

Detection of high-grade neoplasia in air-dried cervical PAP smears by a microRNA-based classifier

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Abstract. Recent studies have shown that changes in the expression levels of certain microRNAs correlate with the degree of severity of cervical lesions. The aim of the present study was to develop a microRNA-based classifier for the detection of high-grade cervical intraepithelial neoplasia (CIN ≥ 2) in cytological samples from patients with different high-risk human papillomavirus (HR-HPV) viral loads. For this purpose, raw RT-qPCR data for 25 candidate microRNAs, U6 snRNA and human DNA in air-dried PAP smears from 174 women with different cervical cytological diagnoses, 144 of which were HR-HPV-positive [40 negative for intraepithelial lesion or malignancy (NILM), 34 low-grade squamous intraepithelial lesions (L-SIL), 57 high-grade squamous intraepithelial lesions (H-SIL), 43 invasive cancers], were statistically processed. The expression level changes of various individual microRNAs were found to be significantly correlated with the cytological diagnosis but the statistical significance of this correlation was critically dependent on the normalization strategy. We developed a linear classifier based on the paired ratios of 8 microRNA concentrations and cellular DNA content. The classifier determines the dimensionless coefficient (DF value),

which increases with the severity of cervical lesion. The high- and low-grade CINs were better distinguished by the microRNA classifier than by the measurement of individual microRNA levels with the use of traditional normalization methods. The diagnostic sensitivity of detecting high-grade lesions (CIN ≥ 2) with the developed microRNA classifier was 83.4%, diagnostic specificity 81.2%, ROC AUC=0.913. The analysis can be performed with the same nucleic acid preparation as used for HPV testing. No statistically significant correlation of the DF value and HR-HPV DNA load was found. The DF value and the HR HPV presence and viral DNA load may be regarded as independent criteria that can complement each other in molecular screening for high-grade cervical intraepithelial neoplasia. Although it has several limitations, the present study showed that the small-scale analysis of microRNA signatures performed by simple PCR-based methods may be useful for improving the diagnostic/prognostic value of cervical screening.

Introduction

Cervical cancer (CC) is one of the most common oncological diseases in women worldwide and one of the leading causes of female cancer mortality. One etiological factor for CC is infection with the human papillomavirus of a high carcinogenic risk (hereafter HR-HPV). Epithelial damage tends to progress slowly (10-20 years from contact with HPV to the development of invasive cancer). Therefore, regular cervical screening can allow the detection of most lesions at early stages and drastically reduce the risk of CC. At present, the preferred method for primary cervical screening is cervical cytology, the efficacy of which as a single screening method is limited due to technical constraints, human factors as well as limitations of the pathomorphological classification itself. This results in relatively low and varying sensitivity (50-80% in different clinical settings) and compromised specificity of cervical cytology (1). Another problem is the ambiguity of the prognosis for patients with mild neoplasia (L-SIL) for possible long- and short-term outcomes, from the complete regression to the rapid development of invasive cancer.

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Abbreviations: CIN, cervical intraepithelial neoplasia; HR-HPV, high-risk human papillomavirus; DF value, decision function value; NILM, negative for intraepithelial lesion or malignancy; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer; PPV, positive predictive value; RT-qPCR, quantitative reverse-transcription real-time PCR; Cq, quantification cycle

Key words: cervical intraepithelial neoplasia, cervical cancer, microRNA, normalization, human papillomavirus

The value of cervical screening can be improved by the additional analysis of molecular biomarkers. At present, the only marker widely used in clinical practice is HR-HPV DNA. HPV testing has a higher diagnostic sensitivity than the cytological method for the detection of cervical neoplasia (2,3). Therefore, it is widely used in cervical screening in combination with cytology (4,5) or as a method of primary screening (6,7). However, HR-HPV infection is frequent in women with no signs of cervical neoplasia even at the age of risk (26-30 years and older) (8). In the vast majority of cases, HPV infection is transient and eliminated spontaneously (9). Therefore, despite the high diagnostic sensitivity and negative predictive value (NPV) of HR-HPV testing, its diagnostic specificity and positive predictive value (PPV) in cervical screening are relatively low. High viral load of HPV DNA in older ages is now considered a surrogate marker of the HPV persistence pointing to an increased risk of malignant transformation but the PPV of this indicator is insufficient.

In multiple studies, diagnostic relevance of the wide range of additional molecular biomarkers of dysplastic changes in the cervix has been reported. These include the integrated form of HR-HPV DNA, the amplification of telomerase gene subunits, the levels of various mRNAs and microRNAs, and the aberrant methylation of the promoters of various genes. Moreover, accumulating evidence indicates that some morphologically indistinguishable subgroups of CIN2 and even CIN3 neoplasms have very different long-term chances of malignant transformation. Such subgroups can be discriminated by analyzing the content of molecular markers of genetic and/or epigenetic changes in affected cells [reviewed in ref. (10)].

MicroRNAs play a significant role in the development of all types of cancer including CC. Cervical lesions are always accompanied by an increase or decrease in the levels of various microRNA which are correlated with the severity of the lesion and/or are characteristic of invasive cancer in comparison with preinvasive stages (11-20).

MicroRNAs are markedly stable in clinical material, including cytology specimens. Therefore, they are regarded as perspective clinical biomarkers. Several recent studies have demonstrated the feasibility of using microRNA profiling in cervical samples for diagnostic purposes (18,21,22). At the same time, the inconsistency of accumulated data concerning changes in microRNA levels in the above-mentioned studies impedes the translation of their results into clinical practice. The reported degree and direction of individual microRNA level changes in cervical lesions can vary substantially and be even contradictory in different studies (23-25). This may be due to differences in the techniques used for quantification, the characteristics of the cohorts of enrolled patients, as well as to the different methods of raw data handling. The importance of proper normalization for quantitative estimates of microRNAs is undoubted (26-29). MicroRNAs represent only a small fraction of the total RNA in the cell; moreover, this fraction can vary significantly between different types of specimens. The extraction efficiency of these small molecules can differ significantly from the extraction efficiency of longer RNAs extracted from sample of the same type by the same method. Thus, traditionally used housekeeper mRNAs are not applicable for the normalization of microRNA expression data. At the same time, profiles of microRNA expression are

characterized by high tissue and cellular specificity (30), and there are no identified microRNA genes expressed as stably as known protein-coding housekeeping genes. Due to the above difficulties, the normalizers for microRNA quantitation in different tissues and specimen types are often chosen empirically. Depending on the method of reference microRNA selection, different researchers choose different normalizers. The use of geometric mean of the group of normalizers instead of a single reference can reduce the bias introduced by normalization. Such an approach, called GeNorm (31) makes it possible to rank candidate reference genes by their expression stability, based on the calculation of an average pairwise variation between all studied genes, and to determine the optimum set of reference genes required for normalization. However, in the case of microRNA analysis this method either requires all possible normalizers to be analyzed or faces the problem of rational selection of the normalizers. The alternative is utilizing the mean expression value of all expressed microRNAs in a given sample as a normalization factor (27). However, this approach requires a large set of microRNAs to be profiled in a single specimen, which may be unacceptable in clinical practice for both technical and economic reasons.

The aim of the present study was to develop a method for detecting high-grade cervical intraepithelial neoplasia and CC in cytological specimens by PCR-based analysis of a small set of microRNAs.

Materials and methods

Clinical material. The present study was approved by the local Ethics Committee of the Federal Government Budgetary Institution 'N.N. Petrov Research Institute of Oncology' as of February 13, 2014 (Internal No. 21). The samples were obtained from patients who underwent examination and treatment at the Oncogynecology Department of the Oncology Research Institute over the period 2010-2016. Cytological examination of cervical smears and histological examination of the surgical material were carried out by specialists at the Cytology Laboratory and Department of Pathomorphology of the Oncology Research Institute, respectively. Cytological specimens were obtained from the archives of the Cytology Laboratory and clinical data were obtained from the database of the Oncology Research Institute. Before the study, the clinical material and information were subjected to anonymization.

