Biological behavior of CIN lesions is predictable by multiple parameter logistic regression models

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Objectives: Progression and regression of premalignant cervical lesions cannot be predicted using conventional cytomorphological or histomorphological parameters. However, markers such as human papillomavirus (hr-HPV) or makers indicating proliferation, genetic instability and chromosomal aberration may be of predictive value assessing short-term biological behavior of cervical intraepithelial neoplasia. In this paper, we have studied the usage of logistic regression models with Ki-67 labeling index (LI), chromosome index for chromosome 1 (CI#1) and aneusomy for chromosome 1 in cervical smears to predict progressive and regressive behavior of premalignant cervical lesions. Methods: Retrospectively, the intake smears of 42 women showing regression in follow-up and of 31 women showing progression in follow-up were assessed. Results: A multiparameter logistic regression model containing the parameters Ki-67 LI, CI#1 and the fraction of cells with four copies of chromosome 1 per nucleus appeared to be the best predicting model, overall correct classification of 93.2% (area under the receiver operating characteristic curve 0.96 ± 0.02). After cross-validation, the model correctly classified 66 of 73 samples (90.4%). Moreover, the model predicted biological behavior perfectly assessing the smear taken subsequently to the intake smear of 46 women. Conclusion: Although measuring parameters indicating proliferation and chromosome 1 aberration is laborious, this study demonstrates that short-term progressive and regressive behavior is highly predictable using a model combing these parameters. We also showed that in the triage management of high-risk human papillomavirus-positive women with minimally abnormal smears applying a model such can be useful.

Introduction

It is generally accepted that cervical intraepithelial neoplasia (CIN) precedes squamous cell carcinoma of the uterine cervix (1). According to the classification of the International Society of Gynecological Pathologists, these premalignant CIN lesions are classified solely by histomorphological criteria, i.e. nuclear atypia, presence, frequency and localization of mitotic figures and the loss of polarity of the nuclei. CIN is hereby subdivided into low-grade CIN 1 (mild dysplasia), CIN 2 (moderate dysplasia) and CIN 3 (severe dysplasia and carcinoma in situ).

Abbreviations: ASC-US, atypical squamous cells of undetermined significance; AUC, area under the receiver operating characteristic curve; BMD, borderline and mild dysplasia; CI, chromosome index; CI#1, chromosome index for chromosome 1; CIN, cervical intraepithelial neoplasia; HPV, human papillomavirus; hr-HPV, high-risk human papillomavirus; HSIL-MD, high-grade squamous intraepithelial lesion of the moderate dysplasia type; LI, labeling index; LLETZ, large-loop excision of the transformation zone of the cervix; LSIL, low-grade squamous intraepithelial lesion; PBS, phosphate-buffered saline; PC, polymerase chain reaction; SSC, standard saline citrate.

High-grade CIN (CIN 2 and 3) can be successfully treated by large-loop excision of the transformation zone of the cervix (LLETZ) (2–4). Unfortunately, substantial overtreatment of low-grade CIN is the penance for the accessibility of this procedure. For achieving adequate and well-deliberate treatment, knowledge on the progressive and regressive behavior of squamous intraepithelial cervical lesions is compulsory. Östör (5) showed in an extensive literature review that CIN 1 will progress to CIN 3 in only 10% of the cases and to invasive cancer in 1%. CIN 3 lesions on the other hand will advance to invasive carcinomas in 12%, whereas spontaneous regression will occur in 33% (6). However, ambiguity persists about the exact timescale of progression and regression of ‘any grade’ cervical intraepithelial lesion. Moreover, neither cytomorphological nor histomorphological markers of cervical intraepithelial abnormalities can predict the biological behavior of CIN.

