Evaluation of microRNA-205 expression as a potential triage marker for patients with low-grade squamous intraepithelial lesions

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Abstract. High-risk human papillomavirus (HPV) testing is a recommended triage approach for females with atypical squamous cells of undetermined significance (ASCUS), but due to its poor specificity this approach is not recommended for patients with low-grade squamous intraepithelial lesions (LSIL). The objective of the current study was to determine microRNA (miR)-205 expression levels in liquid-based cytology (LBC) samples, and evaluate their ability to predict cervical intraepithelial neoplasia grade 2/3 or worse (CIN2/3+) in females with minor cytological abnormalities. LBC samples were obtained from patients attending the Swedish Cervical Cancer Screening Program. The Mann-Whitney U test, one-way analysis of variance, Kruskal-Wallis test, Spearman rank order correlation analysis, and Pearson’s χ2 test were used to assess the results. Accuracy analyses indicated that high miR-205 expression had a significantly higher specificity to high-risk HPV testing, and a sensitivity similar to that of high-risk HPV testing to predict CIN2+ and CIN3+ in women with LSIL, but not those with high-grade squamous intraepithelial lesions. Although further research is required for females with LSIL, miR-205 expression in LBC samples may be a novel triage marker for, or a beneficial supplement to high-risk-HPV testing in these patients.

Introduction

Cervical cancer is a leading cause of cancer-associated mortality among females worldwide. It accounts for 13% of all female cancer cases, with >500,000 new cases and ~275,000 mortalities occurring annually (1). In Sweden, 450 new cases and 150 mortalities occur each year (2). According to reports from the organized Swedish Cervical Cancer Screening Program, ~30,000 women exhibit some form of cellular abnormality and require follow-up with cervical biopsy (3).

Persistent infection with human papillomavirus (HPV) is the causative agent in cervical cancer (4). HPV depends on differentiating keratinocytes; the infection of the squamous epithelia alone is not sufficient for the infection to progress to neoplasia (5). The expression of the HPV oncogenes E6 and E7 is able to inactivate p53 and retinoblastoma proteins, leading to methylation and mutation of the host genome DNA and resulting in the initiation of and progression towards cancer (6,7). The use of high-risk HPV (8) testing in primary screening for cervical disease has exhibited a high

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sensitivity (9), but the specificity of this method is low, and thus a follow-up test must be administered prior to treatment (10).

The implementation of organized cervical cancer screening programs has reduced the incidence of cervical cancer considerably (11). However, several previous studies have demonstrated that conventional cytology has a limited sensitivity (only 50-70%) to detect cervical intraepithelial neoplasia (CIN) (12,13). Liquid-based cytology (LBC) was developed to improve diagnostic reliability (14), as it offers the possibility to use the same sample for HPV testing and triage. Such triage is recommended for women with atypical squamous cells of undetermined significance (ASCUS) due to its high sensitivity, but it is not recommended for women with low-grade squamous intraepithelial lesions (LSIL) due to the high prevalence of high-risk HPV in this population, which generally leads to poor specificity (15). The low predictive value of HPV testing among females with minor cytological abnormalities may create unnecessary concern among healthy patients and contribute to a significant risk of over-diagnosis and over-treatment. The use of predictive biomarkers is a novel approach to improving the diagnosis and management of patients with LSIL.

MicroRNA (miRNA) is a small, non-coding RNA that is ~22 nucleotides in length. miRNA has an important role in pathological processes, including viral infection and cancer development (4). Generally, miRNA negatively regulates gene expression at the post-transcriptional level via transcription inhibition and/or translation suppression (16). Previous studies have identified altered miRNA expression profiles in human cervical cancer tissues and cell lines, and several of them, including miRNA (miR)-145, miR-21 and miR-205, are consistently dysregulated in cervical cancer tissue compared with normal cervical tissue (17-19). In our previous study, it was revealed that miR-205 expression was significantly increased in cervical cancer tissue compared with matched normal cervical tissue, and that miR-205 has an oncogenic role in cervical cancer through the promotion of cell proliferation and migration (20). This prompted the further investigation of the potential value and clinical applications of miR-205 in the present study.

Recently, miRNAs were suggested as potential biomarkers for the diagnosis or prognosis of different cancer types, including cervical cancer (21-24). Due to the requirement for non-invasive detection methods, the majority of the applications focused on serum or plasma samples. For example, serum miR-203 expression was an independent predictive marker for lymph node, peritoneal and distant metastases, and a poor prognosis marker in patients with gastric cancer (8). In patients with colorectal cancer, circulating miR-103, miR-720 and miR-372 were potential novel biomarkers: High serum miR-103 expression levels were significantly associated with histological differentiation grade and lymphatic invasion; high serum miR-720 levels were significantly associated with lymph node metastasis; and high miR-372 levels were significantly associated with tumor size, tumor-node-metastasis stage and poorer overall survival (25,26). Downregulation of miR-205 expression in colorectal cancer predicts the risk of lymph node metastasis (27). Circulating miR-205 and let-7f together were reported to be diagnostic biomarkers for ovarian cancer (28). Serum miR-205 expression was revealed to be significantly downregulated in patients with glioma compared with healthy controls and was a novel and valuable biomarker for the diagnosis of glioma, and a prognostic factor for those with advanced-grade tumors (29). Ma et al (30) reported that upregulated serum miR-205 is a predictive marker for the prognosis of cervical cancer, and Zhao et al (31) reported that high circulating miR-20a expression levels represent a potential marker for detecting lymph node metastasis in early-stage cervical cancer. However, only a limited number of studies have performed miRNA detection in cervical exfoliated cells (32,33).