The cervical epithelial scrapings were obtained and prepared by routine methods (Papanicolaou staining). The samples were classified according to the Bethesda system (32): normal cytology [negative for intraepithelial lesions or malignancy (NILM)] (n=40, mean age 31), low-grade squamous intraepithelial lesion (L-SIL) (n=34, mean age 36), high-grade squamous intraepithelial lesion (H-SIL) (n=57, mean age 44), invasive cervical cancer (CC) (n=43, mean age 53). All H-SIL and CC diagnoses were histologically verified after subsequent surgical treatment. The coincidence of cytological and histological conclusions was observed in 100% of CC cases. Moderate neoplasia (H-SIL) was confirmed histologically in 88% cases, in the remaining cases (7 of 57, 12%) intraepithelial cancer (*Ca in situ*) was revealed. In 5 cases of cytologically diagnosed mild neoplasia (L-SIL), the surgical treatment was prescribed, based on the clinical specifics of

Table I. Raw Cq values for several microRNAs that differ between cervical smears from patients with different cytologic diagnoses.

	Cq, mean			U test, P-value		
	NILM	H-SIL	CC	NILM/H-SIL	NILM/CC	H-SIL/CC
miR-106b	26.13	26.85	24.72	0.13962	0.0037 ^b	0.000131 ^c
miR-1246	25.42	25.53	24.01	0.71362	0.00079 ^c	0.001344 ^b
miR-126	30.99	30.98	27.79	0.905053	0.00048 ^c	0.000458 ^c
miR-196b	30.83	31.03	28.98	0.90506	0.00062 ^c	0.001209 ^b
miR-20a	24.74	24.97	22.52	0.783123	0.00013 ^c	0.000093 ^c
miR-21	21.01	21.16	19.81	0.653008	0.00298 ^b	0.002204 ^b
miR-375	24.55	26.53	27.12	0.001240 ^b	4x10 ^{-6c}	0.216201
miR-145	30.63	31.85	31.68	0.000239 ^c	0.007866 ^b	0.296758

*P<0.05; ^bP<0.01; ^cP<0.001. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion; CC, cervical cancer.

the course of the disease. In all these cases, a histological study revealed Ca *in situ*.

Isolation of total RNA and detection of microRNAs and U6 snRNA by RT-PCR. Isolation of RNA from air-dried cytology preparations was carried out as previously described (33). From the material of cytological preparations, we succeeded in obtaining from 5 to 50 µg of total RNA of satisfactory quality (A 260/280: 1.5-1.8) and in sufficient concentration for quantitative measurements (120-550 ng/µl). The microRNA and U6 snRNA expression were analyzed by stem-loop qPCR as previously described (34). The list of microRNAs was made based on the meta-analysis of related literature data. The following 25 microRNA were selected: hsa-miR-20a-5p (hereinafter referred to as miR-20a), -21-5p, -23a-3p, -31-5p, -34a-5p, -96-5p, -99a-5p, -106b-5p, -125b-5p, -126-3p, -145-5p, -143-3p, -146a-5p, -146b-5p, -155-5p, -181b-5p, -191-5p, -192-5p, -196b-5p, -197-3p, -200b-3p, -203a-3p, -375, -1246, let-7d. For each sample, the content of each marker molecule was measured in single repeat. The results corresponding to Cq >40 were considered negative. Sequences of all oligonucleotides are available upon request.

Identification, genotyping and evaluation of HR-HPV viral DNA load. Identification, genotyping and evaluation of HR-HPV viral DNA load were performed using the 'RealBest HPV genotype, quantitative' kit (AO Vector-Best, Russia) according to the manufacturer's instructions. The kit is designed to quantify the viral load of each of 12 HR-HPV genotypes: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59. The viral DNA load, normalized to the number of copies of the human β-actin gene, was calculated independently for each genotype and the total viral DNA load was provided as the sum of loads for different genotypes in the case of multiple infection. In addition, all samples were tested for HPV DNA of genotypes 26, 53, 66, 68, 73 and 82 using the kits 'RealBest DNA HPV 26/53/66' and 'RealBest DNA HPV 68/73/82' (AO Vector-Best, Russia). In this case, the viral DNA loads were estimated by the ΔΔCq method (35), using the Cq values from amplification of β-actin gene as normalizing factor.

Content of human DNA. In the isolated sample, the number of HMBS gene copies was evaluated using a set of reagents 'RealBest Sample Validation' (AO Vector-Best, Russia) in accordance with the manufacturer's instructions.

Statistical analysis. Data analysis was performed using SciPy library (36) of Python programming language. Differences between groups were assessed using the Mann-Whitney U test. The Bonferroni correction was applied to correct for multiple testing. All P-values of <0.05 were considered statistically significant. The receiver operating characteristic (ROC) curve and logistic regression analysis were used to assess the performance of high-grade CIN detection. Classifications were performed using Scikit-learn library (37) of Python programming language. The linear classification algorithm was used. The stability of reference genes was estimated by geNorm algorithm (31).

Results

Raw microRNA Cq values in samples from different lesions. For some microRNAs, the raw Cq values obtained from amplification curves (shown as box-whisker plots in Fig. 1) differed between the different cytological diagnoses. No statistically significant difference was observed for raw Cq values for any microRNA between the L-SIL group and the NILMs and H-SILs (data not shown). However, for some microRNAs, the differences between the groups in pairs NILM/CC, L-SIL/CC and NILM/H-SIL were significant. Thus, the raw Cq values for 6 microRNAs differed significantly for invasive cancers compared to both NILM and H-SIL specimens. For two microRNAs, the significant difference was observed for the NILM group compared to CCs and/or H-SILs (Table I).

Estimation of expression stability of selected microRNAs and U6 snRNA. The initial list of suggested normalizers included: miR-191 [according to (26), the expression of this microRNA was the most stable in 13 compared tissues]; miR-23a [as a normalizer suitable for the analysis of cervical samples,

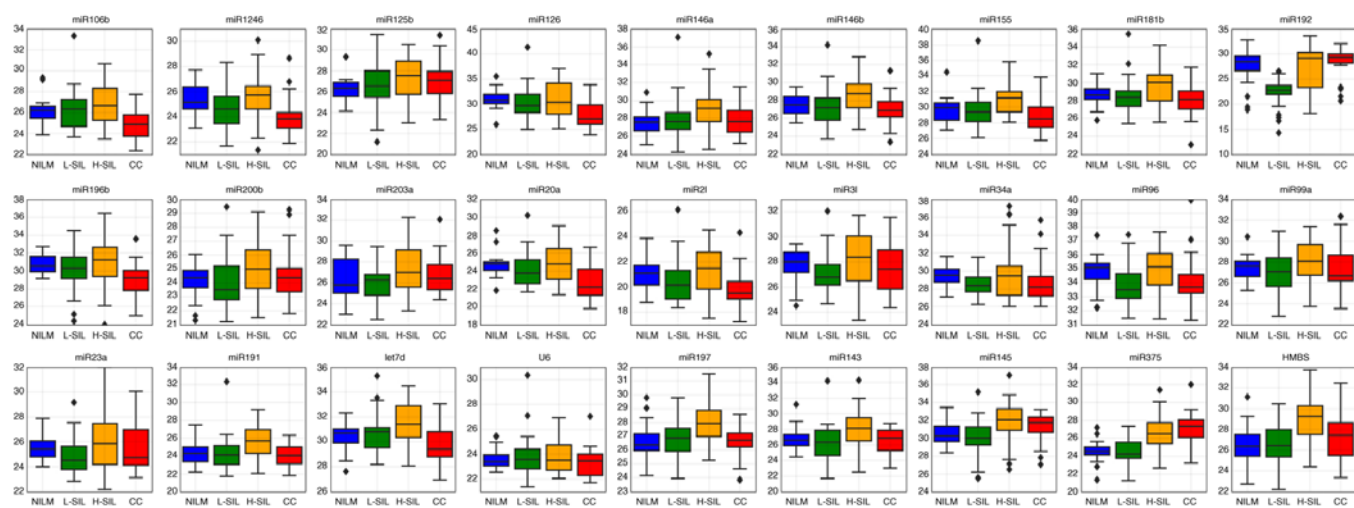


Figure 1. Box-whisker plots for the raw Cq values of selected microRNAs and different cytological diagnoses. Inner lines, median values; box, upper and lower quartiles; whiskers, non-outlier ranges; diamond, outliers. Red, cervical cancer; orange, H-SIL; green, L-SIL; blue, NILM. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion; CC, cervical cancer.

