases of LSIL, 3 of 23 cases of WNL, 5 of 16 cases of ASC-US and 13 of 16 cases of ASC-H. The mean staining intensity was 2.3 and 1.2 for cases of HSIL and LSIL, respectively (table 1).

In samples from the control group that were counterstained conventionally with haematoxylin, $p16^{INK4a}$ was expressed in 7 of 10 cases of LSIL, 9 of 10 cases of ASC-H and 9 of 9 cases of HSIL. The mean staining intensity was 2.1 and 1.2 for cases of HSIL and LSIL, respectively (table 2).

In the study group, all cases of HSIL had a histological follow-up of CIN3 and all cases of LSIL had either a cytological or a histological follow-up of ASC-US (n = 2) or LSIL or CIN1 (n = 4). Follow-up was negative in eight cases of ASC-US, two of which expressed $p16^{INK4a}$. Of the remaining eight cases of ASC-US, three had a CIN3 and five had an LSIL or CIN1 in the follow-up. Overall, two of the cases of CIN3 and three of the cases of LSIL or CIN1 at follow-up were $p16^{INK4a}$ negative. At revision, no residual abnormal cells could be recognised in the two cases of CIN3 with negative $p16^{INK4a}$ immunostaining. Four cases of ASC-H had a histological follow-up of CIN2 or CIN3. All of them

Table 1 Expression of $p16^{INK4a}$ and staining intensity in samples from the study group

Cytological diagnosis	Total cases (n)	$p16^{INK4a}$ positive, n (%)	Mean staining intensity of positive SIL cases
WNL	23	3 (13)	NA
ASC-US	16	5 (31.3)	NA
ASC-H	16	13 (81.3)	NA
LSIL	6	4 (66.6)	1.2
HSIL	20	20 (100)	2.3

ASC-H, abnormal squamous cells, high-grade lesion cannot be excluded; ASC-US, abnormal squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; NA, not assessed; SIL, squamous intraepithelial lesion; WNL, within normal limits.

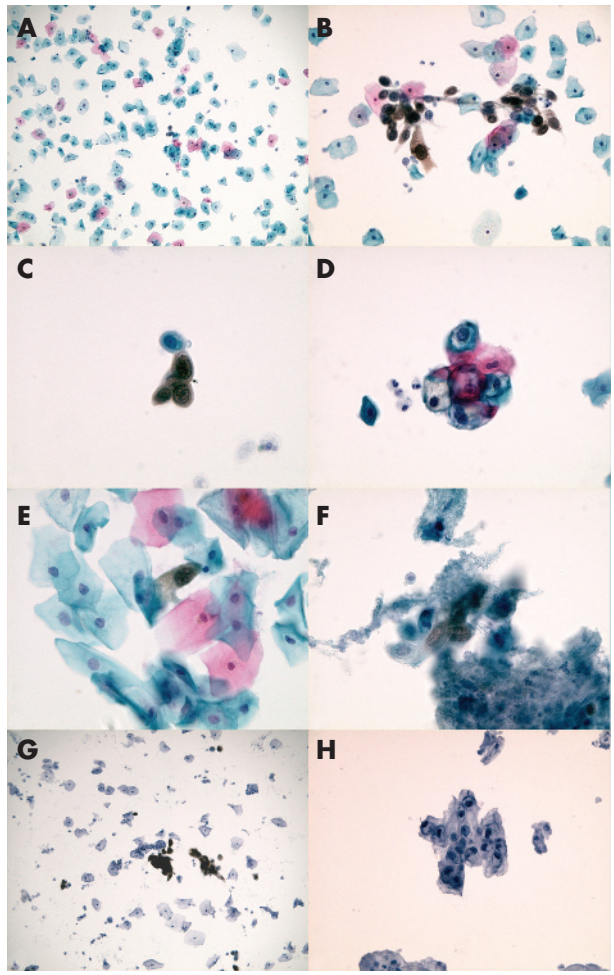

Figure 1 Immunocytochemical analyses with $p16^{INK4a}$ on ThinPrep specimens with the modified Papanicolaou (A–F) and conventional haematoxylin (G,H) counterstain. (A) Low magnification, showing the overall chromatic features of the modified counterstain. (B) High-grade squamous intraepithelial lesion (HSIL). (C) HSIL with one $p16^{INK4a}$negative metaplastic cell. The typical cytoplasmic features in metaplasia are preserved. (D) Low-grade squamous intraepithelial lesion (LSIL), $p16^{INK4a}$negative koilocytes. (E) LSIL. (F) Abnormal squamous cells, high-grade lesion cannot be excluded (ASC-H); this case becomes one of cervical intraepithelial neoplasia (CIN)3 in the follow-up. (G) HSIL, low magnification, showing the overall chromatic features of the conventional counterstain. (H) LSIL, $p16^{INK4a}$negative koilocytes.