The aim of the present study was to investigate whether miR-205 expression may be used as a novel triage approach to predict high-grade CIN in LBC samples from patients attending the population-based Swedish Cervical Cancer Screening Program.

Materials and methods

Study population. Between 2008 and 2012, LBC samples were collected from 140 women with squamous intraepithelial lesions or squamous cell carcinoma detected within the framework of the Swedish Cervical Cancer Screening Program in Stockholm, Sweden (34). Cervical cells for LBC were obtained from the ectocervix and endocervix of the uterus, preserved in PreservCyt medium (ThinPrep™, Hologic, Boxborough, MA, USA) at -20˚C, and evaluated at the Department of Clinical Pathology and Cytology, Karolinska University Hospital (Solna-Stockholm, Sweden). Cytological results were categorized according to the Bethesda classification (35), with modifications based on Swedish recommendations: Samples with atypocytosis, but without cellular atypia, were classified as ‘within normal limits’ (WNL), and LSIL included mild dysplasia only. The diagnosis and staging of CIN was based on colposcopy and histology, and grouped into normal histology (WNL), CIN grade 1 (CINI), CIN grade 2 (CIN2) and CIN2 or worse (CIN2+). Histological information and high-risk-HPV test results were retrieved from the medical and laboratory records at the Karolinska University Hospital.

This study was approved by the Ethical Review Board at Karolinska Institutet (Stockholm, Sweden) and written informed consent was obtained from all participants prior to sample collection.

RNA extraction. Cervical cells were collected by centrifugation and washed with cold PBS twice, followed by total RNA extraction using the mirVana™ miRNA isolation kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), all according to the manufacturer's protocol. RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and stored at -80°C for further use.

TaqMan RT-qPCR. miR-205 expression was quantified by TaqMan reverse transcription quantitative polymerase chain reaction (RT-qPCR) using the StepOne Plus real-time PCR system (Thermo Fisher Scientific, Inc.). cDNA was synthesized from 100 ng of RNA using the TaqMan miRNA reverse transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). The pre-designed TaqMan assays for miR-205
(ID 000509) and the reference material RNU6B (ID 001093) were purchased from Thermo Fisher Scientific, Inc. (20). All reactions were performed in triplicate, according to the manufacturer's protocol. The relative expression of miR-205 was normalized to RNU6B and reported as 2^ΔΔCq (36).

**HPV DNA detection.** HPV testing was performed at Karolinska University Hospital. Briefly, DNA was extracted from the LBC suspensions using the MagNA Pure LC Robot (Roche Diagnostics, Basel, Switzerland). HPV DNA detection and genotyping were carried out using the Linear Array HPV Genotyping test (Roche Diagnostics, Mannheim, Germany) and Cobas 4800 (Roche Diagnostics, Basel, Switzerland), which detects 37 HPV types: High-risk-HPV types (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59/68/73, and 82); probable high-risk-HPV types (HPV26, 53, and 66); and low-risk- or undetermined-risk HPV types (HPV6, 11, 40, 42, 43, 44, 54, 55, 61, 62, 64, 67, 69, 70, 71, 72, 81, 83, 84, IS39, and CP6108).

**Statistical analysis.** Data were entered into Statistica 7.0 (Statsoft, Inc., Tulsa, OK, USA). The difference in miR-205 expression between all HPV-positive and all HPV-negative samples was analyzed using the Mann-Whitney U test. The associations between miR-205 expression levels and diagnoses (including cytology, histology and the final histopathological diagnosis) were analyzed by the Kruskal-Wallis one-way analysis of variance (ANOVA) test. The correlation of miR-205 expression with age was analyzed with the Spearman Rank Order correlation and Pearson's χ² test. Sensitivity and specificity calculations were performed using VassarStats online software (http://vassarstats.net/). P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Cytology, histology, final diagnosis and HPV status.** The median age of the 140 females in the study sample was 32.5 years (range, 23-59 years). Of these patients, 123 (123/140, 87.9%) had histological information available, and 115 (115/140, 82.1%) had HPV test results available in the medical and laboratory records at the Karolinska University Hospital. Among the patients with HPV results, 93 were HPV-positive (93/115, 80.9%) and 22 were HPV-negative (22/115, 19.1%) (Table I).

Of the 93 HPV-positive women, only one (no. 43) was infected with a low-risk HPV type (HPV54). Eighty-seven patients were infected with at least one high-risk HPV type, and 43 (43/93, 46.2%) were infected with either HPV16 or 18, the two most common high-risk HPV types (Table II).

**Sensitivity and specificity of high miR-205 expression levels to predict CIN2+ and CIN3+ in LSIL and HSIL.** Sensitivity and specificity analyses were performed among patients with LSIL and high-grade squamous intraepithelial lesions (HSIL), based on high miR-205 expression levels and HPV positivity. The specificity of HPV testing to predict the absence of CIN2+ and cervical intraepithelial neoplasia grade 3 or worse (CIN3+) was 0.11 [95% confidence interval (CI), 0.03-0.30] and 0.08 (95% CI, 0.02-0.23), respectively, in women with LSIL. The specificity of high miR-205 expression levels was 0.63 (95% CI, 0.42-0.80) and 0.57 (95% CI, 0.40-0.72), which was significantly higher than that of HPV testing. Although positivity for HPV16, HPV18, or HPV16/18 exhibited a higher sensitivity (0.88, 0.96, and 0.85, respectively, to predict CIN2+; 0.83, 0.94, and 0.73, respectively, to predict CIN3+) than high miR-205 expression levels, these values were not statistically significant (Table III).