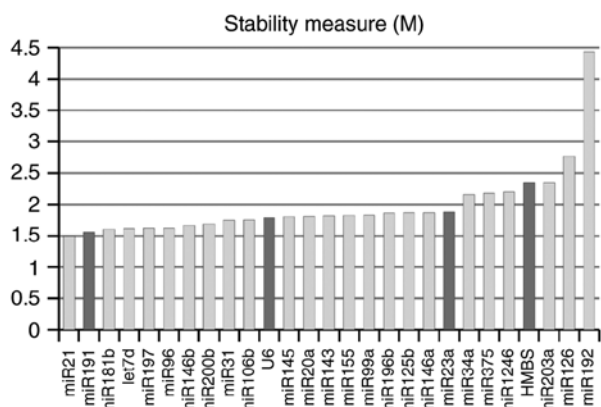


Figure 2. geNorm relative stability plot for analyzed miRNAs, RNU6 and HMBS copy number. Suggested normalizers are denoted by dark grey bars along the x-axis. y-axis shows M-value.

according to (38)]; U6 (traditionally used as a stand-alone reference for normalization of microRNA expression data); and HMBS (as a marker reflecting the input number of epitheliocytes). Surprisingly, according to geNorm stability criteria, the most stably expressed was miR-21, which is widely accepted as an oncomiR, including in CC (39-41). In our sample, its level was increased in invasive cancers. At the same time, suggested normalizers U6 and, particularly, miR-23a, demonstrated relatively low stability, which was comparable to the known oncogenic (miR-34, miR-20a) and onco-suppressor (miR-375, miR-143) microRNAs (Fig. 2). All microRNAs as well as U6 snRNA expectedly demonstrated maximum stability in NILM specimens compared to other cytological diagnoses.

Diagnostic utility of paired marker combinations for detection of cervical lesions in cytological preparations. According to the geNorm criterion, the most ‘stably expressed’ among the selected RNAs was not the supposed normalizer but the oncogene. This suggests that the choice of a normalizer based on the evaluation of the expression stability may be an

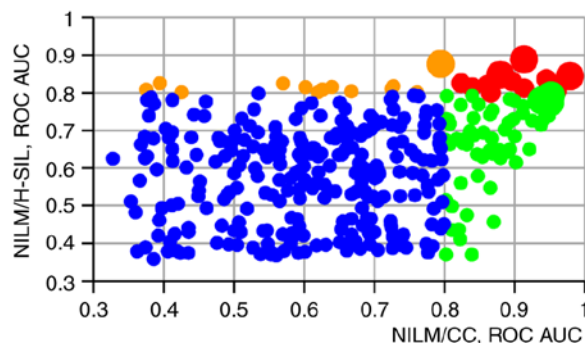


Figure 3. ROC AUC values for 351-paired marker combinations for separating NILM from CC (x-axis) and NILM from H-SIL (y-axis). Each mark on the plot corresponds to a single paired marker combination, for which the ROC AUC corresponding to separation of NILM from CC is plotted on the x-axis and the ROC AUC corresponding to separation of NILM from H-SIL is plotted on the y-axis. ROC AUC values for combinations selected to separate NILMs from H-SILs (orange), NILMs from CCs (green) or NILMs from both H-SILs and CCs (red) are marked by large circles. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

inadequate approach in our case. We evaluated the diagnostic utility of all possible paired combinations of the 27 selected markers (25 microRNAs, U6 snRNA, and HMBS copy number). For each pair of markers A and B, the ΔCq_i value ($\Delta Cq_i = CqA - CqB$) was obtained. The number of possible ΔCq_i values in our case was $27!/(2!25!) = 351$. ΔCq_i value is dimensionless, and, on condition of ~100% PCR efficiency, is equal to \log_2 of the concentration ratio of two marker molecules in the pair. This means that the ΔCq_i value does not depend on the amount of input material as each marker in the pair serves as ‘normalizer’ for the other marker.

For each ΔCq_i value, 6 different areas under the ROC curves (ROC AUCs) were calculated. This was done to estimate the possibility of each paired marker combination to discriminate the specimens by the cytological diagnosis: NILM/L-SIL, NILM/H-SIL, NILM/CC, L-SIL/H-SIL, L-SIL/CC and H-SIL/CC. To calculate the ROC AUCs, the cross-validation

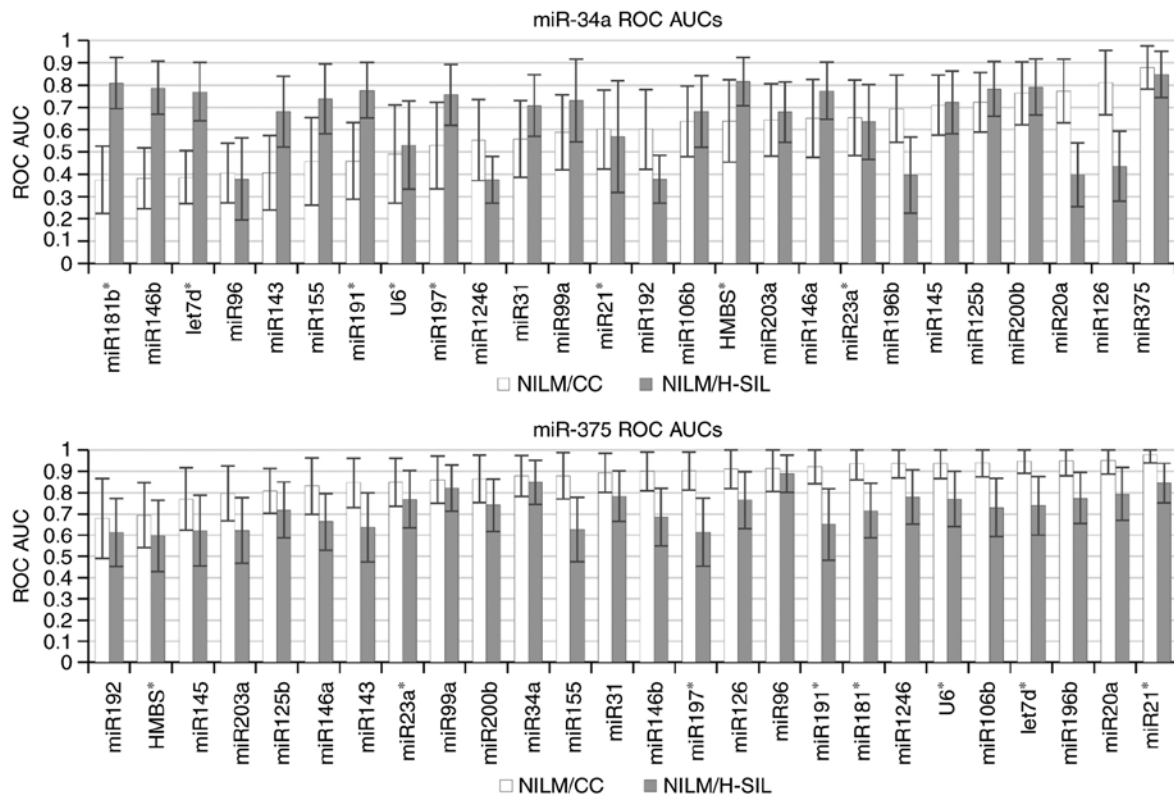


Figure 4. ROC AUCs calculated for $C_{qA} - C_{qB_i}$ values where A is oncogenic microRNA (miR-34a, above) or onco-suppressor microRNA (miR-375, below), and the markers B_i (shown on the x-axis) serve as the normalizers. y-axis, ROC AUC at the separation of H-SIL from NILM (grey) or invasive cancer from NILM (white); 95% confidence intervals are presented. The suggested and/or most stably expressed normalizers are marked by asterisks. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

strategy was used. Each time a random subsample including 80% objects of the original sample was generated. The model was trained on this subsample, and the ROC AUC was evaluated based on the remaining 20% objects of the original sample. In this case, the sample was subdivided into the training and test subsamples in such a way that the proportion of specimens with the cytological diagnoses was the same in both. The procedure was repeated 100 times, that is, the ROC AUC for each ΔC_{q_i} value was obtained as a result of averaging over 100 calculations. The ROC AUC value >0.8 was considered acceptable for the corresponding paired marker combination to discriminate the samples with different cytology.