Genital infection with high-risk human papillomavirus (hr-HPV) is considered the most important factor in the carcinogenesis of cervical cancer (7). This significance is verified by the observations that almost all cervical cancers harbor hr-HPV genotypes (8). Moreover, 74% of CIN 1 and ~84% of high-grade CIN lesions harbor high-risk genotypes (9). The key event of human papillomavirus (HPV)-induced oncogenesis is the integration of viral DNA in the human genome (10). Frequent, hr-HPV16 DNA integrates near common fragile sites of the host genome (11), and this is believed to occur somewhere in the gradual process of progression of a CIN lesion. Integration results in the disruption of the viral E2 region, inducing an over-expression of viral E6 and E7 oncoproteins. Successively, these oncoproteins interfere with two crucial mitosis-regulating pathways of the host cell, the p53 pathway and pRb pathway, respectively. Normally, the p53 pathway induces growth arrest or apoptosis, whereas pRb regulates the passage through the cell cycle. Accordingly, E6/E7-induced inactivation of these pathways results in hyperproliferation (E7 related) and genetic instability, numerical and structural chromosome aberrations and immortalization (all E6 related) [reviewed by Zur Hausen (12)].

Structural aberrations as deletions, translocations and inversions in cervical carcinomas have been reported in chromosomes 1, 3, 6, 9, 11 and 17 (13). E6-induced numerical aberrations of chromosome 1 have been described in 90% of the cervical cancers (14). Moreover, chromosome index (CI)—defined as the mean number of chromosome copies per nucleus—for chromosome 1 shows a significant positive correlation with CIN grading (15,16).

Monoclonal antibody MIB1 recognizes the proliferation-associated Ki-67 antigen (17). The Ki-67 labeling index (LI) (percentage of MIB1-positive cells) has been propound as a promising alternative method for classification of CIN lesions (18–20).

As opposed to cytomorphological or histomorphological markers of a CIN lesion, the parameters related to aberrations of chromosome 1 and those related to cell proliferation are suggested to have predictive value regarding the natural behavior of CIN. The present study was designed to elucidate whether univariate and multivariate linear logistic regression models using Ki-67 LI, chromosome index for chromosome 1 (CI#1) and aneusomy for chromosome 1 assessed in cervical smears were able to predict progressive and regressive behavior of CIN lesions. According to previous studies, only women positive for hr-HPV are at risk for the progression of a CIN lesion and should thus be followed more closely; hence, the results of the predictors were assessed considering hr-HPV-positive samples cytologically indicating atypical squamous cells of undetermined significance (ASC-US) or low-grade squamous intraepithelial lesion (LSIL) [also known as ‘borderline and mild dysplasia (BMD) smears’].

Materials and methods

From a cohort of 800 women, all women referred to the Department of Obstetrics and Gynecology of the Radboud University Nijmegen Medical Centre
having two consecutive smears indicating ASC-US or a LSIL or one smear indicating high-grade squamous intraepithelial lesion of the moderate dysplasia type (HSIL-MD) were eligible for this study. All patients underwent colposcopy within 1 month of the intake. Preceding the colposcopy procedure, a new cervical smear (initial smear) was obtained using the Cervex-Brush® (Rovers Medical Devices B.V., Oss, The Netherlands). The remnants of the cervical smear were fixed with Unifix® and processed into AgarCyto blocks as described previously (21), allowing for multiple analysis. In case colposcopy was suggestive for high-grade CIN, immediate excision of the transformation zone (LLETZ) would follow as described previously (2). These patients were not included in the present study. All referred patients whose colposcopy did not indicate a high-grade CIN were followed using cervical cytology.

In case two consecutive follow-up smears would indicate normal cytology or two grades less severe than the initial smear, the lesions were considered non-progressive or regressive. Women with an initial smear diagnosed ASC-US, LSIL or HSIL-MD having a histologically proven CIN 3 lesion were considered progressive. In the progressive, the follow-up period required to be at least 3 and 6 months in the regressive group. In this latter group, all patients have shown persistence of regression for at least 24 months. These two groups were used to obtain classifiers to facilitate the development of a logistic regression model for assessing the biological behavior of cervical premalignant lesions (Table I). In the present study, the classifiers were considered reliable if they had the ability to predict a negative short-term follow-up correctly within a high percentage of the women who did not develop CIN 3 in follow-up (group I). On the other hand, the classifiers would predict a positive short-term follow-up correctly in very high percentages of the women who developed a CIN 3 lesion in the follow-up (group II).