Although the specificity of HPV testing to predict CIN3+ in patients with HSIL was lower than that of high miR-205 expression levels (0.16, 95% CI: 0.05-0.37; 0.38, 95% CI, 0.23-0.56, respectively), this trend was also not statistically significant (Table IV).

**Cytology (N=140)**

| Characteristic | N | % |
|---------------|---|---|
| WNL           | 18 | 12.86 |
| LSIL          | 45 | 32.14 |
| HSIL          | 74 | 52.86 |
| Cancer        | 3  | 2.40  |

**Histology (N=123)**

| Characteristic | N | % |
|---------------|---|---|
| WNL           | 9  | 7.32  |
| CIN1          | 35 | 28.46 |
| CIN2          | 28 | 22.76 |
| CIN3          | 47 | 38.21 |
| Cancer        | 4  | 3.25  |

**Final histopathological diagnosis (N=140)**

| Characteristic | N | % |
|---------------|---|---|
| Positive      | 93 | 80.87 |
| Negative      | 22 | 19.13 |

**Table I. Summary of clinical features of the study sample (N=140).**

N, number; WNL, within normal limits (normal cytology); LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CIN1, cervical intra-epithelial neoplasia grade 1; CIN2, cervical intra-epithelial neoplasia grade 2; CIN3, cervical intra-epithelial neoplasia grade 3; HPV, human papillomavirus.
Table II. Detailed clinical information and miR-205 expression in 140 patients.