The utility of different paired marker combinations for detecting the lesions of different severity varied greatly. However, the same combinations were among the best at separating the 'neighboring' classes (NILM from L-SIL, L-SIL from H-SIL, H-SIL from CC). The median and mean ΔC_{q_i} values for these combinations increased or decreased stepwise with the severity of the lesion.

For none of the ΔC_{q_i} values, ROC AUC exceeded 0.8 at discriminating 'neighboring' groups (NILM/L-SIL, L-SIL/H-SIL and H-SIL/CC) (data not shown). At the same time, several paired combinations were characterized by high (>0.8) ROC AUC values at separation of NILMs from H-SILs (n=13), NILMs from CCs (n=61) or NILMs from both H-SILs and CCs (n=11). In Fig. 3 a scatter graph is presented reflecting the ratio between the ROC AUC values for discriminating NILM specimens from CCs (x-axis) and H-SILs (y-axis) for each paired marker combination.

For discriminating specimens with different cytological diagnoses, the highest ROC AUC values were obtained when the levels of two markers in a pair tended to change in the opposite way with the increasing lesion severity. At the same time, combination of the suggested normalizer or the 'stably expressed' marker (U6, HMBS, miR-23a, miR-21, miR-191 or miR-181b) with oncogenic or onco-suppressor microRNA (that is, traditional normalization) generally resulted in lower ROC AUC values. This is illustrated in Fig. 4, where ROC AUCs are represented, which are calculated for paired combinations where the first marker in the pair is oncogenic (miR-34a, above) or onco-suppressor microRNA (miR-375, below) and the second marker, serving as 'normalizer', is any of the remaining markers from the selected list. This conclusion remains valid also for the use of geometric mean 2, 3 and 4 for the most stably expressed microRNAs (data not shown).

Dependence of ROC AUCs on the number of markers in linear classifiers. The application of the linear classifier method involving a larger number of ΔC_{q_i} values resulted in a marked improvement in the quality of classification, compared to the use of the best single ΔC_{q_i} values. Up to a certain limit, an increase in the number of ΔC_{q_i} values included in the classifier led to an increase in ROC AUC values, after which the inclusion of additional attributes was no longer significant (Fig. 5). As the ΔC_{q_i} values included in the classifier changed stepwise with the severity of lesion, the accuracy in detection of the lesion also increased with its severity. As can be seen from Fig. 5, the reliability of H-SIL detection in terms of ROC AUC

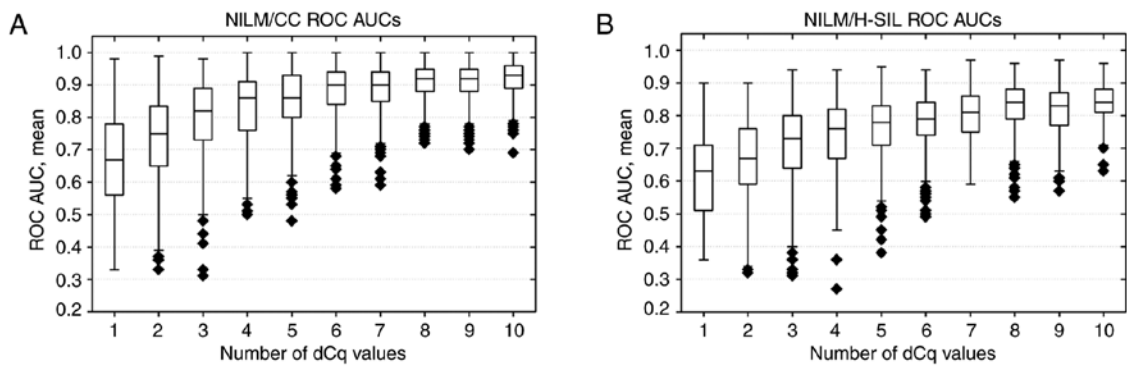


Figure 5. Box-whisker plots for mean ROC AUC values for separation of NILM from (A) CC or NILM from (B) H-SIL using classifiers based on different number of ΔCq_i values (from 1 to 10; shown on the x-axis). Inner lines, the median values; boxes, upper and lower quartiles; whiskers, non-outlier ranges; diamonds, outliers. For each bar corresponding to the classifiers including 2^{10} ΔCq_i values, data are presented for 351 classifiers randomly selected from $351!/(n!(351-n)!)$ possible variants (where 351 is the number of paired marker combinations, and n is the number of ΔCq_i values in the classifier). NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

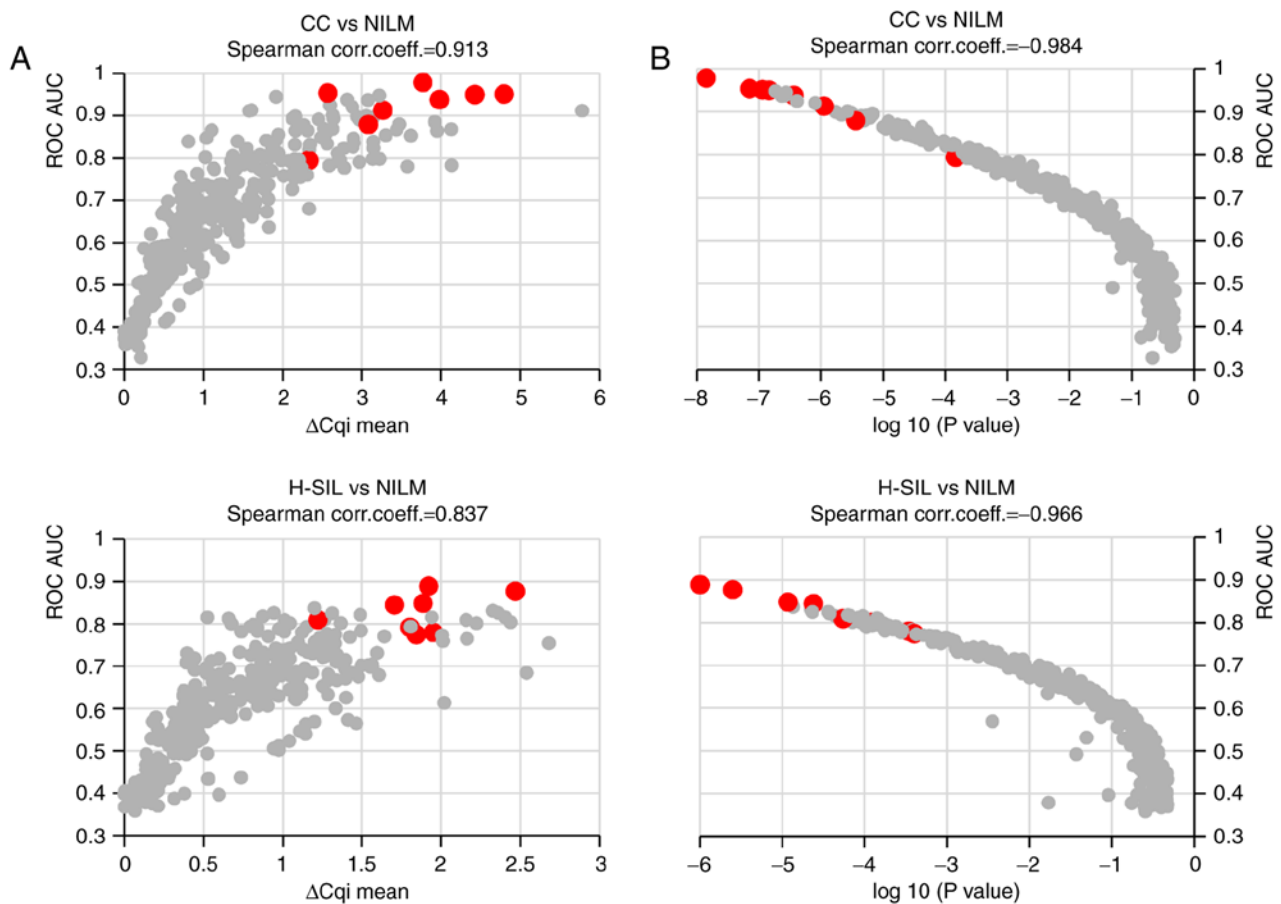


Figure 6. (A) Correlation of ROC AUCs (y-axis) and the mean ΔCq_i values (x-axis) for discriminating NILM from CC samples (above) and H-SIL (below) for each paired marker combination. (B) Correlation of ROC AUCs (y-axis) and \log_{10} (P-value) (x-axis) at discriminating NILM from CC samples (above) and H-SIL (below) for each paired marker combination. Each symbol on the plot matches a single paired combination. Red circles, combinations selected for the classifier. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

was lower, compared to CC detection regardless of the number of ΔCq_i values included in the classifier.

