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 χ^2 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.

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Abbreviations: ASCUS, atypical squamous cells of undetermined significance; CI, confidence interval; CIN, cervical intraepithelial neoplasia; CIN1, cervical intraepithelial neoplasia grade 1; CIN2, cervical intraepithelial neoplasia grade 2; CIN2+, cervical intraepithelial neoplasia grade 2 or worse; CIN3+, cervical intraepithelial neoplasia grade 3 or worse; HPV, human papillomavirus; HSIL, high-grade squamous intraepithelial lesion; LBC, liquid-based cytology; LSIL, low-grade squamous intraepithelial lesions; miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; WNL, within normal limits (normal cytology)

Key words: liquid-based cytology, microRNA-205, specificity, human papillomavirus, cervical intraepithelial lesions

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 colposcopy and biopsy (3).

Persistent infection with human papillomavirus (HPV) is the causative agent in cervical cancer (4). HPV depends on differentiated keratinocytes; the infection of the squamous epithelia alone is not sufficient for the infection to progress to neoplasia (5). The expression of the HPV oncoproteins 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

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 coilocytosis, 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 (CIN1), 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 $2^{-\Delta\Delta Cq}$ (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 χ^2 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).

The sensitivity of high miR-205 expression to predict CIN2+ and CIN3+ was 0.56 (95% CI, 0.31-0.78) and 0.50 (95% CI, 0.17-0.83), respectively, among patients with LSIL, whereas HPV testing had a corresponding sensitivity of 1.0 (95% CI, 0.78-1) and 1.0 (95% CI, 0.60-1), respectively. Furthermore, when divided by HPV type, the individual sensitivity values (0.33, 0.11 and 0.44 for CIN2+; 0.38, 0.12 and 0.50 for CIN3+) were not higher than those for high miR-205 expression levels; the ANOVA test revealed that the differences between HPV testing and high miR-205 expression levels

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

Characteristic (N with results available)	N	%
Cytology (N=140)		
WNL	18	12.86
LSIL	45	32.14
HSIL	74	52.86
Cancer	3	2.40
Histology (N=123)		
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)		
WNL	16	11.43
CIN1	29	20.71
CIN2	44	31.43
CIN3	47	33.57
Cancer	4	2.86
HPV testing (N=115)		
Positive	93	80.87
Negative	22	19.13

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 ($2^{-\Delta\Delta Cq}$)	Cytology diagnosis	Histology diagnosis	Final diagnosis	HPV		
						Status	Subtype	HR/LR-HPV
2	37	61.0439	LSIL	CIN1	CIN1	Positive	31	HR-HPV
3	30	6.7449	WNL	n.a.	WNL	n.a.		
4	29	34.5873	HSIL	CIN3	CIN3	n.a.		
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	n.a.		
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		
12	26	13.9071	HSIL	CIN2	CIN2	Positive	58	HR-HPV
13	34	2.4690	HSIL	CIN3	CIN3	Positive	58	HR-HPV
14	33	4.7576	HSIL	CIN1	CIN2	n.a.		
15	30	29.0087	LSIL	CIN2	CIN2	Positive	39	HR-HPV
19	28	1.2505	LSIL	CIN1	CIN1	Positive		
20	28	20.1998	HSIL	CIN3	CIN3	Positive	16,31	HR-HPV
22	43	7.4901	WNL	n.a.	WNL	Negative		
23	42	2.2771	LSIL	CIN1	CIN1	Positive	16,52,82	HR-HPV
24A	59	2.0112	WNL	n.a.	WNL	Negative		
24B	25	7.2827	LSIL	CIN2	CIN2	Positive	31,51,73	HR-HPV
25	27	39.7380	LSIL	CIN2	CIN2	Positive	31,59	HR-HPV
28	59	17.1738	HSIL	CIN2	CIN2	n.a.		
29	43	0.6120	WNL	CIN1	CIN1	Positive	51	HR-HPV
30	43	53.3738	HSIL	CIN3	CIN3	n.a.		
31	31	12.1099	HSIL	CIN1	CIN2	Negative		
32	44	42.0013	HSIL	CIN3	CIN3	Positive	18	HR-HPV
33	43	7.4764	HSIL	CIN1	CIN2	Positive	45	HR-HPV
34	31	59.1805	HSIL	CIN2	CIN2	Positive		
35	26	3.8242	HSIL	CIN1	CIN2	Negative		
36	28	19.4811	HSIL	CIN3	CIN3	n.a.	16,51	
37	27	2.4561	LSIL	CIN1	CIN1	Positive	53,73	HR-HPV
38	32	1.4752	LSIL	WNL	CIN1	Positive	82	HR-HPV
39	26	14.5685	HSIL	WNL	CIN2	Positive	31,56	HR-HPV