| Sample ID | Age | miR-205 (2^ΔΔCq) | Cytology diagnosis | Histology diagnosis | Final diagnosis | Status | Subtype | HR/LR-HPV |
|-----------|-----|------------------|-------------------|--------------------|----------------|--------|---------|-----------|
| 2         | 37  | 61.0439          | LSIL              | CIN1               | CIN1           | Positive | 31      | HR-HPV    |
| 3         | 30  | 6.7449           | WNL               | n.a.               | WNL            |         |         |           |
| 4         | 29  | 34.5873          | HSIL              | CIN3               | CIN3           | Positive | 16      | HR-HPV    |
| 5         | 35  | 2.2973           | HSIL              | CIN3               | CIN3           | n.a.    |         |           |
| 6         | 34  | 19.0510          | LSIL              | CIN3               | CIN3           | Positive | 16      | HR-HPV    |
| 7         | 32  | 23.3276          | HSIL              | CIN3               | CIN3           | Positive | 16      | HR-HPV    |
| 8         | 39  | 25.2251          | HSIL              | CIN2               | CIN2           |         |         |           |
| 9         | 41  | 3.8929           | LSIL              | CIN1               | CIN1           | Negative |         |           |
| 10        | 30  | 3.3291           | HSIL              | CIN2               | CIN2           | Positive | 18      | HR-HPV    |
| 11        | 37  | 20.8132          | HSIL              | CIN2               | CIN2           | Positive | 58      | HR-HPV    |
| 12        | 26  | 13.9071          | HSIL              | CIN2               | CIN2           | Positive | 58      | HR-HPV    |
| 13        | 34  | 2.4690           | HSIL              | CIN3               | CIN3           | Positive | 16      | HR-HPV    |
| 14        | 33  | 4.7576           | HSIL              | CIN1               | CIN1           | Positive | 39      | HR-HPV    |
| 15        | 30  | 29.0087          | LSIL              | CIN2               | CIN2           | Positive | 16,31   | HR-HPV    |
| 16        | 28  | 1.2505           | LSIL              | CIN1               | CIN1           | Positive | 16,52,82| HR-HPV    |
| 17        | 43  | 7.4901           | WNL               | n.a.               | WNL            | Negative |         |           |
| 18        | 42  | 2.2771           | LSIL              | CIN1               | CIN1           | Positive | 31,51,73| HR-HPV    |
| 19        | 59  | 7.2827           | LSIL              | CIN2               | CIN2           | Positive | 31,59   | HR-HPV    |
| 20        | 28  | 20.1998          | HSIL              | CIN3               | CIN3           | Positive | 51      | HR-HPV    |
| 21        | 43  | 0.1200           | WNL               | CIN1               | CIN1           | Positive |         |           |
| 22        | 31  | 2.4038           | HSIL              | CIN3               | CIN3           | Positive | 18      | HR-HPV    |
| 23        | 44  | 2.4038           | HSIL              | CIN3               | CIN3           | Positive | 45      | HR-HPV    |
| 24        | 31  | 7.4764           | HSIL              | CIN1               | CIN2           | Positive |         |           |
| 25        | 43  | 59.1805          | LSIL              | CIN2               | CIN2           | Positive |         |           |
| 26        | 26  | 3.8242           | HSIL              | CIN1               | CIN2           | Positive |         |           |
| 27        | 27  | 19.4811          | HSIL              | CIN3               | CIN3           | Positive | 53,73   | HR-HPV    |
| 28        | 32  | 2.4561           | LSIL              | CIN1               | CIN1           | Positive | 82      | HR-HPV    |
| 29        | 26  | 1.4752           | LSIL              | WNL                | CIN1           | Positive | 31,56   | HR-HPV    |
| Sample ID | Age | miR-205 (2^ΔΔCq) | Cytology diagnosis | Histology diagnosis | Final diagnosis | HPV | Status | Subtype | HR/LR-HPV |
|-----------|-----|------------------|-------------------|-------------------|----------------|-----|--------|---------|----------|
| 40        | 30  | 14.2268          | HSIL              | CIN3              | CIN3           |     | n.a.   |         |          |
| 41        | 28  | 6.1169           | HSIL              | WNL               | CIN3           |     | Negative|         |          |
| 42        | 33  | 12.0710          | HSIL              | n.a.              | CIN3           |     | n.a.   |         |          |
| 43        | 39  | 3.5259           | HSIL              | CIN3              | CIN3           |     | Positive| 54      | LR-HPV   |
| 44        | 35  | 10.6758          | HSIL              | CIN2              | CIN2           |     | Positive| 16      | HR-HPV   |
| 45        | 43  | 7.5600           | HSIL              | CIN3              | CIN3           |     | Positive| 56,     | HR-HPV   |
| 46        | 26  | 72.3169          | LSIL              | CIN2              | CIN2           |     | Positive| 39,51,58,73 | HR-HPV |
| 47        | 26  | 41.7024          | HSIL              | CIN3              | CIN3           |     | Positive| 16      | HR-HPV   |
| 48        | 43  | 22.4166          | WNL               | n.a.              | WNL            |     | Positive|         |          |
| 49        | 45  | 16.2120          | WNL               | n.a.              | WNL            |     | Positive|         |          |
| 50        | 41  | 7.5508           | WNL               | n.a.              | WNL            |     | Negative|         |          |
| 51        | 35  | 7.5375           | WNL               | n.a.              | WNL            |     | Negative|         |          |
| 52        | 26  | 32.8494          | HSIL              | CIN3              | CIN3           |     | Positive| 18,31,51,52,66,68 | HR-HPV |
| 53        | 39  | 14.3435          | LSIL              | CIN1              | CIN1           |     | Positive| 18,51   | HR-HPV   |
| 54        | 39  | 8.1765           | HSIL              | CIN2              | CIN2           |     | Negative|         |          |
| 55        | 29  | 54.1454          | HSIL              | CIN1              | CIN2           |     | Positive| 16,33,59 | HR-HPV   |
| 56        | 43  | 33.0009          | HSIL              | CIN1              | CIN2           |     | Positive| 59      | HR-HPV   |
| 57        | 33  | 6.3544           | HSIL              | CIN3              | CIN3           |     | n.a.   |         |          |
| 58        | 34  | 3.6957           | WNL               | n.a.              | WNL            |     | Positive| 18      | HR-HPV   |
| 59        | 43  | 2.4636           | HSIL              | CIN3              | CIN3           |     | Positive| 52      | HR-HPV   |
| 60        | 54  | 28.7410          | WNL               | n.a.              | WNL            |     | Negative|         |          |
| 61        | 46  | 18.0521          | WNL               | n.a.              | WNL            |     | Negative|         |          |
| 62        | 27  | 7.9717           | HSIL              | CIN2              | CIN2           |     | Positive| 16      | HR-HPV   |
| 63        | 51  | 6.7104           | WNL               | n.a.              | WNL            |     | Negative|         |          |
| 64        | 41  | 0.7032           | HSIL              | CIN3              | CIN3           |     | Positive| 52      | HR-HPV   |
| 65        | 29  | 12.8313          | HSIL              | CIN3              | CIN3           |     | n.a.   |         |          |
| 66        | 42  | 6.9052           | HSIL              | CIN2              | CIN2           |     | Positive| 16      | HR-HPV   |
| 67        | 32  | 1.3904           | LSIL              | WNL               | CIN1           |     | Negative| n.a.    |          |
| 68        | 28  | 0.3772           | HSIL              | CIN2              | CIN2           |     | Positive| 16      | HR-HPV   |
| 69        | 47  | 6.7330           | Cancer            | Cancer            | Cancer         |     | n.a.   |         |          |
| 70        | 29  | 7.7228           | WNL               | CIN1              | CIN1           |     | Positive| 16      | HR-HPV   |
| 71        | 28  | 9.6434           | HSIL              | CIN2              | CIN2           |     | Positive| 16      | HR-HPV   |
| 72        | 26  | 6.9220           | HSIL              | CIN2              | CIN2           |     | Positive| 16,33   | HR-HPV   |
| Sample ID | Age | miR-205 \(2^{-\Delta\Delta Cq}\) | Cytology | Histology | Final Diagnosis | HPV |
|-----------|-----|-------------------------------|----------|-----------|----------------|-----|
|           |     |                               | diagnosis| diagnosis | Status          | Subtype |
| 74        | 41  | 7.0546                        | HSIL     | CIN3      | CIN3            | n.a. |
| 75        | 32  | 4.2851                        | LSIL     | CIN2      | CIN2            | Positive |
| 76        | 44  | 11.3330                       | HSIL     | WNL       | WNL             | n.a.   |
| 78        | 51  | 2.6267                        | WNL      | n.a.      | WNL             | Negative |
| 79        | 32  | 8.7506                        | LSIL     | CIN1      | CIN1            | Positive |
| 80        | 28  | 1.6829                        | HSIL     | CIN2      | CIN2            | n.a.   |
| 81        | 30  | 3.4284                        | WNL      | n.a.      | WNL             | Negative |
| 82        | 48  | 17.9020                       | LSIL     | WNL       | CIN1            | Positive |
| 84        | 30  | 13.8683                       | LSIL     | CIN1      | CIN1            | Positive |
| 85        | 48  | 6.9998                        | WNL      | n.a.      | WNL             | Negative |
| 86        | 31  | 6.0450                        | HSIL     | CIN3      | CIN3            | n.a.   |
| 87        | 30  | 3.0692                        | HSIL     | CIN2      | CIN2            | n.a.   |
| 88        | 56  | 12.7754                       | WNL      | n.a.      | WNL             | Negative |
| 89        | 28  | 16.8543                       | WNL      | WNL       | WNL             | Negative |
| 90        | 33  | 2.0479                        | HSIL     | WNL       | CIN2            | Positive |
| 91        | 27  | 40.2667                       | LSIL     | CIN1      | CIN1            | Negative |
| 93        | 31  | 28.9839                       | HSIL     | CIN3      | CIN3            | Positive |
| 94        | 29  | 45.6632                       | HSIL     | CIN2      | CIN2            | n.a.   |
| 95        | 37  | 6.2884                        | LSIL     | CIN1      | CIN1            | Positive |
| 97        | 28  | 38.5117                       | HSIL     | CIN1      | CIN2            | Positive |
| 98        | 29  | 2.4868                        | HSIL     | CIN2      | CIN2            | Positive |
| 99        | 28  | 8.1134                        | HSIL     | CIN1      | CIN2            | Positive |
| 100       | 31  | 1.6449                        | HSIL     | CIN3      | CIN3            | Positive |
| 101       | 29  | 21.7971                       | HSIL     | CIN1      | CIN2            | Positive |
| 111       | 31  | 8.9870                        | HSIL     | CIN3      | CIN3            | Positive |
| 113       | 51  | 7.1208                        | Cancer   | CIN3      | CIN3            | Positive |
| 115       | 26  | 5.0528                        | HSIL     | CIN3      | CIN3            | Positive |
| 116       | 45  | 2.5974                        | HSIL     | n.a.      | CIN2            | Positive |
| 117       | 30  | 8.9810                        | HSIL     | CIN3      | CIN3            | Positive |
| 119       | 58  | 8.5443                        | LSIL     | Cancer    | Cancer          | Positive |
| 121       | 36  | 0.6870                        | HSIL     | CIN3      | CIN3            | Positive |
| 124       | 29  | 3.5774                        | HSIL     | CIN3      | CIN3            | Positive |
| 126       | 29  | 10.9771                       | HSIL     | CIN3      | CIN3            | Positive |

