Immunocytochemistry in liquid-based cervical cytology: analysis of clinical use following a cross-sectional study

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Cytological screening for cervical cancer is hampered by imperfect sensitivity and low inter-observer reproducibility. Human papillomavirus (HPV) testing lacks specificity as a primary screening method. Studies indicate that immunocytochemical detection of alterations caused by HPV in the host cells can optimise screening. Here, the potential of p16INK4a (cyclin-dependent kinase inhibitor p16) and MIB-1 (Ki-67 proliferation marker) as adjunct molecular markers for cervical lesions was investigated in a prospective, cross-sectional study of 500 samples from the framework of opportunistic screening in Flanders, Belgium. A consecutive series of 200 samples and 100 samples from the cytological categories ASC, LSIL and HSIL were investigated. Surepath samples were interpreted according to the Bethesda 2001 reporting system. HPV testing was done with MY09/MY11 consensus PCR. Immunocytochemistry for p16INK4a and MIB-1 was performed with an automated staining protocol. The number of immunoreactive cells/1,000 cervical cells was assessed. There was a higher mean number of p16INK4a and MIB-1 immunoreactive cells (1,000 cells in HSIL, 4.06 ± 1.93 and 11.13 ± 2.83, respectively) compared to other cytological categories. Both markers showed a large spread in counts, for all categories. In cases of HSIL without immunoreactive cells for either marker, low cellularity and long-term storage in water were often the cause of false negativity. This study confirms that positive staining for p16INK4a and MIB-1 is highly correlated with presence of high-grade lesions. These markers could be used as adjuncts to increase the sensitivity of cytological screening as well as the specificity of the HPV test. However, clear methodological standards are needed for optimal performance of immunocytochemistry in a clinical setting.

Key words: cervical cancer; human papillomavirus; molecular markers

The application of screening programmes for cervical cancer has achieved a reduction of 80% in incidence and mortality in some countries. Nevertheless, screening is hampered by the relatively low sensitivity of the cytological Pap smear, which has high false-negative rates and high inter-observer variability. Even after the introduction of liquid-based cytology (LBC), the rate of atypical squamous cells (ASC) of undetermined significance diagnosis has not decreased and screening remains a labour-intensive complex method.

Persistent infection with a genital high-risk human papillomavirus (HR-HPV) type is necessary in the development of dysplasia and cervical cancer.6–3 HPV DNA testing appears very useful in the triage of difficult or unequivocal cases. However, studies could not demonstrate a high level of specificity of HPV DNA testing for clinically significant cervical disease.7–13

A clinically relevant HR-HPV infection is manifested by deregulation of function or expression level of host genes and associated proteins. The viral E6 and E7 oncoproteins bind to host regulatory proteins, which leads to degradation of the p53 tumour suppressor gene product and the inactivation of the retinoblastoma (Rb) gene protein. The subsequent disruptions in the cell cycle are manifested by the abnormal expression of cell cycle-associated proteins like MIB-1 (Ki-67 proliferation marker) and p16INK4a (cyclin-dependent kinase inhibitor p16).14–17 Studies indicate that the detection of these alterations, with the help of molecular markers, can facilitate and optimise screening and diagnosis.18–23 It may be possible to detect clinically important disease with risk of progression towards dysplasia and carcinoma, and consequently, improve patient care by combining test results from molecular markers with either cytology or HPV test or both.14,16

To be of clinical diagnostic relevance, detection techniques for molecular markers and interpretation of results need to be standardised.

In the present prospective study, we investigated the potential of p16INK4a and MIB-1 as adjunct markers for cervical lesions in a screening setting. Results of immunocytochemistry for both markers were correlated with the cytological diagnosis and HPV DNA testing and typing results by PCR. During the course of the study, several important methodological issues surfaced, which have important implications for the application of these markers as well as for the use of markers in cytology, in general. These issues are highlighted and a standardised algorithm for the application of molecular markers is introduced.

Material and methods

Study population

Pap smears, taken by general practitioners and gynaecologists in Flanders, between June 2002 and December 2003, in the framework of opportunistic cervical screening, made up the sample frame for this cross-sectional study.19,20 Reflex testing for HPV DNA is performed by PCR on all cytological abnormal samples. In practice, HPV testing is only performed on negative samples at explicit request from the clinician. The mean age of women in this population is 40.3 years (SD = 13.1) with an age range of 13–95 years. In normal samples, HR-HPV prevalence is 7%.20 The prevalence of cytological abnormalities in Belgium is 1–2% for ASC of undetermined significance, 0.8–1% for low-grade squamous intraepithelial lesions (LSIL) and 0.3–0.5% for high-grade squamous intraepithelial lesions (HSIL).20,24

A prospective series of 500 samples was assembled, consisting of 200 consecutive samples and the first 100 samples in each of the cytological categories ASC, LSIL and HSIL. The entire series was randomised and tested for HR-HPV DNA. The cytological diagnosis as well as HPV testing results were unknown to the investigators of the immunostaining and evaluation. Immunocyto-

Abbreviations: ASC, atypical squamous cells; CIN, cervical intraepithelial neoplasia; DAB, diaminobenzidine; HPV, Human papillomavirus; HR-HPV, high-risk HPV type; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesion; MIB-1, Ki-67 proliferation marker; p16INK4a, cyclin-dependent kinase inhibitor p16; Rb, retinoblastoma gene protein; SEM, standard error of mean.