Table II. Continued.

Sample ID	Age	miR-205 ($2^{-\Delta\Delta Cq}$)	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	CIN2	Negative		
42	33	12.0710	HSIL	CIN3	CIN3	n.a.		
43	39	3.5259	HSIL	n.a.	CIN2	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
64	51	6.7104	WNL	n.a.	WNL	Negative		
65	41	0.7032	HSIL	CIN3	CIN3	Positive	52	HR-HPV
66	29	12.8313	HSIL	CIN2	CIN2	n.a.		
67	42	6.9052	HSIL	CIN2	CIN2	Positive	16	HR-HPV
68	32	1.3904	LSIL	WNL	CIN1	Negative		
69	28	0.3772	HSIL	CIN2	CIN2	n.a.		
70	47	6.7330	Cancer	Cancer	Cancer	n.a		
71	29	7.7228	WNL	CIN1	CIN1	Positive	16	HR-HPV
72	28	9.6434	HSIL	CIN2	CIN2	Positive	16	HR-HPV
73	26	6.9220	HSIL	CIN2	CIN2	Positive	16,33	HR-HPV

Table II. Continued.

Sample ID	Age	miR-205 ($2^{-\Delta\Delta Cq}$)	Cytology diagnosis	Histology diagnosis	Final diagnosis	HPV		
						Status	Subtype	HR/LR-HPV
74	41	7.0546	HSIL	CIN3	CIN3	n.a.		
75	32	4.2851	LSIL	CIN2	CIN2	Positive	33,73	HR-HPV
76	44	11.3330	HSIL	WNL	CIN2	n.a.		
78	51	2.6267	WNL	n.a.	WNL	Negative		
79	32	8.7506	LSIL	CIN1	CIN1	Positive	73	HR-HPV
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	16	HR-HPV
84	30	13.8683	LSIL	CIN1	CIN1	Positive	33	HR-HPV
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	51	HR-HPV
91	27	40.2667	LSIL	CIN1	CIN1	Negative		
93	31	28.9839	HSIL	CIN3	CIN3	Positive	HR-HPV not 16,18	HR-HPV
94	29	45.6632	HSIL	CIN2	CIN2	n.a.		
95	37	6.2884	LSIL	CIN1	CIN1	Positive	31,39,56,53	HR-HPV
97	28	38.5117	HSIL	CIN1	CIN2	Positive	18	HR-HPV
98	29	2.4868	HSIL	CIN2	CIN2	Positive	45,51	HR-HPV
99	28	8.1134	HSIL	CIN1	CIN2	Positive	51	HR-HPV
100	31	1.6449	HSIL	CIN3	CIN3	n.a.		
101	29	21.7971	HSIL	CIN1	CIN2	Positive	16	HR-HPV
111	31	8.9870	HSIL	CIN3	CIN3	n.a.		
113	51	7.1208	Cancer	CIN3	CIN3	Positive	16	HR-HPV
115	26	5.0528	HSIL	CIN3	CIN3	n.a.		
116	45	2.5974	HSIL	n.a.	CIN2	Positive	51,52	HR-HPV
117	30	8.9810	HSIL	CIN3	CIN3	Positive	16	HR-HPV
119	58	8.5443	LSIL	Cancer	Cancer	Positive	18	HR-HPV
121	36	0.6870	HSIL	CIN3	CIN3	Positive	51	HR-HPV
124	29	3.5774	HSIL	CIN3	CIN3	Positive	16	HR-HPV
126	29	10.9771	HSIL	CIN3	CIN3	Positive	31	HR-HPV

Table II. Continued.