In order to test the classifiers, the model was supposed to predict initial smears as progressive in seven underdiagnosed women who had had a LLETZ performed indicating a CIN 3 lesion, but whose initial smear taken 6 weeks prior to LLETZ procedure was underdiagnosed as HSIL-MD (test group). Moreover, classifiers that correctly predicted the biological behavior would perform as good or even better if applied to the follow-up cervical smears taken subsequent to the initial smears whereupon the model is based, i.e. a shorter time–distance to the surrogate end point of progression or regression. Therefore, 46 patients from groups I and II who had a subsequent follow-up smear taken after the initial smear were selected from the local pathology database and assessed in a similar way as the initial smears. These samples were used to validate the performance of the model.

Since cervical cancer screening programs can benefit most from any triage of minimally abnormal cervical smears, the logistic regression model best predicting biological behavior was applied to the HPV-positive minimally abnormal smears, the borderline and LSIL smears.

**HPV genotyping**

For HPV detection, a highly sensitive short-fragment polymerase chain reaction (PCR) (SPF10 INNO LIPA HPV genotyping assay; Labo Bio-medical Products B.V., Rijswijk, The Netherlands) was performed on a section of the AgarCyto cellblock. A serial 6 µm thick tissue section was put into a reaction tube and incubated overnight at 56°C in 200 µl of 10 mM Tris–HCl with 1 mM ethylenediaminetetraacetic acid, 0.2% Tween-20 and proteinase K (0.3 mg/ml) at 56°C for 10 min and the slides were rinsed three times in phosphate-buffered saline (PBS, pH 7.4) for 5 min. The slides were placed in a citrate buffer (0.01 M, pH 6.0), heated in a household microwave oven (3 min at 850 W until boiling, followed by 10 min at 180 W). The sections were allowed to cool down to room temperature and washed in PBS (10 min). The primary antibody was diluted in PBS and incubated overnight at 4°C. All following antibodies were diluted in PBS with 1% bovine serum albumin (Sigma, St Louis, MO) and incubated for 30 min at room temperature. All intermediate wash steps were performed in PBS. Ki-67 was detected by monoclonal antibody MIB1 (1:50; Dianova, Hamburg, Germany), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse (1:100; DAKO SA, Glostrup, Denmark). The slides were developed with 0.05% diaminobenzidine (Sigma) with 0.15% H2O2 in PBS for 5 min at room temperature. Specimens were counterstained with Mayer’s hematoxylin, dehydrated in ethanol and xylene and finally mounted in Permout (Fisher Scientific, Fair Lawn, NJ). The Ki-67 LI is defined as the fraction of Ki-67-positive nuclei. This LI was assessed in at least 100 non-overlapping nuclei.  

**Immunohistochemistry**

Detection of Ki-67 in AgarCyto cervical smear samples was performed using a standard immunocytochemical procedure, described in detail previously (25). Four micrometer thick paraffin sections of the tissue samples were mounted onto polylysine-coated slides and dried overnight at 58°C. The sections were dewaxed in xylene and endogenous peroxidase was blocked using H2O2 in methanol for 15 min and the slides were rinsed three times in phosphate-buffered saline (PBS, pH 7.4) for 5 min. The slides were placed in a citrate buffer (0.01 M, pH 6.0), heated in a household microwave oven (3 min at 850 W until boiling, followed by 10 min at 180 W). The sections were allowed to cool down to room temperature and washed in PBS (10 min). The primary antibody was diluted in PBS and incubated overnight at 4°C. All following antibodies were diluted in PBS with 1% bovine serum albumin (Sigma, St Louis, MO) and incubated for 30 min at room temperature. All intermediate wash steps were performed in PBS. Ki-67 was detected by monoclonal antibody MIB1 (1:50; Dianova, Hamburg, Germany), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse (1:100; DAKO SA, Glostrup, Denmark). The slides were developed with 0.05% diaminobenzidine (Sigma) with 0.15% H2O2 in PBS for 5 min at room temperature. Specimens were counterstained with Mayer’s hematoxylin, dehydrated in ethanol and xylene and finally mounted in Permout (Fisher Scientific, Fair Lawn, NJ). The Ki-67 LI is defined as the fraction of Ki-67-positive nuclei. This LI was assessed in at least 100 non-overlapping nuclei.