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chemistry was evaluated by an investigator with neither screening experience nor knowledge about the cytological diagnosis.

All investigations were conducted respecting the advice of the ethical committee of the University of Antwerp. Study-specific patient identification codes were assigned and transmitted in such a way that patient confidentiality was preserved.

Laboratory assays

Cytology. Cervical cells were collected with the Cervex brush (Rovers, Oss, the Netherlands) and placed in preservative fluid (Tripath Imaging, Burlington, NC). LBC preparations were made with the AutoCyte Prep System (Tripath Imaging). From a starting volume of 1,000 μl per sample 200 μl were used to prepare a Pap slide. During this automated process, the initial alcohol-based preservative fluid was replaced by water.

All slides were screened manually by cytotechnologists with extensive training and experience in the evaluation of LBC. Diagnosis was based on the Bethesda 2001 classification system. Five percent of all LBCs, as well as all abnormal and suspicious cases, were reviewed by senior cytotechnologists. Abnormal or equivocal cases were forwarded to a pathologist for final diagnosis.

HPV DNA testing and typing by PCR. Of the remaining 800 μl cell suspension, 400 μl was used for DNA extraction. DNA extracts were stored at −20°C until PCR was performed. Samples were tested with MY09/MY11 consensus PCR primers and type-specific PCR primers for HR-HPV types, according to a previously developed algorithm. Samples that were negative for the type-specific primers but positive for the consensus PCR were classified as unidentified HPV genotypes of unknown malignant potential (HPV X).

The quality of the sample was assessed by testing for β-globin.

Immunocytochemistry for MIB-1 and p16INK4a. Of the remaining 400 μl of cell suspension (after HPV DNA detection), 50 μl was used for each staining. This volume was determined in a pilot study as the lowest volume with which it was possible to prepare an adequate cytospin. Thinnerlayer preparations were made with a cytocentrifuge on poly-L-lysine-coated glass slides and fixed in 50% ethanol for 10 min, followed by epitope retrieval for 40 min at 95–99°C in citrate buffer. After cooling for 20 min at room temperature, preparations were stained with an Autostainer (DakoCytomation, Glostrup, Denmark). Reagents were used from the CInTec and Envision kits (DakoCytomation) according to the manufacturer’s instructions. Stainings were performed at room temperature, with washing in Tris/Tween buffer. The primary murine monoclonal antibody was incubated for 30 min (MIB-1: 1:50 and prediluted anti-p16INK4a clone E6H4). The secondary goat-antimouse antibody was incubated for 30 min. After incubation with diaminobenzidine (DAB) for 10 min, slides were counterstained manually with haematoxylin.

Evaluation of immunocytochemistry was done by light microscopy according to a previously developed protocol based on the Bethesda System 2001 concerning the assessment of LBC cellularity. All cells in 1 high-power field (10× ocular, 40× objective, field diameter 500 μm, viewed surface area = 1/144th of total cell area of slide), which was considered to be representative of the whole cell area of the slide, were counted and the total number of cells on the slide was assessed by multiplication. All positively stained cells in the whole cell area of the slide were counted and the fraction of positive cells on the slide was calculated. This fraction was expressed as the number of immunopositive cells/1,000 cells so as to compare results of all samples. Samples were excluded when the number of cells on the slide was less than 1 per high-power field (400×).

In MIB-1 immunostaining, cells were considered positive if the nucleus showed homogenous or punctate staining. In p16INK4a immunostaining, cells were considered positive if both nucleus and cytoplasm were stained. Cytoplasmatic staining without nuclear staining was not considered positive in either staining, nor was staining intensity graded. In both stainings, enlarged naked nuclei that stained positive were included in the count. Immunopositive staining of inflammatory cells, mucus or bacilli was detected occasionally but not included in the count.

Statistical analysis

After data collection was completed, blinded data were disclosed to the evaluators. All analyses were performed with Prism 4 for Mac OS X (GraphPad Software, San Diego, CA). A Kruskal–Wallis test, followed by a Dunn’s multiple comparison posttest was used, with a p-value ≤ 0.05 considered to be statistically significant. Results were expressed as the mean number of immunopositive cells/1,000 cells ± standard error of mean (SEM).