Sample ID	Age	miR-205 ($2^{-\Delta\Delta Cq}$)	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		
138	51	37.3282	HSIL	WNL	CIN2	Positive	16	HR-HPV
139	26	42.2245	HSIL	CIN3	CIN3	Positive	16,68	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		
150	23	2.2068	LSIL	CIN1	CIN1	Positive	51	HR-HPV
151	34	0.1408	LSIL	CIN1	CIN1	Positive	51	HR-HPV
152	40	22.2206	LSIL	CIN1	CIN1	Positive	51,52,82,83	HR-HPV
153	39	3.6158	LSIL	CIN3	CIN3	Positive	31	HR-HPV
155	39	2.4600	LSIL	CIN2	CIN2	Positive	16	HR-HPV
156	23	4.3218	LSIL	CIN1	CIN1	Positive	53	HR-HPV
157	49	2.9542	LSIL	CIN1	CIN1	Positive	52,73	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

Table II. Continued.

Sample ID	Age	miR-205 ($2^{-\Delta\Delta Cq}$)	Cytology diagnosis	Histology diagnosis	Final diagnosis	HPV		
						Status	Subtype	HR/LR-HPV
166	23	7.9566	LSIL	CIN3	CIN3	Positive	31	HR-HPV
167	23	29.6942	LSIL	CIN1	CIN1	Positive	31,33,53	HR-HPV
168	44	2.9572	LSIL	CIN1	CIN1	Positive	56	HR-HPV
169	25	4.1910	LSIL	CIN1	CIN1	Positive	51	HR-HPV
170	34	85.2947	LSIL	CIN3	CIN3	Positive	35	HR-HPV
171	23	42.8047	LSIL	CIN2	CIN2	Positive	51	HR-HPV
172	38	0.4877	LSIL	CIN3	CIN3	Positive	16	HR-HPV

HPV, human papillomavirus; LR, low-risk; HR, high-risk; LSIL, low-grade squamous intraepithelial lesion; CIN1, cervical intra-epithelial neoplasia grade 1; WNL, within normal limits; n.a., not applicable; HSIL, high-grade squamous intraepithelial lesion; CIN3, cervical intra-epithelial neoplasia grade 3; CIN2, cervical intra-epithelial neoplasia grade 2; miR, microRNA.