**In situ hybridization**

DNA probe pUC17.77 for the centromere region of chromosome 1 was labeled by nick translation with biotin-16-dUTP according to the supplier’s instructions (Boehringer, Mannheim, Germany). The hybridization protocol applied to AgarCyto sections has been described previously in detail (25). In short, AgarCyto sections were consecutively dewaxed, blocked for endogenous peroxidase and pretreated with 1 M NaSCN for 10 min at 80°C. Protein digestion was performed using 4000 U/ml pepsin (Sigma) in 0.2 M HCl for 35 min at 57°C and the sections were dehydrated through an alcohol series and air-dried. DNA probe (2 ng/µl) was dissolved in 15 µl hybridization mix containing 60% formamide, 2 × standard saline citrate (SSC), pH 7.0, 10% dextran sulfate (Sigma) and 50 ng/µl herring sperm DNA (Boehringer). The probe mix was applied to the sections, covered with a coverslip and sealed with rubber cement. Probe and target DNA were heat denatured simultaneously for 10 min at 80°C and hybridized overnight at 37°C in a moist chamber. Coverslips were removed by immersing the slides at 42°C in 2× SSC, pH 7.0. Post-hybridization washes at 42°C were carried out twice for 5 min in 60% formamide/2× SSC, pH 7.0, and twice for 5 min in 2× SSC, pH 7.0. The slides were rinsed in PBS/0.05% Tween-20. Hybridized DNA probes were detected immunohistochemically using mouse anti-biotin (1:100; DAKO SA), biotinylated horse anti-mouse and peroxidase-ABC as described for immunochemistry. Evaluation of ISH signals of non-overlapping and morphologically good preserved nuclei was performed as described previously (16). At least 100 nuclei per sample were assessed.

The CI is defined as the mean number of chromosomes copies per nucleus in the sample of measured nuclei. The CI measured in non-treated diploid nuclei has a theoretical value of 2. In truncated nuclei, the CI measured in diploid cells will be always smaller than two. As pointed out in an earlier study, an aberrant CI was defined as any value exceeding 1.4 (mean value CI ± 2.58 × standard deviation, obtained from normal cervical epithelium in control subjects) (15,16). In this study, the CI#1 was assessed.

**Statistics**

Between groups I and II, t-tests for unpaired independent observations were performed to assess differences in the means of the various parameters measured. In order to select a subset of features to discriminate between the patients in groups I and II, a forward likelihood ratio stepwise logistic regression analysis was performed. Logistic regression analyses were also used to find the best multiparameter linear predictor to allocate women in these groups.

### Table I. Classification of the two study groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Selection criteria</th>
<th>Considered to be</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Initial smear: ASC-US, LSIL or HSIL-MD Follow-up: two successive normal smears or two grades less severe than the initial smear</td>
<td>Lesions with a negative short-term follow-up with a low risk to progress toward CIN 3—‘regressive lesions’</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>II Initial smear: ASC-US, LSIL or HSIL-MD Treated by LLETZ &gt;13 weeks and diagnosed CIN 3</td>
<td>Lesions with a positive short-term follow-up, corresponding with a high risk to progress into CIN 3—‘progressive lesions’</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>
as is given by the formula: $\eta = a_0 + a_1 \cdot x_1 + \ldots + a_n \cdot x_n$, in which $a_i$ are the regression coefficients of the corresponding predictor $x_i$, which are selected using forward stepwise logistic regression analysis. The conditional probability that a low-grade cervical lesion will progress to a CIN 3 if the value of the linear predictor $\eta$ (as described above) is known is given by the formula: $P(\text{progression} | \eta) = \frac{1}{1 + e^{-\eta}}$. In case the progression probability is $\geq 0.5$, the sample is classified progressive and a probability $<0.5$ means the sample is classified regressive. The same samples were used to construct as well as to evaluate the performance of selection criteria.