Selection of the best paired marker combinations for the linear classifier. The results presented in Fig. 5 indicate that the reasonable number of ΔCq_i values for inclusion in the final classifier in our case did not exceed 8. Thus, we decided to

select 8 ΔCq_i values for the construction of the final classifier. The selection was based on the statistical significance of the observed differences at separation of NILM from CC and NILM from H-SIL. The non-parametric Mann-Whitney criterion was used. Taking into account that the statistical criterion was applied twice (for the separation of the classes NILM/CC and NILM/H-SIL), the selected significance level

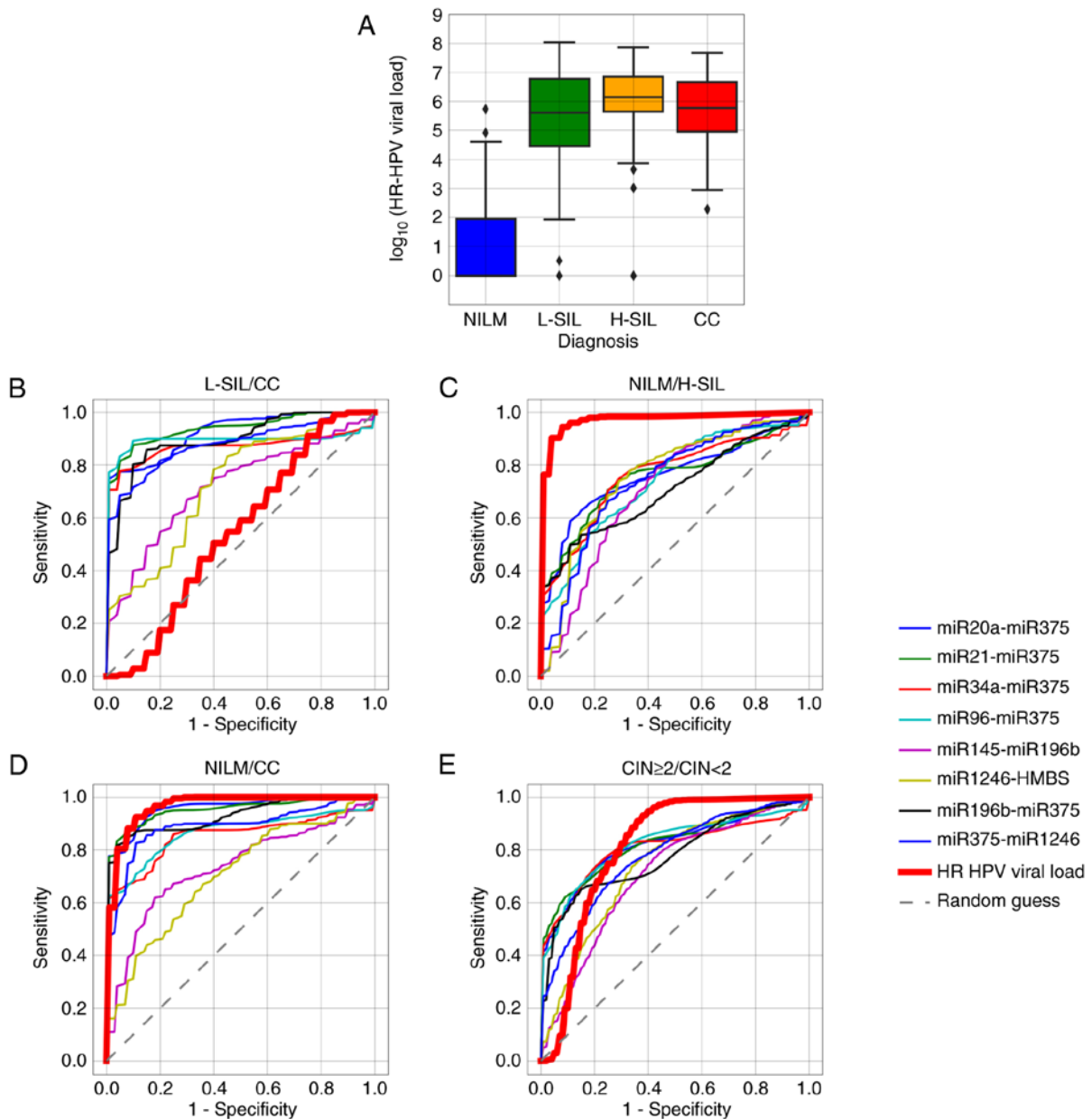


Figure 7. (A) Box-whisker plots for the HR-HPV DNA load in samples with different cytological diagnoses. Box, upper and lower quartiles; inner line, median value; whisker, non-outlier range; diamond, outliers. Red, invasive cancer; orange, H-SIL; green, L-SIL; blue, NILM. (B-E) ROC curves corresponding to 8 ΔCq_i values selected for the classifier and to the HR-HPV DNA viral load, for discriminating different classes of cytological diagnoses. HR-HPV, high-risk human papillomavirus; NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion; CC, cervical cancer; CIN, cervical intraepithelial neoplasia.

was $0.05/2=0.025$. Since we dealt with multiple hypothesis testing (the number of validations in the case of each classification was 351), we applied the Bonferroni correction, which is the most conservative for multiple verification. With this correction, statistically significant differences at a significance level of 0.025 were observed for 53 ΔCq_i values at separating NILM from CC and for 14 values at separating NILM from H-SIL (8 ΔCq_i values were simultaneously present in these two groups).

We also accepted the required effect size (the difference in the mean ΔCq_i values in groups of specimens from different cytology diagnoses) to be ≥ 1 . This was done since in the case when the ΔCq_i value did not meet this requirement (effect size < 1) it is comparable to the characteristic RT-qPCR bias.

In this case, despite the statistical significance of the ΔCq_i value, its practical significance was limited by the analytical variation of the method.

In Fig. 6, the correlations between the ROC AUC and the effect size for each ΔCq_i value (Fig. 6A) as well as between the ROC AUC and the achieved significance level (ASL) of each ΔCq_i value (Fig. 6B) are represented as scatter diagrams.

It can be seen from the figure that the ROC AUC values were correlated to a greater extent with the ASLs than with the effect sizes. The highest ROC AUCs corresponded to ΔCq_i values with the lowest ASLs at discriminating the specimens according to their cytological diagnoses. Thus, the ASL value was accepted as the primary criterion for selection of the paired combination. Among the combinations with the lowest

Table II. Detection of HPV DNA in the different groups of cytological specimens.

	NILM	L-SIL	H-SIL	CC
No of samples	40	29	62	43
HR-HPV(+), n (%)	14 (35%)	26 (89.66%)	61 (98.39%)	43 (100%)
HPV16(+) or HPV18(+), n (%)	4 (10%)	20 (69.0%)	56 (90.32%)	40 (93.02%)
Median (mean) viral load ^a in HR-HPV(+) samples	0.34 (39.9)	436.2 (15,687.3)	1,405.4 (6,341.8)	500.3 (4,515.3)

^aCopies of viral DNA/10⁸ copies of epithelial cell DNA. HR-HPV, high-risk human papillomavirus; NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion; CC, cervical cancer.