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 ($2^{-\Delta\Delta Cq}$), 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 χ^2 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 χ^2 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 χ^2 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 miR-205	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 miR-205	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	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)
HSIL	CIN2+	high miR-205	41	0	33	0	74	1 (0.94-1.00)	0.55 (0.43-0.67)	n.a.	1 (0.89-1.00)	0 (0-0.13)	n.a.	n.a.
HSIL	CIN2+	HPV+	46	0	7	0	53	1 (0.92-1.00)	0.87 (0.74-0.94)	n.a.	1 (0.90-1.00)	0 (0-0.44)	n.a.	n.a.
HSIL	CIN2+	HPV16+	22	0	29	0	51	1 (0.91-1.00)	0.43 (0.30-0.58)	n.a.	1 (0.82-1.00)	0 (0-0.15)	n.a.	n.a.
HSIL	CIN2+	HPV18+	5	0	46	0	51	1 (0.91-1.00)	0.10 (0.04-0.22)	n.a.	1 (0.46-1.00)	0 (0-0.10)	n.a.	n.a.
HSIL	CIN2+	HPV16+18+	27	0	24	0	51	1 (0.91-1.00)	0.53 (0.39-0.67)	n.a.	1 (0.84-1.00)	0 (0-0.17)	n.a.	n.a.
HSIL	CIN3+	high miR-205	20	21	20	13	74	0.54 (0.42-0.66)	0.50 (0.34-0.66)	0.38 (0.23-0.56)	0.49 (0.33-0.65)	0.39 (0.23-0.58)	0.81 (0.54-1.22)	1.31 (0.87-1.96)
HSIL	CIN3+	HPV+	25	21	3	4	53	0.53 (0.39-0.66)	0.89 (0.71-0.97)	0.16 (0.05-0.37)	0.54 (0.39-0.69)	0.57 (0.20-0.88)	1.06 (0.86-1.32)	0.67 (0.15-2.94)
HSIL	CIN3+	HPV16+	14	8	14	15	51	0.55 (0.40-0.69)	0.50 (0.31-0.69)	0.65 (0.43-0.83)	0.64 (0.41-0.82)	0.52 (0.33-0.70)	1.44 (0.73-2.81)	0.77 (0.51-1.16)
HSIL	CIN3+	HPV18+	3	2	25	21	51	0.55 (0.40-0.69)	0.11 (0.03-0.29)	0.91 (0.70-0.98)	0.60 (0.17-0.93)	0.46 (0.31-0.61)	1.23 (0.22-6.76)	0.98 (0.85-1.12)
HSIL	CIN3+	HPV16+18+	17	10	11	13	51	0.55 (0.40-0.69)	0.61 (0.41-0.78)	0.57 (0.35-0.76)	0.63 (0.42-0.80)	0.54 (0.33-0.74)	1.40 (0.80-2.43)	0.70 (0.41-1.18)

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.

Table V. Correlation of clinical features of LBC samples with miR-205 expression levels.

Characteristics	All cases	High miR-205 (>median)	Low miR-205 (<median)	P-value[a]
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	

[a]Two-tailed χ^2 test (without Yates correlation). 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.

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 a 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

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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References