The goodness-of-fit of logistic regression models were tested by the widely used Hosmer–Lemeshow test (26,27). This test is based on grouping predicted probabilities into deciles and performing a $\chi^2$-test for the mean predicted probability against the observed fraction of events.

The predictive ability of the logistic regression models were quantified or ranked by the area under the receiver operating characteristic curve (AUC) and Brier scores (26). The receiver operating characteristic curve is a graphical plot of the sensitivity versus 1—specificity of a classifier. The AUC varies from 0 to 1, and the greater the AUC, the better the prediction by the logistic regression model. The Brier score is defined as: $B = \frac{1}{n} \sum_i (P_i - Y_i)^2$, where $P_i$ is the predicted probability by the regression model and $Y_i$ is the corresponding observed response for the $i$th observation (26). The Brier score varies from 0 to 1, and the lower the Brier score the better the predictive ability.

In addition, the percentages of correctly classified cases of the different logistic regression models are given. The same data were used both to develop the logistic regression models and to classify the cases in regressive and non-regressive lesions. Therefore, the classification results may be too optimistically biased. The leave-one-out cross-validation procedure was used in the logistic regression analyses to reduce this bias (26). In this procedure, one of the $n$ cases is omitted from the analysis and the obtained regression model was used to classify the excluded case. This procedure will be repeated $n$ times so that none of the classified cases were used to construct the logistic regression model. The statistical analyses were performed with SPSS version 12.0.1 for Microsoft Windows.

Results

From a cohort of 800 women, 73 were allocated meeting the criteria defined in Table I. The mean age at initial smear was 37.0 ($\pm$4.4) years. Thirty-five of the 73 initial smears showed HSIL-MD (47.9%), 14/73 (19.2%) of the women had a smear indicating LSIL and in 24/73 (32.9%) of the women the initial cytology was diagnosed ASC-US. Details of the distribution of the initial cytology per group are given in Table II. Twenty-one of the 24 women having ASC-US (87.5%) showed regression toward normal epithelium in the consecutive smears, whereas progression to CIN 3 appeared in 3/24 cases (12.5%). Eleven of the 14 women (78.6%) initially having LSIL regressed to a normal smear, whereas progressive behavior was observed in 3/14 cases (21.4%). Finally, of the 35 women demonstrating HSIL-MD in their initial smear, 4/35 (11.4%) regressed to a normal smear, 6/35 (17.1%) regressed toward an ASC-US smear and 26/35 (74.3%) showed progression.

The indicated follow-up time between initial smears and subsequent normal or two grades less severe smears (group I) was at least 6 months and between initial smears and LLETZ procedures (group II) was at least 3 months. However, the actual mean ($\pm$standard deviation) follow-up time in months for the two groups was prolonged, group I 8.7 ($\pm$6.1) months and group II 5.9 ($\pm$5.0) months.

Table II. Cytological classification of the initial smears in the two groups assessed

<table>
<thead>
<tr>
<th>Initial smear</th>
<th>Total</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I, n (%)</td>
<td>II, n (%)</td>
</tr>
<tr>
<td>ASC-US</td>
<td>24 21 (50)</td>
<td>3 (12.5)</td>
</tr>
<tr>
<td>LSIL</td>
<td>14 11 (26.2)</td>
<td>3 (21.4)</td>
</tr>
<tr>
<td>HSIL-MD</td>
<td>35 10 (23.8)</td>
<td>25 (59.5)</td>
</tr>
<tr>
<td>Total</td>
<td>73 42</td>
<td>31</td>
</tr>
</tbody>
</table>

hr-HPV detection

Forty-four of the 73 initial cervical smears (60.3%) harbored hr-HPV genotypes; in the remaining 29 (39.7%), no hr-HPV types were detected using the SPF10-line probe assay. Considering the initial cyto-logical results 11/24 (45.8%) ASC-US smears, 7/14 (50%) LSIL smears and 26/35 (74.3%) HSIL-MD smears contained hr-HPV. Using the presence or absence of an hr-HPV infection to classify women in, respectively, group II (progression) and group I (regression) yielded a poor overall classification percentage of 63.0% (46/73), 52.4% (22/42) was correctly classified in group I and 77.4% (24/31) in group II.