HPV status:
- Positive
- Negative

Subtype:
- 33,73 HR-HPV
- 33 HR-HPV
- 51 HR-HPV
- HR-HPV

HR/LR-HPV:
- 16 HR-HPV
- 18 HR-HPV
- 31,39,56,53 HR-HPV
Table II. Continued.

| Sample ID | Age | miR-205 (2^(-ΔΔCq)) | Cytology diagnosis | Histology diagnosis | Final diagnosis | HPV | Status | Subtype | HR/LR-HPV |
|-----------|-----|---------------------|--------------------|--------------------|----------------|-----|--------|---------|-----------|
| 127       | 38  | 0.4523              | HSIL               | CIN3               | CIN3           |     | Negative |         |           |
| 129       | 30  | 2.6331              | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 130       | 37  | 0.1385              | HSIL               | CIN3               | CIN3           |     | Positive | 58      | HR-HPV    |
| 132       | 29  | 9.6822              | HSIL               | CIN3               | CIN3           |     | Positive | 16,45   | HR-HPV    |
| 133       | 30  | 3.2601              | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 135       | 28  | 7.0403              | HSIL               | CIN3               | CIN3           |     | Positive | 16,66   | HR-HPV    |
| 136       | 28  | 16.9954             | HSIL               | CIN3               | CIN3           |     | Positive | 18      | HR-HPV    |
| 137       | 44  | 0.2899              | HSIL               | Cancer             | Cancer         | n.a | Positive | 16      | HR-HPV    |
| 138       | 51  | 37.3282             | HSIL               | WNL                | CIN2           |     | Positive | 16,68   | HR-HPV    |
| 139       | 26  | 42.2245             | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 140       | 36  | 7.2177              | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 141       | 42  | 13.0473             | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 142       | 30  | 5.8978              | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 143       | 34  | 3.6293              | LSIL               | CIN2               | CIN2           |     | Positive | 16,52   | HR-HPV    |
| 144       | 31  | 10.5154             | LSIL               | CIN2               | CIN2           |     | Positive | 18,31,58| HR-HPV    |
| 145       | 27  | 2.4491              | HSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 146       | 44  | 4.5693              | Cancer             | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 147       | 30  | 2.1592              | LSIL               | CIN2               | CIN2           |     | Positive | 16,31,33,39| HR-HPV |
| 148       | 55  | 4.4372              | HSIL               | Cancer             | Cancer         | n.a | Positive | 51      | HR-HPV    |
| 150       | 23  | 2.2068              | LSIL               | CIN1               | CIN1           |     | Positive | 51      | HR-HPV    |
| 151       | 34  | 0.1408              | LSIL               | CIN1               | CIN1           |     | Positive | 51,52,82,83| HR-HPV |
| 152       | 40  | 22.2206             | LSIL               | CIN1               | CIN1           |     | Positive | 31      | HR-HPV    |
| 153       | 39  | 3.6158              | LSIL               | CIN3               | CIN3           |     | Positive | 16      | HR-HPV    |
| 155       | 39  | 2.4600              | LSIL               | CIN2               | CIN2           |     | Positive | 53      | HR-HPV    |
| 156       | 23  | 4.3218              | LSIL               | CIN1               | CIN1           |     | Positive | 52,73   | HR-HPV    |
| 157       | 49  | 2.9542              | LSIL               | CIN1               | CIN1           |     | Positive | 52      | HR-HPV    |
| 158       | 47  | 19.4705             | LSIL               | CIN1               | CIN1           |     | Positive | 52      | HR-HPV    |
| 159       | 47  | 1.5333              | LSIL               | CIN3               | CIN3           |     | Positive | 52      | HR-HPV    |
| 160       | 23  | 5.2855              | LSIL               | CIN1               | CIN1           |     | Positive | 31,73   | HR-HPV    |
| 161       | 24  | 1.7701              | LSIL               | CIN1               | CIN1           |     | Positive | 59      | HR-HPV    |
| 162       | 37  | 8.7852              | LSIL               | CIN1               | CIN1           |     | Positive | 68      | HR-HPV    |
| 163       | 23  | 0.7902              | LSIL               | CIN3               | CIN3           |     | Positive | 16,39,58,73| HR-HPV |
| 164       | 33  | 5.0789              | LSIL               | CIN1               | CIN1           |     | Positive | 31      | HR-HPV    |
| 165       | 39  | 4.6425              | LSIL               | CIN1               | CIN1           |     | Positive | 31      | HR-HPV    |
were not statistically significant (Table III). Similar results were obtained in the HSIL group, in which the sensitivity of HPV testing to predict CIN2+ and CIN3+ was 0.87 (95% CI, 0.74-0.94) and 0.89 (95% CI, 0.71-0.97), respectively, which was higher than that of high miR-205 expression levels (0.55, 95% CI, 0.43-0.67 for CIN2+ and 0.50, 95% CI, 0.34-0.66 for CIN3+; Table IV).