Table 2 Expression of p16^{INK4a} and staining intensity in the control group

Cytological diagnosis	Total cases (n)	p16^{INK4a} positive, n (%)	Mean staining intensity of positive cases of SIL
ASC-H	10	9 (90)	NA
LSIL	10	7 (70)	1.2
HSIL	9	9 (100)	2.1

ASC-H, atypical squamous cells, high-grade lesion cannot be excluded; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion, NA, not assessed; SIL, squamous intraepithelial lesion; WNL, within normal limits.

expressed p16^{INK4a}, as well as the four cases with a cytological follow-up of ASC-US or ASC-H. Of the eight cases of ASC-H with negative follow-up, five were p16^{INK4a} positive (table 3).

In both groups, dysplastic cells showed a nuclear or nuclear and cytoplasmic p16^{INK4a} staining (fig 1). Most non-dysplastic cells that expressed p16^{INK4a} were metaplastic or endocervical cells. The overall concordance of study group, conventional immunostaining alone and conventional immunostaining together with the respective liquid-based cytology was 80.0%, 58.6% and 65.5%, respectively. The statistical analysis confirmed that interobserver agreement was significantly better in the study group than in the control group with immunostaining alone (κ 0.77 *v* 0.55; $p = 0.0237$), whereas for conventional immunostaining and the respective liquid-based cytology together, the interobserver agreement showed an intermediate value (κ 0.64, not significant). Concordance was higher in cases of SIL than in those of ASC in all groups (table 4).

DISCUSSION

The low sensitivity of conventional smear test and the frequent occurrence of indeterminate cell changes in cervico-vaginal samples is still an unresolved problem. Markers of dysplasia may therefore be very interesting, potentially permitting an increase in sensitivity and specificity of cytological screening. Among the various proposed ancillary tests, p16^{INK4a} staining is one of the most promising. The specificity of p16^{INK4a} for cervical lesions is a consequence of the oncogenic mechanism of HPV. The oncogenic influence of HPV depends on the interaction of viral gene products, particularly E6 and E7, with specific host proteins. Whereas E6 inactivates p53, E7 binds and thus inactivates the tumour suppressor retinoblastoma protein. As the transcription of the cyclin-dependent kinase inhibitor p16^{INK4a} underlies a negative feedback control by the retinoblastoma protein, its inactivation due to HPV results in an overexpression of p16^{INK4a} in replication-competent epithelial cells.[1] Several studies have already described the possibility of p16^{INK4a} staining in liquid-based cytology samples.[6–12] Although p16^{INK4a} immunocytochemistry is characterised by a high sensitivity for high-grade lesions, some cases of WNL may show a positive staining in non-dysplastic cells.[6 7 12] Thus, the interpretation of immunocytochemical results requires a careful evaluation of the morphology of the stained cells. Unfortunately, the usual counterstain with haematoxylin produces a poor chromatic contrast that may result in difficulty in evaluating the cytological features.

Table 3 Expression of p16^{INK4a} and follow-up in the study group

Cytological diagnosis	Total cases (n)	p16^{INK4a} positive (n)	Follow-up	P16^{INK4a} negative (n)	Follow-up
WNL	23	3	3 WNL*	20	20 WNL*
ASC-US	16	5	2 WNL* 2 LSIL* 1 CIN3	11	5 WNL* 1 LSIL* 1 CIN0 2 CIN1 2 CIN3†
ASC-H	16	13	2 WNL* 1 ASC-US* 3 ASC-H* 3 CIN0 2 CIN2 2 CIN3	3	1 WNL* 2 CIN0
LSIL	6	4	1 ASC-US* 2 LSIL* 1 CIN1	2	1 ASC-US* 1 CIN1
HSIL	20	20	20 CIN3	0	–

ASC-H, abnormal squamous cells, high-grade lesion cannot be excluded; ASC-US, abnormal squamous cells of undetermined significance; CIN, cervical intraepithelial neoplasia; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; WNL, within normal limits.
*Cytological follow-up.
†No residual abnormal cells in the immunocytochemistry samples.