Ki-67 LI and numerical chromosome aberrations

The mean values and standard deviations of the Ki-67 LI, the fraction of cells with four copies of chromosome 1 per nucleus and of the CI#1 for groups I and II are given in Table III. In group II, the fraction of MIB1 positively stained nuclei (e.g. Ki-67 LI) was more than two times greater than in group I ($P < 0.001$, Student’s $t$-test). Also the CI#1 was significantly greater in group II than in group I ($P < 0.001$, Student’s $t$-test). Moreover, compared with group I, the fraction cells with four copies of chromosome per nucleus was significantly greater in group II ($P < 0.01$, Student’s $t$-test). The difference between regressive and progressive lesions with respect to Ki-67 LI and CI#1 are visualized in the AgarCyto specimens in Figure 1A–D.

Logistic regression model

Using forward likelihood ratio stepwise logistic regression analysis with Ki-67 LI, CI#1 and the fractions of cells with zero, one, two, three, four, five, six, seven and eight copies of chromosome 1 per nucleus, a linear predictor ($\eta_1$) based on three parameters was identified (data not shown). The CI#1 was the best discriminating biomarker, followed by Ki-67 LI and the fraction of cells with four copies of chromosome 1 per nucleus (Fr4ISH). This led to the following logistic regression models: $\eta_1$ was based on the best discriminating biomarkers, respectively, Ki-67 LI, CI#1 and Fr4ISH and bivariate linear prediction model $\eta_2$, was based on Ki-67 LI and CI#1. Univariate linear prediction models $\eta_3$ and $\eta_4$ were solely based on CI#1 and Ki-67 LI, respectively.

The results of the tests validating the models, i.e. Hosmer and Lemeshow test, the AUC and the Brier score, are listed in Table IV. The highest AUC score and the lowest Brier score indicate that model $\eta_1$ is the best prediction model. Applying the models to the initial cervical smears of groups I and II multivariate logistic regression model, $\eta_1$ generated correct predictions of $>90\%$ in both groups, whereas the other three models $\eta_1(2)–\eta_1(4)$ predicted overall correct group membership substantially less, Table IV. This was especially true for the samples in the progressive group II.

Reducing optimistically biased results using the leave-one-out cross-validation procedure in model $\eta_1$, led to correct classification of 27/31 (87.1%) progressive samples and 39/42 (92.9%) regressive samples; all other models performed less good, Table IV.

Validating model $\eta_1$ using the additional moderate dysplasia samples of the test group lead to correct classification of all seven samples.
Thus, using regression model $g(1)$, all smears in the additional group were correctly classified progressive. Subsequent smears Since the cervical smears following the initial smear are taken within a shorter time-distance to the surrogate end point of progression or regression, the parameters indicating proliferation and chromosomal aberrations will have shown development toward the final diagnoses, either progression toward CIN 3 or regression toward normal cytology. Therefore, the classifiers described in our logistic regression model should perform as good or even better if applied to these follow-up cervical smears.

Using the results of the biomarkers assessed in the selected 46 subsequent smears (see also Materials and methods section), we were once more able to assess and validate the logistic regression models. Applying the initial samples of the 46 selected smears to the best predicting model $g(1)$, the percentages correctly classified samples were comparable with the percentages in the total group of 73 samples (93% versus 95% and 88% versus 90% for groups I and II, respectively). The overall correct classification in these 46 initial smears using the other models was substantially less. Using the biomarkers assessed in the subsequent smears led to correct prediction of all members of the two groups using the prediction models $g(1)$, $g(2)$ and $g(3)$, Table V.