ASLs, the combinations with a larger effect size were further selected. In our case, for the top 5 paired combinations with the lowest ASL values (from 1.4×10^{-8} to 1.8×10^{-7}) at discriminating NILM from CC, the effect size ranged from 2.6 to 4.8. The top 5-paired combinations with the lowest ASLs (from 1.0×10^{-6} to 2.2×10^{-5}) at discriminating NILM from H-SIL the effect size was smaller (ranged from 1.2 to 2.5) but, nevertheless, exceeded 1, which suggests the feasibility of their use for H-SIL detection by a qPCR-based method.

Thus, we selected 6 paired marker combinations with a high statistical significance. Three of them performed best at discrimination of NILM from CC: miR21-miR375 (AUC=0.978±0.038; P=1.42×10⁻⁸), miR145-miR196b (AUC=0.954±0.069; P=7.08×10⁻⁸), miR20a-miR375 (AUC=0.951±0.064; P=1.14×10⁻⁷). The other three were selected to separate NILM from H-SIL: miR96-miR375 (AUC=0.889±0.088; P=1.00×10⁻⁶), miR1246-HMBS DNA (AUC=0.877±0.09; P=2.51×10⁻⁶), miR34a-miR375 (AUC=0.848±0.104; P=1.17×10⁻⁵). ΔC_q value for each of these combinations differed significantly between the specimens with different diagnoses even with the most conservative Bonferroni correction, and also had satisfactory effect size at discriminating these specimens. Two additional combinations were included in the classifier: miR196b-miR375 and miR375-miR1246. The calculation of these did not require involvement of additional markers but enabled further improvement in the classification quality.

In total, 9 markers were selected for the classifier: 8 microRNAs (miR-20a, miR-21, miR-34a, miR-96, miR-145, miR-196b, miR-375 and miR-1246) and cellular DNA content. The difference between invasive cancers and NILMs was statistically significant even for the raw C_q values for most of these microRNAs (except for miR-34a, see Table I).

Absence of correlation between the microRNA level changes and HR-HPV viral load in cytological specimens. The proportion of HR-HPV-positive samples with different cytological diagnoses, as well as the median and mean viral DNA loads, are provided in Table II. In all invasive cancers, except three, HPV16 and/or HPV18 DNA was detected. In one of these, only HPV73 DNA was found, in another - only HPV45, in the third - HPV73 and HPV45 simultaneously. Viral DNA loads in all these cases exceeded 10⁷/10⁸ copies of epithelial cell DNA. It should be noted that in our sample there were no NILM specimens with high HR-HPV DNA viral loads, although this is a fairly common situation even in the risk age group.

For none of the microRNAs, regardless of the normalization method, statistically significant correlation with the HR-HPV DNA load, genotype, or the number of genotypes in case of multiple infections was observed. The levels of several microRNAs notably differed between the HR-HPV-positive L-SILs and invasive cancers, while the viral loads did not differ significantly between these groups of specimens (Fig. 7A and B). Important to note, in our sample the viral load differed significantly in H-SILs and CCs compared to HR-HPV-positive NILMs (Fig. 7C and D). This may be partly due to the above-mentioned absence of NILMs with high HR-HPV viral loads. Nevertheless, for discrimination between subsamples of the CIN ≥2 specimens from the rest of the sample (CIN <2) some single ΔC_q values performed as well as the viral DNA load or even surpassed it (Fig. 7E).

Training of the linear classifier. Training of the linear classifier based on 8 ΔC_q values was performed with a sample of 171 specimens. Three CC samples with a highly degraded biomaterial (C_q values for HMBS and most marker microRNAs close to 40) were excluded from analysis. The decision function value (hereafter referred to as DF value) calculated by the classifier is dimensionless. In Fig. 8, the diagnostic characteristics of the trained classifier for discrimination between different groups of cervical specimens depending on the selected cut-offs are presented.

Fig. 9 presents box-whisker plots for 8 ΔC_q values included in the classifier and a box-whisker plot for DF values obtained for discriminating high-grade lesions (CIN ≥2) from the rest of the sample.

As the marker of high-grade lesions (CIN ≥2), the DF value performed better than the viral load or any ΔC_q value (ROC AUC=0.913, diagnostic sensitivity=83.4%, diagnostic specificity=81.2% at maximum Youden index) (Fig. 10B). Nevertheless, the DF value ranges sufficiently overlapped between groups of samples with different pathomorphological diagnoses (Fig. 10A). This overlap may result from the combined effect of the analytical biases, biological variation in the marker RNA levels, the cellular heterogeneity of cervical specimens, and the cytology misclassification.

Estimation of method reproducibility. The accuracy of DF value measuring results obviously depends on the analytical variation of RT-PCR. To evaluate the possible contribution of RT-PCR bias, we re-analyzed extracted nucleic acid preparations from two cytological specimens (from NILM and CC

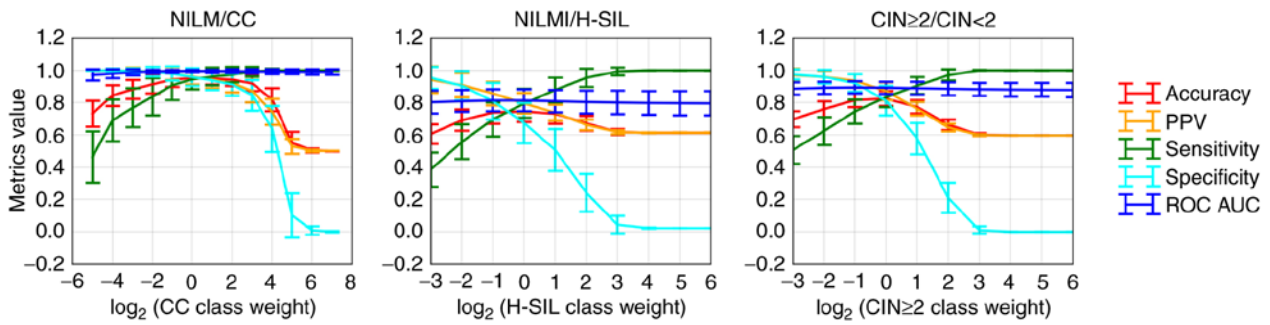


Figure 8. Discrimination of cervical samples with different cytology by the microRNA classifier. x-axis, \log_2 of the weighting factor for the group corresponding to the higher degree of lesion in the pair (the change in this coefficient is identical to the change in cut-off for the sample fitting into a particular class). y-axis, metrics values. Standard deviations corresponding to different weighting coefficient values are presented. NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; CIN, cervical intraepithelial neoplasia; CC, cervical cancer; PPV, positive predictive value.