The modified Papanicolaou counterstain that we propose in this study can be helpful in resolving this issue. To enable the detection of even faint immunostains and at the same time warrant good cytological details, we modified the conventional Papanicolaou stain and used it as a counterstain. As shown in fig 1, although the modified counterstain is slightly weaker than a conventional Papanicolaou stain, the usual chromatic pattern is conserved. This is particularly important in cases of immature metaplasia or an HPV-induced cytopathic effect, in which the chromatic features of the cytoplasm are often a key to distinguishing dysplastic changes from reactive ones. Overall, diagnostic concordance was higher in the study group than in the control group, which used conventional counterstaining (80.0% *v* 58.6%), with a significantly higher interobserver agreement in the study group (combined κ 0.773 *v* 0.549; $p<0.05$). Not surprisingly, as the observers share the same extensive experience and common diagnostic criteria with respect to the use of Papanicolaou stains, adding the respective liquid-based cytology to conventional immunostaining partially increased the interobserver agreement. In fact, concordance with conventional immunostaining and the respective liquid-based cytology together showed an intermediate value (κ 0.64) that was still lower than that in the study group, even if not significant.

The relatively low overall concordance may be owing to the high percentage of cases of ASC-H in our study. Indeed, not surprisingly, in each group, concordance was considerably better in cases of SIL than in those of ASC. Furthermore, the three observers who evaluated the slides for the agreement analysis had little experience with immunostainings. An improvement in interpretation performance may occur over time as the cytologists gain more experienced with p16^{INK4a}.

Although, theoretically, a weak cytoplasmic immunostaining may be masked by the modified counterstain, we found no meaningful difference in p16^{INK4a} staining pattern and intensity between study and control groups. The mean staining intensity for cases of HSIL was 2.3 and 2.1 in the study and control groups, respectively, and 1.2 for LSIL in both groups. As in our study the overall p16^{INK4a} results agree with those of previous studies,[6–12] we conclude that the modified Papanicolaou counterstain does not mask or interfere with the p16^{INK4a} immunostaining. All cases of HSIL were stained with p16^{INK4a}, and even those with relatively weak immunocytochemical expression could be readily recognised. Considering cases of HSIL and ASC together, all but two cases of a CIN2 or CIN3 histological follow-up were positive for p16^{INK4a} and only two cases with

Table 4 Analysis of concordance between three observers in the study and control groups

	Modified counterstain			Conventional counterstain			Conventional counterstain and smear test		
Diagnosis	κ*	95% CI	p Value†	κ*	95% CI	p Value†	κ*	95% CI	p Value†
WNL	0.842	0.616 to 1.069	<0.001	0.435	0.225 to 0.645	<0.001	0.284	0.127 to 0.440	0.004
ASC-US	0.306	0.079 to 0.532	0.004	0.174	−0.036 to 0.384	0.052	0.380	0.224 to 0.537	<0.001
ASC-H	−0.042	−0.268 to 0.185	0.641	−0.088	−0.298 to 0.123	0.793	−0.061	−0.218 to 0.096	0.715
LSIL	0.781	0.554 to 1.007	<0.001	0.731	0.521 to 0.942	<0.001	0.839	0.682 to 0.995	<0.001
HSIL	0.945	0.719 to 1.171	<0.001	0.761	0.551 to 0.971	<0.001	0.815	0.658 to 0.971	<0.001
Combined‡	0.773	0.622 to 0.923	<0.001	0.549	0.427 to 0.671	<0.001	0.642	0.544 to 0.739	<0.001

ASC-H, abnormal squamous cells, high-grade lesion cannot be excluded; ASC-US, abnormal squamous cells of undetermined significance; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; WNL, within normal limits.
*Landis–Koch extension.
†p Value for the test z (for $\kappa = 0$).
‡Combined κ (Fleiss–Nee–Landis test).

Take-home messages

- The modified Papanicolaou counterstain improves the interobserver agreement of $p16^{INK4a}$ immunocytochemistry on liquid-based cervicovaginal samples.
- Immunocytochemistry with the modified Papanicolaou counterstain may add the full potentiality of $p16^{INK4a}$ without the need of a second slide and the risk of loss of dysplastic cells, yet maintaining the typical morphological features of the test.