miR-205 expression is not associated with HPV status, but may differ by HPV type. Using the relative quantification method (2^(-ΔΔCq)) as normalized to RNU6B, the relative miR-205 expression in all 140 LBC samples was calculated, and the associations between miR-205 expression and HPV positivity in the 115 samples that had this information available were analyzed using the Mann-Whitney U test. No statistically significant difference in miR-205 expression was observed between HPV-positive (n=93) and HPV-negative (n=22) samples (P=0.97; Z-score=0.039; two-tailed), indicating that miR-205 expression was not associated with HPV positivity. Similar results were obtained using the χ² test (Table V). A univariate test for miR-205 expression in all 140 samples revealed significant differences (P=1x10^-6), indicating the role of an unknown variable. Therefore, the association between miR-205 expression and HPV type, particularly HPV16 and 18, was investigated using the ANOVA Kruskal-Wallis test. Although the mean miR-205 expression levels in HPV18-positive samples (mean value, 18.98; n=9) were higher than those in HPV16-positive samples (mean value, 12.27; n=34), due to small sample size and large variation between samples, they were not statistically significant (P=0.279).

miR-205 expression and age. Spearman Rank Order correlation analyses did not reveal any significant correlations between miR-205 expression and age (R=0.0836; P=0.324); similar results were obtained using χ² tests (Table V).

miR-205 expression and cervical cancer progression. No significant difference between the LSIL and the HSIL group was observed based on cytology diagnosis, histology diagnosis or final histopathological diagnosis (P=0.64, 0.70 and 0.32, respectively), indicating that miR-205 expression alone was not able to distinguish the progression of cervical cancer in LBC samples. Based on the median expression levels of miR-205 in the 140 LBC samples, the correlations between miR-205 expression and different characteristics, including age, HPV positivity, HPV type, and final histopathological diagnosis were evaluated using a two-tailed χ² test; however, no significant differences were observed (Table V).

Discussion

Cervical cancer develops from well-recognized, pre-malignant forms. The detection of these forms through population-based screening programs is able to reduce the number of cases of cervical cancer dramatically (37). However, more robust and reliable molecular markers are required in current screening programs in order to distinguish between lesions with invasive potential and lesions that will spontaneously regress.

miRNAs are well described non-coding RNAs involved in human cancer, which typically negatively regulate gene...
Table III. Overview of the sensitivity and specificity, PPV, NPV and risk of disease in the LSIL group.