**Discussion**

This study shows the possibility to predict biological behavior of cervical lesions using a logistic regression model based on Ki-67 LI and numerical aberrations for chromosome 1 assessed in cervical

**Table III. Mean and SD for the biomarkers in the initial smear which appeared to be most suited for logistic regression analysis**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group I ($N = 42$), mean ± SD</th>
<th>Group II ($N = 31$), mean ± SD</th>
<th>$P$-value Student’s t-test</th>
<th>Test group ($N = 7$), mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki-67 LI</td>
<td>0.19 ± 0.15</td>
<td>0.43 ± 0.15</td>
<td>&lt;0.001</td>
<td>0.59 ± 0.14</td>
</tr>
<tr>
<td>CI#1</td>
<td>1.32 ± 0.21</td>
<td>1.86 ± 0.35</td>
<td>&lt;0.001</td>
<td>2.11 ± 0.33</td>
</tr>
<tr>
<td>Fr 4 copies #1</td>
<td>0.01 ± 0.03</td>
<td>0.04 ± 0.05</td>
<td>&lt;0.01</td>
<td>0.04 ± 0.06</td>
</tr>
</tbody>
</table>

SD, standard deviation; Fr 4 copies #1, fractions of cells with four copies of chromosome 1 per nucleus.

**Fig. 1.** (A–D) Cell groups in AgarCyto specimens of a regressive and a progressive (or non-regressive) cervical lesion with a low Ki-67 LI (1A) and a high Ki-67 LI (1B), respectively. Cell groups in AgarCyto specimens of a regressive and a progressive (or non-regressive) cervical lesion with a low (1C) and a high (1D) CI#1.
smears. Taking all samples into account, our model containing the proliferation marker Ki-67 LI, the CI#1 and the fractions of cells with four copies of chromosome 1 per nucleus predicted 40/42 (95.2%) of the samples would have been correctly classified; 22 (of 46) of the samples taken subsequent to the initial smear led to correct prediction of all 17 progressive and all 29 regressive samples. Since the 46 smears were obtained closer to the end point of progression or regression, the smears of the seven additional HSIL-MD samples (test group) were obtained closer to the end point of progression or regression, the additional medical societies. This triage will improve the management of patients having these minimal abnormal cervical smear would be referred to the gynecologist. In our follow-up, the proposed hr-HPV triage, only 50% of the minimally abnormal hr-HPV-positive samples progressive, whereas 27.4% (20/73) would have been incorrectly predicted to be progressive. If patient management would be solely based on the best predicting model the false negative results would have possibly lead to undertreatment. After reducing optimistically biased results using the leave-one-out cross-validation procedure, the biological behavior of 66/73 (90.4%) samples was correctly predicted. Validating the model using the 46 follow-up smears taken subsequent to the initial smear led to correct prediction of all 17 progressive and all 29 regressive samples. Since the 46 smears were obtained closer to the end point of progression or regression, the parameters assessed were indeed involved in progression. The initial smears of the seven additional HSIL-MD samples (test group) were also predicted correctly progressive by the model. Since, the time to progression was short (i.e. <6 weeks), these initial samples ought to be considered cytologically underdiagnosed CIN 3.

hr-HPV is associated with an increased risk in the development of cervical carcinoma. Supposing regressive or progressive behavior of the lesions assessed in this study was predicted solely based on the absence or presence of hr-HPV genotypes, respectively, only 63% (46/73) of the samples would have been correctly classified; 22 (of 42) hr-HPV-negative samples were predicted regressive and 24 (of 31) hr-HPV-positive samples progressive, whereas 27.4% (20/73) would have been incorrectly predicted to be progressive and 9.6% (7/73) would have been incorrectly predicted to be regressive. This suggests that hr-HPV testing is inappropriate as a prediction marker for progressive behavior and that this parameter can be merely valued as a risk indicator in case of screening patients susceptible to developing high-grade CIN and/or cervical cancer. Previously, high negative predictive values of HPV DNA tests for identifying high-grade CIN and cancer and the questionable clinical relevance of a single positive hr-HPV test have been extensively reviewed and debated in literature (28,29).