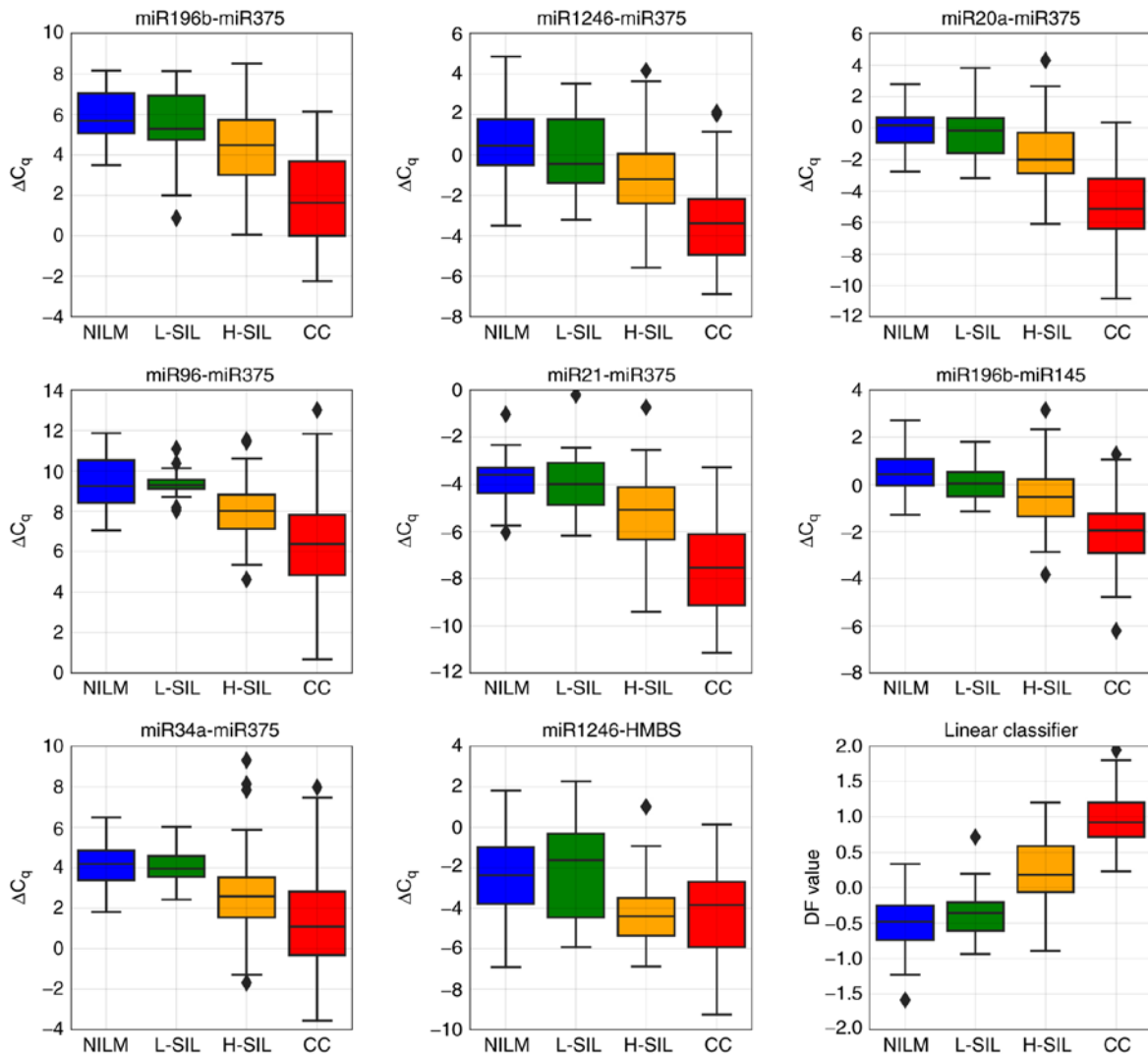


Figure 9. Box-whisker plots for the ΔCq_i values selected for the classifier and for DF value calculated by the classifier using these ΔCq_i values. Upper and lower quartiles are shown by the box. Inner line, median value; whisker, non-outlier range; diamond, outlier. Red, invasive cancer; orange, H-SIL; green, L-SIL; blue, NILM. NILM, negative for intraepithelial lesions or malignancy; L-SIL, low-grade squamous intraepithelial lesion; H-SIL, high-grade squamous intraepithelial lesion; CC, cervical cancer.

patients), in which each marker was analyzed 4-fold and each Cq value obtained for each marker in the sample was used in repeated calculations of DF value for this sample. In Fig. 11A, the histograms of DF values for each specimen (calculated for

10,000 randomly selected combinations of the obtained Cq values from the possible 4.3×10^9 combinations) and the histograms of DF values for the NILMs and CCs are presented. The histograms of DF values for specimens are several times

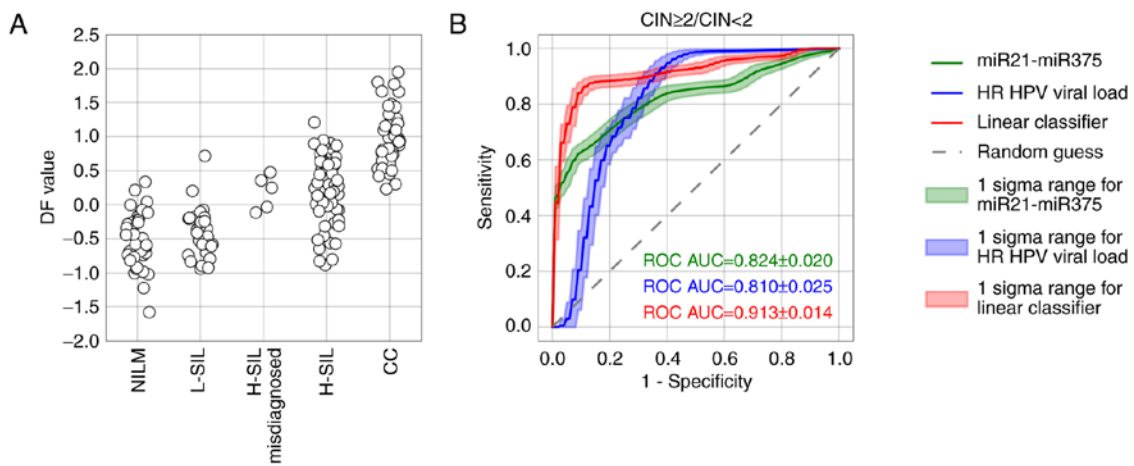


Figure 10. (A) Scatter plot of DF values calculated by microRNA classifier for different groups of cytological specimens. Five carcinomas *in situ* initially misdiagnosed as L-SILs are highlighted as a separate group. (B) ROC curves for the detection of CIN ≥ 2 by the miR21-miR375 ΔCq value (green), HR-HPV DNA load (blue) and DF value (red). HR-HPV, high-risk human papillomavirus; NILM, negative for intraepithelial lesions or malignancy; H-SIL, high-grade squamous intraepithelial lesion; L-SIL, low-grade squamous intraepithelial lesion; CC, cervical cancer; CIN, cervical intraepithelial neoplasia.

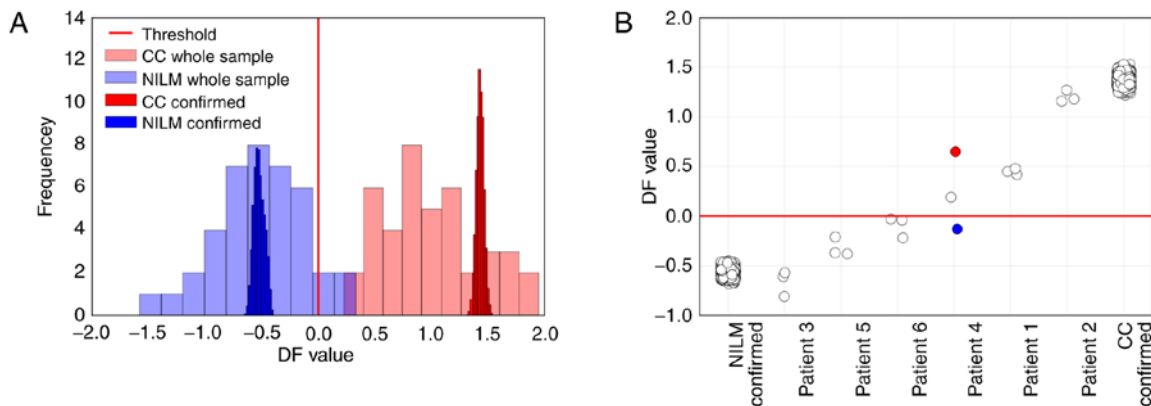


Figure 11. (A) blue and red, the histograms of 10,000 decision function (DF) values calculated for randomly selected combinations of Cq values derived from multiple RT-PCRs performed for each marker in the classifier; light blue and light red, the histograms of DF values calculated for parts of the whole sample corresponding to the NILM and CC classes, respectively. (B) DF values calculated for multiple PAP slides from patients with different cytology diagnoses (patients 1-6) and for multiple RT-PCR repeats made for single samples from patients with confirmed NILM and CC. Vertical red line (left) and horizontal red line (right), accepted cut-off value for detecting CIN ≥ 2 . NILM, negative for intraepithelial lesions or malignancy; CC, cervical cancer; CIN, cervical intraepithelial neoplasia.

narrower than the histograms of DF values for the NILM and CC classes, which means that the contribution of RT-PCR bias into the accuracy of the DF value calculation was not high.