Results

Description of study population

The consecutive series of 200 samples consisted of 189 negatives, 3 ASC, 5 LSIL and 3 HSIL. After follow-up of the 300 abnormal samples, 2 samples were reclassified from ASC to LSIL (Table I). In cytologically negative samples, HR-HPV was present in 6.35%. From all HSILs, 1 sample was HPV negative and HPV X was found in 3 others. After evaluation for immunocytochemistry, 13 samples were excluded. In 12 cases, low cellularity and weak counterstaining prevented an appropriate evaluation of both the MIB-1 and the p16INK4a stainings. In 1 case, the slide for MIB-1 staining broke, but the p16INK4a slide could be evaluated.

Immunocytochemical staining

In MIB-1 immunostaining, positive cells showed a dark brown staining limited exclusively to the nucleus (Fig. 1a). MIB1-immunopositive cells could be identified as (para-)basal cells, but there was also staining of intermediate or superficial cells. In p16INK4a immunostaining, positive cells showed brown cytoplasm with a

<table>
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<th>TABLE I – STUDY POPULATION ACCORDING TO CYTOLOGICAL DIAGNOSIS BASED ON THE BETHESDA SYSTEM 2001</th>
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<tbody>
<tr>
<td>Consec: negative</td>
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<tr>
<td>HR-HPV (%)</td>
</tr>
<tr>
<td>Consec: negative</td>
</tr>
<tr>
<td>ASC</td>
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<td>LSIL</td>
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<td>HSIL</td>
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<td>Total</td>
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The consecutive series is shown in the upper part. For each category, the numbers of HR-HPV infections and HPV negative samples are given. The mean number of p16INK4a and MIB-1 immunopositive cells per 1,000 cervical cells is given for the 4 main cytological groups (mean ± SEM). The HR-HPV and % HPV neg. is calculated against the total number of samples in that category.
evaluation, there was a large spread in the number of immunoreactive cells for both markers, with considerable overlap between the different groups.

**Evaluation of immunocytochemistry protocol**

From a total of 103 HSIL cases, 38 were biopsy-proven CIN2+ (CIN, cervical intraepithelial neoplasia; Table II). Only 1 sample was HPV negative. 22 HSIL cases did not show staining for either p16INK4a or MIB-1, 8 of which contained CIN2 or more on biopsy. When comparing the cellularity of the cytological slides used in the immunocytochemical assays however, this group of false-negative HSIL had a significantly lower number of cells (3,305 ± 551, Fig. 5) than the HSIL cases with positive immunostaining (7,457 ± 851). Cellularity of the false-negative HSILs was increased in subsequent experiments by doubling the amount of sample used to prepare a cytopsin (100 μl). These samples showed immunopositivity in 10 out of 22.

For 13 false-negative HSIL cases, the original vials were still available, in which cells are kept in preservative fluid (instead of water). In only 1 sample, false-negativity was still observed when immunostaining was performed using cells from the original vial. In this sample, there was a low cellularity.

**Discussion**

This cross-sectional study confirms, that the molecular markers p16INK4a and MIB-1 can be used as adjuncts to LBC to identify high-grade lesions in a screening setting for cervical cancer. p16INK4a can also act either as a complement or as a surrogate marker for HR-HPV infections. Due to the large spread in the number of immunoreactive cells, there is considerable overlap between categories. The importance of a standardised protocol for the application of molecular markers in cervical cytology is highlighted.

In the selection of the study population, the consideration to emulate the screening process was important. There was a very high degree of automation and standardisation, of all techniques used. All tests were performed on the same sample so as to avoid split sample bias. Immunocytochemistry is a fast, simple and relatively inexpensive technique, which retains much of the morphological information available, facilitating integration into the final pathological diagnosis and keeping the budget low.

We used 2 markers to increase sensitivity and specificity. In the case of MIB-1, part of the spread and overlap in different categories is caused by the fraction of normal proliferating cells. This cell fraction should not be stained by p16INK4a. It is also known that staining for p16INK4a can be present in metaplasia, which will not be seen in MIB-1 staining.

There was no routine biopsy follow-up in this study. In an opportunistic screening setting this is very difficult to organise and we could not justify taking biopsies from women without cytological lesions. Furthermore, due to sampling bias, biopsy diagnosis is not always a reliable golden standard. As in previous studies, immunocytochemistry was compared with both cytology and HPV testing in a screening setting, with the conventionally available material.

To our knowledge, this is the largest study up to date in which 2 molecular markers were evaluated in parallel and combined with both cytology as well as HPV DNA testing. Most studies concern histology without inclusion of any screening material or consist of a rather smaller collection of cytological samples. Some of the latter do not provide HPV testing data or are split sample studies.