Previous studies have already shown a significant positive correlation between numerical aberrations of chromosome 1 and CIN lesions (15,16,30,31) and between MIB1 and CIN lesions (18,19,32). This is the fist study successfully assessing CIs and Ki-67 LI simultaneously in serial sections of cervical smears. Although MIB 1 is suggested an excellent proliferation marker and aneusomy for chromosome 1 seems to be a promising surrogate marker for the prediction of biological behavior of low-grade CIN lesions, the combination of parameters appears an even better method predicting CIN behavior. Large-scale implementation, e.g. population-based screening programs, might, however, be restricted. Especially since assessments of Ki-67 LI and of numerical aberrations of chromosomes are time consuming, require expertise, are expensive and need to be performed under strict standardized conditions and laboratory settings. Moreover, the thickness of the dissected sample is influenced by various uncontrollable factors, e.g. barometric pressure. Since the CI measured in truncated nuclei is dependent on the fraction of the nuclear volume that is enclosed in the tissue section, reliable measurement of CI requires standardized thickness of the assessed sections (33).

The CI measured in truncated nuclei is dependent on the fraction of the nuclear volume that is enclosed in the tissue section, reliable measurement of CI requires standardized thickness of the assessed sections (33). Although, all parameters in this study were measured in AgarCyto blocks, assessing the parameters in liquid-based cytology samples is also possible. MIB-1, for instance, has lately shown to be of promising value as surrogate marker in a cervical cancer screening setting using liquid-based cytology (34). In addition to a clinical use, our multiple parameter logistic regression model can be used for the validation of and correlation to new biomarkers that might be discovered in the future and are associated with progressive or regressive behavior of CIN.

However, the model could also be used more practically, i.e. in the triage of women with minimally abnormal cervical cytology, like ASC-US and LSIL [in various international studies also known as BMD smears]. Approximately 5% of the women participating in the population-based screening for cervical cancer are diagnosed having a BMD smear. The management of patients having these minimal abnormal cervical smears has been a clinical problem for a long time (35). Currently, women with two consecutive BMD smears are referred to the gynecologist for colposcopic assessment. Since cytology is not able to predict the progressive or regressive behavior and HPV DNA testing alone has a too low positive predictive value for identifying high-grade CIN and cancer, additionally assessing HPV in the triage of repetitive BMD smears is recommended by the American Society for Colposcopy and Cervical Pathology and other (inter)national medical societies. This triage will improve the management of BMD women at risk for developing cervical cancer (36–38). Further, triaging the HPV-positive BMD smears, using the progression markers described in our model, could improve management even more. Indeed, in the present study, 18 of the 38 BMD (47.4%) smears were hr-HPV positive, which is consistent with other studies (38,39). So, using the proposed hr-HPV triage, only 50% of the minimally abnormal cervical smear would be referred to the gynecologist. In our follow-up,
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References


4/18 hr-HPV-positive BMD smears (22.2%) showed progression, whereas the remaining 14 hr-HPV-positive smears showed regression. Using the multiple parameter prediction model (\(\eta_{1,3}\)), all progressive lesions were predicted correctly and only one observed regressive lesion was misclassified. This suggests that our prediction model works very well and that subsequent to the hr-HPV triage in borderline and LSIL, the hr-HPV-positive smears can be assessed using Ki-67 LI and numerical aberrations of chromosome 1 to reliably predict the behavior of the lesion. In this (small numbered) study, it would mean that of a total number of 38 BMD smears, only 4 (10%) should have been referred to the gynecologist since the model correctly predicted progression. However, of the 20 hr-HPV-negative BMD smears, two showed progression. According to the American Society for Colposcopy and Cervical Pathology triage guidelines for hr-HPV in BMD smears, these women would not have been referred to the gynecologist, nevertheless our model did predict these progressive lesions correctly. Since the general dogma states that progression to ultimately cervical cancer will only occur in hr-HPV-positive lesions, the behavior of the lesions in these two women should, however, be considered non-progressive.

Our data demonstrate that short-term progressive and regressive behavior is highly predictable. In case hr-HPV is detected in a borderline and LSIL smear, one should consider to determine the biological behavior of the lesion. In this (small numbered) study, it would work very well and that subsequent to the hr-HPV triage in borderline lesions, this suggests that our prediction model works very well and that subsequent to the hr-HPV triage in borderline and LSIL, 1. Akin,N.B. et al (1990) Chromosome changes in 43 carcinomas of the cervix uteri. Cancer Genet. Cytogenet., 44, 229–241.

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