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893-2917, 2010.
2. The National Board of Health and Welfare: Cancer incidence in Sweden 2008. The National Board of Health and Welfare, Stockholm, 2009.
3. National Quality Register for Cervical Screening: Prevention of cervical cancer in Sweden. Annual report 2014 with data to 2013. National Quality Register for Cervical Screening, Stockholm, Swedish 2014.
4. Bosch FX and Muñoz N: The viral etiology of cervical cancer. Virus Res 89: 183-190, 2002.
5. Woodman CB, Collins SI and Young LS: The natural history of cervical HPV infection: Unresolved issues. Nat Rev Cancer 7: 11-22, 2007.
6. Münger K: Disruption of oncogene/tumor suppressor networks during human carcinogenesis. Cancer Invest 20: 71-81, 2002.
7. Pett M and Coleman N: Integration of high-risk human papillomavirus: A key event in cervical carcinogenesis? J Pathol 212: 356-367, 2007.
8. Imaoka H, Toiyama Y, Okigami M, Yasuda H, Saigusa S, Ohi M, Tanaka K, Inoue Y, Mohri Y and Kusunoki M: Circulating microRNA-203 predicts metastases, early recurrence and poor prognosis in human gastric cancer. Gastric Cancer 19: 744-753, 2016.
9. Naucler P, Ryd W, Törnberg S, Strand A, Wadell G, Elfgren K, Rådberg T, Strander B, Johansson B, Forslund O, *et al*: Human papillomavirus and Papanicolaou tests to screen for cervical cancer. N Engl J Med 357: 1589-1597, 2007.
10. Giorgi-Rossi P, Franceschi S and Ronco G: HPV prevalence and accuracy of HPV testing to detect high-grade cervical intraepithelial neoplasia. Int J Cancer 130: 1387-1394, 2012.
11. Bergström R, Sparén P and Adami HO. Trends in cancer of the cervix uteri in Sweden following cytological screening. Br J Cancer 81:159-166, 1999.
12. Cuzick J, Clavel C, Petry KU, Meijer CJ, Hoyer H, Ratnam S, Szarewski A, Birembaut P, Kulasingam S, Sasieni P and Iftner T: Overview of the European and North American studies on HPV testing in primary cervical cancer screening. Int J Cancer 119: 1095-1101, 2006.
13. Nanda K, McCrory DC, Myers ER, Bastian LA, Hasselblad V, Hickey JD and Matchar DB: Accuracy of the Papanicolaou test in screening for and follow-up of cervical cytologic abnormalities: A systematic review. Ann Intern Med 132: 810-819, 2000.
14. Strander B, Andersson-Ellström A, Milsom I, Rådberg T and Ryd W: Liquid-based cytology versus conventional Papanicolaou smear in an organized screening program: A prospective randomized study. Cancer 111: 285-291, 2007.
15. Persson M, Elfström KM, Brismar Wendel S, Weiderpass E and Andersson S: Triage of HR-HPV positive women with minor cytological abnormalities: A comparison of mRNA testing, HPV DNA testing and repeat cytology using a 4-year follow-up of a population-based study. PLoS One 9: e90023, 2014.
16. Bartel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
17. Lui WO, Pourmand N, Patterson BK and Fire A: Patterns of known and novel small RNAs in human cervical cancer. Cancer Res 67: 6031-6043, 2007.
18. Wang X, Tang S, Le SY, Lu R, Rader JS, Meyers C and Zheng ZM: Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth. PLoS One 3: e2557, 2008.
19. Witten D, Tibshirani R, Gu SG, Fire A and Lui WO: Ultra-high throughput sequencing-based small RNA discovery and discrete statistical biomarker analysis in a collection of cervical tumours and matched controls. BMC Biol 8: 58, 2010.
20. Xie H, Zhao Y, Caramuta S, Larsson C and Lui WO: MiR-205 expression promotes cell proliferation and migration of human cervical cancer cells. PLoS One 7: e46990, 2012.
21. Hagrass HA, Sharaf S, Pasha HF, Tantawy EA, Mohamed RH and Kassem R: Circulating microRNAs-a new horizon in molecular diagnosis of breast cancer. Genes Cancer 6: 281-287, 2015.
22. He Y, Lin J, Kong D, Huang M, Xu C, Kim TK, Etheridge A, Luo Y, Ding Y and Wang K: Current state of circulating microRNAs as cancer biomarkers. Clin Chem 61: 1138-1155, 2015.
23. Lin XJ, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, Li SP, Xiong Y, Yuan Y, Min J, *et al*: A serum microRNA classifier for early detection of hepatocellular carcinoma: A multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. Lancet Oncol 16: 804-815, 2015.
24. Mu YP, Sun WJ, Lu CW and Su XL: MicroRNAs may serve as emerging molecular biomarkers for diagnosis and prognostic assessment or as targets for therapy in gastric cancer. Asian Pac J Cancer Prev 16: 4813-4820, 2015.
25. Nonaka R, Miyake Y, Hata T, Kagawa Y, Kato T, Osawa H, Nishimura J, Ikenaga M, Murata K, Uemura M, *et al*: Circulating miR-103 and miR-720 as novel serum biomarkers for patients with colorectal cancer. Int J Oncol 47: 1097-1102, 2015.
26. Yu J, Jin L, Jiang L, Gao L, Zhou J, Hu Y, Li W, Zhi Q and Zhu X: Serum miR-372 is a diagnostic and prognostic biomarker in patients with early colorectal cancer. Anticancer Agents Med Chem 16: 424-431, 2016.
27. Orang AV, Safaralizadeh R, Hosseinpour Feizi MA and Somi MH: Diagnostic and prognostic value of miR-205 in colorectal cancer. Asian Pac J Cancer Prev 15: 4033-4037, 2014.
28. Zheng H, Zhang L, Zhao Y, Yang D, Song F, Wen Y, Hao Q, Hu Z, Zhang W and Chen K: Plasma miRNAs as diagnostic and prognostic biomarkers for ovarian cancer. PLoS One 8: e77853, 2013.
29. Yue X, Lan F, Hu M, Pan Q, Wang Q and Wang J: Downregulation of serum microRNA-205 as a potential diagnostic and prognostic biomarker for human glioma. J Neurosurg 124: 122-128, 2016.
30. Ma Q, Wan G, Wang S, Yang W, Zhang J and Yao X: Serum microRNA-205 as a novel biomarker for cervical cancer patients. Cancer Cell Int 14: 81, 2014.
31. Zhao S, Yao D, Chen J and Ding N: Circulating miRNA-20a and miRNA-203 for screening lymph node metastasis in early stage cervical cancer. Genet Test Mol Biomarkers 17: 631-636, 2013.
32. Malta M, Ribeiro J, Monteiro P, Loureiro J, Medeiros R and Sousa H: Let-7c is a candidate biomarker for cervical intraepithelial lesions: A pilot study. Mol Diagn Ther 19: 191-196, 2015.
33. Tian Q, Li Y, Wang F, Li Y, Xu J, Shen Y, Ye F, Wang X, Cheng X, Chen Y, *et al*: MicroRNA detection in cervical exfoliated cells as a triage for human papillomavirus-positive women. J Natl Cancer Inst 106: pii: dju241, 2014.
34. Fröberg M, Johansson B, Hjerpe A and Andersson S: Human papillomavirus 'reflex' testing as a screening method in cases of minor cytological abnormalities. Br J Cancer 99: 563-568, 2008.
35. Solomon D, Davey D, Kurman R, Moriarty A, O'Connor D, Prey M, Raab S, Sherman M, Wilbur D, Wright T Jr, *et al*: The 2001 bethesda system: Terminology for reporting results of cervical cytology. JAMA 287: 2114-2119, 2002.

36. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
37. Canavan TP and Doshi NR: Cervical cancer. Am Fam Physician 61: 1369-1376, 2000.
38. Esquela-Kerscher A and Slack FJ: Oncomirs-microRNAs with a role in cancer. Nat Rev Cancer 6: 259-269, 2006.
39. Lee YS and Dutta A: MicroRNAs in cancer. Annu Rev Pathol 4: 199-227, 2009.
40. Qin AY, Zhang XW, Liu L, Yu JP, Li H, Wang SZ, Ren XB and Cao S: MiR-205 in cancer: An angel or a devil? Eur J Cell Biol 92: 54-60, 2013.
41. Martinez I, Gardiner AS, Board KF, Monzon FA, Edwards RP and Khan SA: Human papillomavirus type 16 reduces the expression of microRNA-218 in cervical carcinoma cells. Oncogene 27: 2575-2582, 2008.
42. Wang X, Wang HK, Li Y, Hafner M, Banerjee NS, Tang S, Briskin D, Meyers C, Chow LT, Xie X, *et al*: MicroRNAs are biomarkers of oncogenic human papillomavirus infections. Proc Natl Acad Sci USA 111: 4262-4267, 2014.
43. Nielsen BS: MicroRNA in situ hybridization. Methods Mol Biol 822: 67-84, 2012.
44. Ge J, Zhang LL, Liu SJ, Yu RQ and Chu X: A highly sensitive target-primed rolling circle amplification (TPRCA) method for fluorescent in situ hybridization detection of microRNA in tumor cells. Anal Chem 86: 1808-1815, 2014.
45. Wu M, Piccini M, Koh CY, Lam KS and Singh AK: Single cell microRNA analysis using microfluidic flow cytometry. PLoS One 8: e55044, 2013.
46. Wu M, Piccini ME and Singh AK: MiRNA detection at single-cell resolution using microfluidic LNA flow-FISH. Methods Mol Biol 1211: 245-260, 2014.