Staining intensity was not graded in immunocytochemistry, since this would introduce subjective findings. The evaluation simply yields the number of immunopositive cells. We feel that till now there are no clear-cut arguments for establishing threshold values above which a sample becomes "positive." Integration of immunocytochemistry results into a larger evaluation is necessary to establish the final diagnosis.
The 2 investigated markers may be of great help in identifying clinically important HR-HPV infections and cervical lesions. The ideal molecular marker is a direct surrogate marker of the oncogenes of HR-HPV and its degree of distribution in the different grades of SIL should reflect the degree of disruption of the cell cycle as caused by HPV. However, in practice, expression of molecular markers is also modified by other factors. Abundance, expression, temporal and spatial distributions vary in normal, dysplastic and reactive epithelium. Furthermore, the presence of a HR-HPV type does not by itself represent dysregulation...
lation of the cell cycle. Consequently, molecular markers tied to the cell cycle will not be dysregulated either, which may indicate that the HR-HPV infection is of no clinical importance. Not only the HR-HPV type itself, but also integration of the viral genome, viral load, immunological and hormonal factors play an important part.\textsuperscript{14,16}

Despite the effort to attain a high level of standardisation, variability in the time interval between the collection of the sample and processing in the lab for each of the 3 tests may occur. This is a matter of quality control. The process of rendering a cytological slide with LBC has been standardised and almost fully automated. However, there are significant differences between LBC systems currently available. Contrary to the use of ethanol-based preservative fluid in the present study, a methanol-based preservative fluid is used in another system. This causes a significantly different treatment of samples for immunocytochemistry. Furthermore, the system used in this study replaces the preservative fluid with water, while preparing a cytological slide. When surplus of the samples are kept in storage for a longer period of time, this replacement may influence the reactivity of the samples for some molecular markers. In this study, immunocytochemistry tests were repeated for false-negative HSIL cases on cells from the original vials containing preservative fluid, with positive results in 12 out of 13 cases. A solution to this problem would be to manually substitute preservative fluid for the added water. This can be done in a clinical setting, but is more expensive, and as yet, not a standard practice. Another option may be the use of other fixatives, such as acetone or formaldehyde. These, however, are not routinely used in LBC systems, and little information on their use in this setting is available. In a prior study concerning p16\textsuperscript{INK4a}, cold acetone was used in an extra fixation step in the immunocytochemistry protocol.\textsuperscript{19} However, prior to immunocytochemistry, these samples were also kept in preservative fluid (followed by water storage). Usage of different fixatives would therefore introduce an important nonstandardised stage in an otherwise highly standardised clinical method, which is not warranted at this stage.

Out of 103 HSIL cases, 22 did not show any staining for either MIB-1 or p16\textsuperscript{INK4a}. This phenomenon was in profound disagreement with current theoretical assumptions and experimental data.\textsuperscript{14,16,17} It is reasonable to assume that diagnostically important cells did not end up on the slides, because of low cellularity. The same problem was encountered in a large prospective study concerning the use of telomerase in the detection of cervical dysplasia and cancer.\textsuperscript{25} In our study, 12 samples had to be excluded because of low cellularity. The causes of low cellularity may vary and are linked to several steps in the retrieval and processing of the sample. It may be that the initial sample received by the laboratory did not contain enough cells (specimen acquisition), or it may be that cells are lost during processing in the laboratory (laboratory failure).

It becomes apparent that clear methodological standards have to be set for the various steps in the screening protocol, for immunocytochemistry to perform at its best. LBC would be well suited for a multitest protocol in which many steps could be automated. In this study, cytospins were used to prepare slides for immunocytochemistry, in order to expend as little of each sample as possible, making the sample last for several tests. In a clinical setting, with the application of a limited number of tests, 1 or more slides could be prepared at once for Pap staining and immunocytochemistry by the same LBC system. This will consume more of a sample and may be more expensive, but it would also be standardised. The surplus of the sample could then be used for HPV testing.

\begin{table}
\centering
\caption{Follow-up data from HSIL samples}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
 & n & HR-HPV & Biopsy follow-up & Confirmed \(\geq\)CIN2 & Lost to follow-up \\
\hline
All HSIL & 103 & 99 & 52 & 38 & 51 \\
HSIL false negatives & 22 & 21 & 13 & 8 & 9 \\
\hline
\end{tabular}
\end{table}
Quality control at various levels needs to be impeccable. To yield a useful clinical diagnosis, all morphological information may be integrated. This will require a standardised evaluation method for both cytological specimen as well as immunocytochemistry in which thresholds and definitions are stated for positivity and negativity of the test. At present, these definitions vary according to different published studies. This hampers implementation of immunocytochemistry for molecular markers in a clinical setting. Standardised evaluation will also facilitate automated screening.

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