| Triage group | Outcome  | Test  | TP  | FP  | FN  | TN  | N  | Prevalence (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | PLR (95% CI) | NLR (95% CI) |
|--------------|---------|------|-----|-----|-----|-----|----|---------------------|---------------------|----------------------|----------------|-------------|-------------|------------|
| LSIL         | CIN2+   | high| 10  | 10  | 8   | 17  | 45 | 0.40 (0.26-0.55)    | 0.56 (0.31-0.78)    | 0.63 (0.42-0.80)    | 0.50 (0.28-0.72) | 0.68 (0.46-0.84) | 1.50 (0.79-2.85) | 0.71 (0.40-1.23) |
| LSIL         | CIN2+   | HPV+ | 18  | 24  | 0   | 3   | 45 | 0.40 (0.26-0.55)    | 1.00 (0.78-1.00)    | 0.11 (0.03-0.30)    | 0.43 (0.28-0.59) | 1.00 (0.31-1.00) | 1.12 (0.98-1.29) | 0           |
| LSIL         | CIN2+   | HPV16+ | 6   | 3   | 12  | 23  | 44 | 0.41 (0.27-0.57)    | 0.33 (0.14-0.59)    | 0.88 (0.69-0.97)    | 0.67 (0.31-0.91) | 0.66 (0.48-0.80) | 2.89 (0.83-10.07) | 0.75 (0.54-1.06) |
| LSIL         | CIN2+   | HPV18+ | 2   | 1   | 16  | 25  | 44 | 0.41 (0.27-0.57)    | 0.11 (0.02-0.36)    | 0.96 (0.78-1.00)    | 0.67 (0.13-0.98) | 0.61 (0.45-0.75) | 2.89 (0.28-29.51) | 0.92 (0.78-1.09) |
| LSIL         | CIN2+   | HPV16+18+ | 8   | 4   | 10  | 22  | 44 | 0.41 (0.27-0.57)    | 0.44 (0.22-0.69)    | 0.85 (0.64-0.95)    | 0.67 (0.35-0.89) | 0.69 (0.50-0.83) | 2.89 (1.02-8.16) | 0.66 (0.43-1.01) |
| LSIL         | CIN3+   | high| 4   | 16  | 4   | 21  | 45 | 0.18 (0.09-0.33)    | 0.50 (0.17-0.83)    | 0.57 (0.40-0.72)    | 0.20 (0.07-0.44) | 0.84 (0.63-0.95) | 1.16 (0.53-2.54) | 0.88 (0.42-1.83) |
| LSIL         | CIN3+   | HPV+ | 8   | 34  | 0   | 3   | 45 | 0.18 (0.09-0.33)    | 1.00 (0.60-1.00)    | 0.08 (0.02-0.23)    | 0.19 (0.09-0.35) | 1.00 (0.31-1.00) | 1.09 (0.99-1.20) | 0           |
| LSIL         | CIN3+   | HPV16+ | 3   | 6   | 5   | 30  | 44 | 0.18 (0.09-0.33)    | 0.38 (0.10-0.74)    | 0.83 (0.66-0.93)    | 0.33 (0.09-0.69) | 0.86 (0.69-0.95) | 2.25 (0.71-7.14) | 0.75 (0.43-1.30) |
| LSIL         | CIN3+   | HPV18+ | 1   | 2   | 7   | 34  | 44 | 0.18 (0.09-0.33)    | 0.12 (0.01-0.53)    | 0.94 (0.80-0.99)    | 0.33 (0.02-0.87) | 0.83 (0.67-0.92) | 2.25 (0.23-21.89) | 0.93 (0.71-1.21) |
| LSIL         | CIN3+   | HPV16+18+ | 4   | 8   | 4   | 28  | 44 | 0.18 (0.09-0.33)    | 0.50 (0.17-0.83)    | 0.78 (0.60-0.89)    | 0.33 (0.11-0.65) | 0.88 (0.70-0.96) | 2.25 (0.89-5.67) | 0.64 (0.32-1.31) |

PPV, positive predictive value; NPV, negative predictive value; LSIL, low-grade squamous intraepithelial lesions; TP, true positive; FP, false positive; FN, false negative; TN, true negative; CI, confidence interval; PLR, positive likelihood ratio; NLR, negative likelihood ratio; N, number; WNL, within normal limits (normal cytology); CIN2+, cervical intra-epithelial neoplasia grade 2 or worse; CIN3+, cervical intra-epithelial neoplasia grade 3 or worse; HPV, human papillomavirus.
Table IV. Overview of the sensitivity and specificity, PPV, NPV and risk of disease in the HSIL group.

| Triage group | Outcome      | Test        | Prevalence (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | PLR (95% CI) | NLR (95% CI) |
|--------------|--------------|-------------|---------------------|----------------------|----------------------|-------------|-------------|-------------|-------------|
| HSIL         | CIN2+ high   | miR-205     | 0.94-1.00           | 0.43-0.67            | 0.89-1.00            | 0.0-0.13    | n.a.        | n.a.        | n.a.        |
| HSIL         | CIN2+ HPV+   | miR-205     | 0.92-1.00           | 0.74-0.94            | 0.90-1.00            | 0-0.44      | n.a.        | n.a.        | n.a.        |
| HSIL         | CIN2+ HPV16+ | miR-205     | 0.91-1.00           | 0.30-0.58            | 0.82-1.00            | 0-0.15      | n.a.        | n.a.        | n.a.        |
| HSIL         | CIN2+ HPV18+ | miR-205     | 0.91-1.00           | 0.04-0.22            | 0.46-1.00            | 0-0.10      | n.a.        | n.a.        | n.a.        |
| HSIL         | CIN2+ HPV16+18+ | miR-205 | 0.91-1.00           | 0.39-0.67            | 0.84-1.00            | 0-0.17      | n.a.        | n.a.        | n.a.        |
| HSIL         | CIN3+ high   | miR-205     | 0.42-0.66           | 0.34-0.66            | 0.33-0.65            | 0.23-0.58   | 0.54-1.22   | 0.87-1.96   | 0.87-1.96   |
| HSIL         | CIN3+ HPV+   | miR-205     | 0.39-0.66           | 0.71-0.97            | 0.39-0.69            | 0.20-0.88   | 0.86-1.32   | 0.15-2.94   | 0.15-2.94   |
| HSIL         | CIN3+ HPV16+ | miR-205     | 0.40-0.69           | 0.31-0.69            | 0.41-0.82            | 0.33-0.70   | 0.73-2.81   | 0.51-1.16   | 0.77        |
| HSIL         | CIN3+ HPV18+ | miR-205     | 0.40-0.69           | 0.03-0.29            | 0.17-0.93            | 0.31-0.61   | 0.22-6.76   | 0.85-1.12   | 0.98        |
| HSIL         | CIN3+ HPV16+18+ | miR-205 | 0.40-0.69           | 0.41-0.78            | 0.42-0.80            | 0.33-0.74   | 0.80-2.43   | 0.41-1.18   | 0.70        |