negative $p16^{INK4a}$ expression showed a high-grade lesion in the follow-up. At revision, in the two cases of CIN3 with negative $p16^{INK4a}$ immunostaining, no residual dysplastic cells were present. Because subsequent slides always show cells different from those in the original one, the loss of dysplastic cells in immunocytochemical samples may be possible. In particular, cases with a small number of abnormal cells in the original slide may be at risk of losing diagnostic cells. Combining the Papanicolaou stain with immunocytology may avoid this risk, making a further slide unnecessary. This may be particularly interesting in the follow-up of cases with previous abnormal cytology, in which the high probability of dysplastic cells may make such an alternative approach attractive. Also, the results in cases of LSIL and WNL are in accordance with those from previous studies.[6–12] In the present study, $p16^{INK4a}$ was expressed in 4 of 6 cases of LSIL and 3 of 23 cases of WNL. In cases of LSIL with negative immunostaining, the features of the HPV-induced cytopathic effect were obvious (fig 1D), particularly in comparison with the conventionally counterstained control group (fig 1H). In cases of WNL, the modified counterstain facilitated the differentiation between dysplastic and metaplastic cells, in which the typical features of the cytoplasm were conserved (fig 1C).

In conclusion, the modified Papanicolaou counterstain can help in interpreting $p16^{INK4a}$ immunostainings. This technique may be an alternative approach particularly in high-risk cases, for instance in the follow-up of cases of previous abnormal cytology. Immunocytochemistry with $p16^{INK4a}$ and modified Papanicolaou counterstain can replace the routine smear in these cases, adding the full potentiality of $p16^{INK4a}$ without the need of a second slide or the risk of affecting the morphological features of the sample.

Authors' affiliations
G Negri, G Moretto, E Menia, F Vittadello, A Kasal, C Mian, E Egarter-Vigl, Department of Pathology, Central Hospital Bolzano, Bolzano, Italy

Competing interests: None.

REFERENCES

1 **Klaes R**, Friedrich T, Spitkowsky D, *et al*. Overexpression of p16INK4a as a specific marker for dysplastic and neoplastic epithelial cells of the cervix uteri. *Int J Cancer* 2001;**92**:276–84.
2 **Sano T**, Oyama T, Kashiwabara K, *et al*. Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia. *Pathol Int* 1998;**48**:580–5.
3 **Negri G**, Egarter-Vigl E, Kasal A, *et al*. $p16^{INK4a}$ is a useful marker for the diagnosis of adenocarcinoma of the cervix uteri and its precursors. An immunohistochemical study with immunocytochemical correlations. *Am J Surg Pathol* 2003;**27**:187–93.
4 **Agoff NS**, Lin P, Morihara J, *et al*. $p16^{INK4a}$ expression correlates with degree of cervical neoplasia: a comparison with Ki-67 expression and detection of high-risk HPV types. *Mod Pathol* 2003;**16**:665–73.
5 **Tringler B**, Gup CJ, Singh M, *et al*. Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia. *Hum Pathol* 2004;**35**:689–96.
6 **Bose S**, Evans H, Lantzy L, *et al*. p16(INK4A) is a surrogate biomarker for a subset of human papilloma virus-associated dysplasias of the uterine cervix as determined on the Pap smear. *Diagn Cytopathol* 2005;**32**:21–4.
7 **Yoshida T**, Fukuda T, Sano T, *et al*. Usefulness of liquid-based cytology specimens for the immunocytochemical study of p16 expression and human papillomavirus testing: a comparative study using simultaneously sampled histology materials. *Cancer* 2004;**102**:100–8.
8 **Nieh S**, Chen SF, Chu TY, *et al*. Expression of p16 INK4A in Papanicolaou smears containing atypical squamous cells of undetermined significance from the uterine cervix. *Gynecol Oncol* 2003;**91**:201–8.
9 **Pientong C**, Ekalaksananan T, Swadpanich U, *et al*. Immunocytochemical detection of p16INK4a protein in scraped cervical cells. *Acta Cytol* 2003;**47**:616–23.
10 **Bibbo M**, DeCecco J, Kovatich AJ. P16INK4A as an adjunct test in liquid-based cytology. *Anal Quant Cytol Histol* 2003;**25**:8–11.
11 **Murphy N**, Ring M, Killalea AG, *et al*. p16INK4A as a marker for cervical dyskaryosis: CIN and cGIN in cervical biopsies and ThinPrep smears. *J Clin Pathol* 2003;**56**:56–63.
12 **Trunk MJ**, Dallenbach-Hellweg G, Ridder R, *et al*. Morphologic characteristics of p16INK4a-positive cells in cervical cytology samples. *Acta Cytol* 2004;**48**:771–82.
13 **Fleiss JL**, Nee CM, Landis JR. Large sample variance of kappa in the case of different sets of raters. *Psychol Bull* 1979;**86**:974–7.
14 **Fleiss JL**. *Statistical methods for rates and proportions*, 2nd edn. New York: Wiley, 1981:222.