For 6 patients with different cytological diagnoses, 3 PAP slides prepared from a single smear were tested. In Fig. 11B, the DF values calculated for the nucleic acid preparations extracted from each slide are provided compared to 10,000 DF values for NILM and CC samples calculated as described above. For 5 out of 6 patients, the variation between the slides was comparable to the RT-PCR bias. Nevertheless, for patient no. 4, different classification results for the different slides were obtained. We suggest that the observed variation was determined to a greater extent by the procedure of preparing the slide (which affects the amount and integrity of the material analyzed) than by the nucleic acid extraction procedure. Thus, in the case of patient no. 4, the maximum DF value (marked red in Fig. 11B) corresponded to the slide for which the HMBS DNA Cq value was ~ 39 and most Cq values for microRNA markers were beyond the linear range

of qPCR. The minimum DF value (marked blue) corresponded to the slide from the same patient, where HMBS DNA Cq was ~ 33 (~ 100 -fold greater concentration of input cellular DNA) and Cq values for microRNA markers were within the qPCR linear range, which suggest that the classification results for this slide were more reliable.

When comparing the ROC AUCs for DF values calculated for subsamples of specimens with very different nucleic acid concentrations (the difference between the max and min Cq values for HMBS=11.5, the difference between the max and min Cq values for the most stably expressed miR-21=9.5), we observed no difference. In addition, we re-classified 3 samples after the 4-fold dilution of nucleic acid preparation. The differences in the obtained DF values were within the limits of the variation evaluated in the previous experiment and in no case led to reclassification of the sample compared to non-diluted preparation (data not shown). Taken together, these facts support the lack of a significant contribution made by PCR efficiency to the classification reliability.

Discussion

Recent research demonstrates the feasibility of using microRNAs as biomarkers in cervical cancer (CC) screening and follow-up. However, the reliable detection of biologically relevant changes in microRNA levels may be compromised by the variability introduced by the methodology of analysis and the cellular heterogeneity of clinical specimens. It is this problem that may account for the fact that none of the numerous but diverse design and methodology methods has achieved acceptable diagnostic characteristics when using microRNA profiling for the diagnosis of cervical neoplasia.

As mentioned above, the lists of the microRNA markers, deregulation of which accompanies cervical lesions of different degrees, as well as the direction of their concentration changes, often differ between different studies. This can certainly be attributed to the peculiarities of the microRNA isolation and quantitation techniques used. Each technology of microRNA profiling (microarrays, NanoString counting, RNA-seq, TaqMan low density array) has its own sources of bias. Even the widely used techniques for microRNA analysis (Exiqon and TaqMan), based on similar RT-qPCR techniques, demonstrate serious discrepancies in the efficiency of detecting various microRNAs (42-46). To search for the most suitable markers for the further design of microRNA diagnostic tools, researchers typically analyze a limited sample of patients by microarrays or RNAseq, which allows one to choose from the great number of microRNAs; then candidate microRNAs are validated by real-time PCR. This approach may result in neglecting some relevant microRNAs due to lower efficiency of their detection by chip-based methods or RNA-seq biases.

The normalization method is another apparent factor affecting the reliability of microRNA quantification. In cases where the disease-associated microRNA level changes are relatively small (as in high-grade CINs), the choice of the normalization strategy is particularly important, since in such cases the biologically relevant microRNA expression changes may be comparable to the biases introduced by the method. Some authors have proposed individual normalizers for microRNA level measurements in cervical epithelium by real-time qPCR, e.g., U6 snRNA (18,21,23,47), miR-23 (22,38) and miR-92a (48). The use of such normalizers by our research group did not lead to favorable results for the classification of cervical lesions in cytological preparations. Moreover, classification results were strongly normalizer-dependent. With some normalizers, 'wave-like' changes in the content of particular microRNAs with the increase in lesion severity from NILM to CC were observed (data not shown). Such changes were not consistent with the known biological functions of these microRNAs and can be considered artifacts of the analysis. On the contrary, the use of the classifier based on paired microRNA combinations selected as described above led to better classification results than any single normalizer or geometrical mean of the 3-5 most 'stably expressed' microRNAs. This can be attributed to the following. i) The amplitudes of disease-related microRNA level changes may be comparable to the analytical variation of the method used, which hinders the reliable registration of such changes. The paired marker approach (where the concentrations of two markers in the pair, the levels of which change in opposite directions at neoplastic transformation, are

reciprocally normalized) helps to better distinguish biologically relevant change in microRNA profile from the 'noise' (the fluctuation of measurement results due to analytical variation). This approach, compared to traditional normalization, better compensates for the biases caused by the analytical variation of the method and/or the cellular heterogeneity of the specimen. The lower the level of physiological changes in the content of the marker is, the greater the contribution of this compensation. Similarly to the traditional normalization, this approach also compensates for the variation associated with the amount and degradation level of input biomaterial. ii) The use of normalizers selected by formal criteria can generate a system error. Thus, in the present study, the most 'stably expressed' microRNA was miR-21. For this microRNA, known as oncogenic in multiple cancers including CC, one could expect the increase in concentration accompanying cervical neoplastic transformation. In our case, this was most likely, as even without any normalization the decline in raw miR-21 Cq values in H-SIL and CC compared to NILM was statistically significant (Table I). The use of a normalizer whose concentration itself *de facto* increases with the severity of the lesion, will inevitably lower or even mask the statistically significant concentration changes of the relevant microRNAs, the levels of which also tend to decline in precancerous lesions and cancer. iii) The cell heterogeneity of the analyzed preparation may result in 'watering down' the observed microRNA level changes. The specimen may contain cells corresponding to the different degree of neoplasia in different proportions. In addition, the cytological preparation may contain an admixture of cells not related to the lesion. Thus, the resulting microRNA profile may appear 'intermediate', which will complicate the classification. In the analysis of cytological specimens, it is particularly difficult to take into account and compensate for this source of biases. In addition, it cannot be ruled out that neoplasms considered as belonging to a single class based on pathomorphological classification can in fact represent different subclasses for which microRNA expression profiles differ.

In the present study, the best individual microRNA marker of cervical neoplasia was miR-375, which was present in 6 of 8-paired combinations included into the classifier. This was the only microRNA, for which the median ΔCq_i values changed monotonically in the range NILM/L-SIL/H-SIL/CC regardless of the second marker ('normalizer') in the paired combination. The mean ΔCq_i values calculated for the combinations including this microRNA also demonstrated the greatest difference between the lesions of different severity. miR-375 is a known tumor suppressor involved in the development of CC (21,49). The other microRNAs included in the classifier displayed a tendency for increase or decrease with the degree of lesion, which, however, was significantly dependent on the choice of the normalizer.

Thus, we developed a technique for detecting cervical precancerous lesions and cancer in cytological specimens using a microRNA-based classifier. The method demonstrated acceptable diagnostic characteristics and analytical reproducibility. The analysis can be performed with the same nucleic acid preparation as used for HPV testing, genotyping, and the measurement of the HR-HPV viral DNA load. It is now generally accepted that the detection of HR-HPV DNA has a high NPV for precancerous cervical lesions while its PPV is low due

to the high frequency of transient HPV carriage without cellular transformation. The differences in microRNA expression more likely reflect cellular events related to transformation and, therefore, may provide a higher PPV if used as a diagnostic marker. Nevertheless, the NPV of such an analysis can also remain significant. In our case, in sample no. 129 with a cytological diagnosis of H-SIL and a histologically confirmed CIN3, in which HPV DNA was not detected, the DF value corresponded to high-grade CIN. Our results support the feasibility of using small-scale microRNA profiling for detection of high-grade cervical intraepithelial neoplasia. Taking into account the very high NPV of HPV-testing in cervical screening, the microRNA profiling and the HR-HPV DNA testing may serve as complementary tools in the molecular testing for cervical lesions.

Some limitations of our research should be emphasized. First, the choice of microRNA markers was made from a limited set, which in itself could fail to be optimal. Secondly, the sample was relatively small and enriched with invasive cancers, which could have led to overestimating the diagnostic characteristics of the classifier. Thirdly, the study was a single-setting. A separate issue in our case is the interpretation of the results of the analysis of samples cytologically characterized as NILM. Since in our case this diagnosis was not verified, we cannot exclude misclassification of some preparations from this group. Further clinical validation of the developed microRNA-based classifier and its use in multicenter and follow-up studies will additionally substantiate the conclusion concerning the prospects of its clinical use.

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