PPV, positive predictive value; NPV, negative predictive value; HSIL, high-grade squamous intraepithelial lesions; TP, true positive; FP, false positive; FN, false negative; TN, true negative; CI, confidence interval; PLR, positive likelihood ratio; NLR, negative likelihood ratio; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; HPV, human papillomavirus; n.a., not available.
expression by transcription repression or translation inhibition (38). Dysregulated miRNA profiles have been identified in various human cancer types, including cervical cancer (17,39). However, the majority of previous studies were based on tissue samples or serum samples; there is a lack of knowledge concerning miRNA expression in LBC samples. miR-205 is frequently dysregulated in many cancer types and functions as either a tumor suppressor or an oncogene, depending on the cellular context (20). miR-205 expression in tumor tissue or serum is associated with the development and progression of tumors (40). Our previous studies revealed that miR-205 is highly expressed in cervical tumor tissue compared with matched normal cervical tissue, and further demonstrated that miR-205 has an oncogenic role by promoting cell proliferation and migration in cervical cancer cells (17,20). In the present study, miR-205 was selected as an example to evaluate the possibility of miRNA detection by RT-qPCR in LBC samples and to assess the potential value of miR-205 in clinical applications.

The preliminary results revealed that high miR-205 expression levels had a significantly higher specificity than HPV testing to predict the absence of CIN2+ or CIN3+ in women with LSIL, whereas the corresponding sensitivities were not significantly different. This demonstrates that there may be promising clinical applications for miR-205 expression. HPV testing is not recommended to triage women with LSIL due to its poor specificity, but this may be improved by the addition of the evaluation of miR-205 expression in these patients.

Certain miRNAs have been associated with HPV infection in cervical cancer. For example, miR-218 was specifically underexpressed in HPV16-positive cervical cancer cell lines, cervical lesions and cancer tissues when compared with HPV-negative C33A cells and normal cervical cells (41). Wang et al (42) revealed that HPV16 E6 expression is regulated via the histone acetyltransferase p300 and reported that increases in the expression of miR-16, miR-25, miR-92a and miR-378, and decreased expression of miR-22, miR-27a, miR-29a and miR-100 may be attributed to the HPV oncoproteins E6 and E7. In the present study, the association between high miR-205 expression and the presence of HPV was also analyzed, but no significant differences were observed, indicating that miR-205 expression is not associated with HPV infection.

In addition, no significant association between miR-205 expression and cancer stage was detected based on cytology, histology or final histopathological diagnosis. This may indicate that miR-205 expression levels do not increase at specific stages, but may increase continually during cancer progression. To better address this question, analyses are required to be performed on more than one sample from the same patient, on specially paired samples or on series of samples.

The present study cohort was taken from patients attending the population-based organized cervical cancer screening program in Sweden, and the majority of the samples were pre-malignant. However, the majority of the cells in the samples were normal, and thus it was difficult to distinguish if the

| Characteristics | All cases | High miR-205 (>median) | Low miR-205 (<median) | P-value* |
|-----------------|-----------|------------------------|----------------------|----------|
| Age (n=140)     |           |                        |                      |          |
| <32.5           | 70        | 39                     | 31                   | 0.1763   |
| >32.5           | 70        | 31                     | 39                   |          |
| HPV (n=115)     |           |                        |                      |          |
| Positive        | 93        | 47                     | 46                   | 0.7352   |
| Negative        | 22        | 12                     | 10                   |          |
| HPV subtypes (n=90) |       |                        |                      |          |
| HPV16, HPV18    | 43        | 23                     | 20                   | 0.5267   |
| Non HPV16, non HPV18 | 47    | 22                     | 25                   |          |
| Cytology (n=140) |           |                        |                      |          |
| LSIL            | 45        | 20                     | 25                   | 0.3093   |
| HSIL            | 74        | 40                     | 34                   |          |
| Histology (n=123) |         |                        |                      |          |
| CIN1            | 35        | 17                     | 18                   | 0.8391   |
| CIN2+           | 79        | 40                     | 39                   |          |
| Final diagnosis (n=140) |     |                        |                      |          |
| CIN1            | 29        | 11                     | 18                   | 0.1657   |
| CIN2+           | 95        | 50                     | 45                   |          |

*Two-tailed χ² test (without Yates correction). High or low miR-205 expression based on the median expression level. LBC, liquid-based cytology; HPV, human papillomavirus; LSIL, low-grade squamous intraepithelial lesion; HSIL, high-grade squamous intraepithelial lesion; CIN1, cervical intra-epithelial neoplasia grade 1; CIN2+, cervical intra-epithelial neoplasia grade 2 or worse.
miR-205 molecules extracted were from abnormal or normal cells. Theoretically, other single-cell-based detection methods, such as in situ hybridization (43,44) or microfluidic flow cytometry (45,46) are practical and ideal methods for LBC.

In conclusion, the findings from this screening-based population study revealed that high miR-205 expression levels in patients with LSIL provided statistically higher specificity than HPV testing to predict the absence of CIN2+ and CIN3+. Therefore, the data suggest that miRNA detection in LBC samples may have a potential application as an adjunct to HPV testing in the triage of women with LSIL. Further studies in larger cohorts or testing for a panel of miRNAs is required before recommendations may be suggested.

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