Ⅲ. 研究成果の公開に関する一覧表

雑誌

著者氏名	論文タイトル	発表誌名	巻・頁・出版年
Abe S, Miura K, Kinoshita A, Mishima H, Miura S, Yamasaki K, Hasegawa Y, Higashijima A, Jo O, Yoshida A, Kaneuchi M, Yoshiura KI, Masuzaki H.	Single human papillomavirus 16 or 52 infection and later cytological findings in Japanese women with NILM or ASC-US.	J. Hum. Genet	Epub ahead of print
Tsukamoto O, Miura K, Mishima H, Abe S, Kaneuchi M, Higashijima A, Miura S, Kinoshita A, Yoshiura K, Masuzaki H.	Identification of endometrioid endometrial carcinoma-associated microRNAs in tissue and plasma.	Gynecol Oncol.	PubMed - in process
Miura K, Higashijima A, Miura S, Mishima H, Yamasaki K, Abe S, Hasegawa Y, Kaneuchi M, Yoshida A, Kinoshita A, Yoshiura KI, Masuzaki H.	Predominantly placenta-expressed mRNAs in maternal plasma as predictive markers for twin-twin transfusion syndrome.	Prenat Diagn.	Epub ahead of print
Hamaguchi D, Miura K, Abe S, Kinoshita A, Miura S, Yamasaki K, Yoshiura K, Masuzaki H.	Initial viral load in cases of single human papillomavirus 16 or 52 persistent infection is associated with progression of later cytopathological findings in the uterine cervix.	J Med Virol.	85: 2093-2100, 2013

著者氏名	論文タイトル	発表誌名	巻・頁・出版年
Abe S, Miura K, Kinoshita A, Mishima H, Miura S, Yamasaki K, Hasegawa Y, Higashijima A, Jo O, Sasaki K, Yoshida A, Yoshiura K, Masuzaki H.	Copy number variation of the antimicrobial-gene, defensin beta 4, is associated with susceptibility to cervical cancer.	J Hum Genet.	58: 250-253, 2013
Higashijima A, Miura K, Mishima H, Kinoshita A, Jo O, Abe S, Hasegawa Y, Miura S, Yamasaki K, Yoshida A, Yoshiura K, Masuzaki H.	Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy.	Prenat Diagn.	33: 214-222, 2013
Shimizu K, Wakui K, **Kosho T (corresponding author)**, Okamoto N, Mizuno S, Itomi K, Hattori S, Nishio K, Samura O, Kobayashi Y, Kako Y, Arai T, Oishi T, Kawame H, Narumi Y, Ohashi H, Fukushima Y.	Microarray and FISH-based genotype-phenotype analysis of 22 Japanese patients with Wolf-Hirschhorn syndrome.	Am J Med Genet Part A	in press

著者氏名	論文タイトル	発表誌名	巻・頁・出版年
Nishi E, Takamizawa S, Iio K, Yamada Y, Yoshizawa K, Hatata T, Hiroma T, Mizuno S, Kawame H, Fukushima Y, Nakamura T, Kosho T (corresponding author).	Surgical intervention for esophageal atresia in patients with trisomy 18.	Am J Med Genet Part A	Epub ahead of print
Kosho T (corresponding author), Kuniba H, Tanikawa Y, Hashimoto Y, Sakurai H.	Natural history and parental experience of children with trisomy 18 based on a questionnaire given to a Japanese trisomy 18 parental support group.	Am J Med Genet Part A	161A(7): 1531-1542, 2013.
Kosho T (corresponding author), Okamoto N, Ohashi H, Tsurusaki Y, Imai Y, Hibi-Ko Y, Kawame H, Homma T, Tanabe S, Kato M, Hiraki Y, Yamagata T, Yano S, Sakazume S, Ishii T, Nagai T, Ohta T, Niikawa N, Mizuno S, Kaname T, Naritomi K, Narumi Y, Wakui K, Fukushima Y, Miyatake S, Mizuguchi T, Saitsu H, Miyake N, Matsumoto N.	Clinical correlations of mutations affecting six components of the SWI/SNF complex: detailed description of 21 patients and a review of the literature.	Am J Med Genet Part A	161A(6): 1221-1237, 2013.

著者氏名	論文タイトル	発表誌名	巻・頁・出版年
Tsurusaki Y, Kosho T (equal contribution, corresponding author), Hatasaki K, Narumi Y, Wakui K, Fukushima Y, Doi H, Saitsu H, Miyake N, Matsumoto N.	Exome sequencing in a family with an X-linked lethal malformation syndrome: clinical consequences of hemizygous truncating OFD1 mutations in male patients.	Clin Genet.	83(2): 135-144, 2013.
Higashimoto K, Jozaki K, Kosho T, Matsubara K, Fuke T, Yamada D, Yatsuki H, Maeda T, Ohtsuka Y, Nishioka K, Joh K, Koseki H, Ogata T, Soejima H.	A novel de novo point mutation of the OCT-binding site in the IGF2/H19-imprinting control region in a Beckwith-Wiedemann syndrome patient.	Clin Genet	Epub ahead of print
Sugiura K, Takeichi T, Tanahashi K, Ito Y, Kosho T, Saida K, Uhara H, Okuyama R, Akiyama M.	Lamellar ichthyosis in a collodion baby caused by CYP4F22 mutations in a non-consanguineous family outside the Mediterranean.	J Dermatol Sci.	72(2): 193-195, 2013.
Nitta H, Unoki M, Ichiyanagi K, Kosho T, Shigemura T, Takahashi H, Velasco	hree novel ZBTB24 mutations identified in Japanese and Cape Verdean type 2	J Hum Genet.	58(7): 455-460, 2013.

著者氏名	論文タイトル	発表誌名	巻・頁・出版年
G, Francastel C, Picard C, Kubota T, Sasaki H.	ICF syndrome patients.		
Miyake N, Koshimizu E, Okamoto N, Mizuno S, Ogata T, Nagai T, Kosho T, Ohashi H, Kato M, Sasaki G, Mabe H, Watanabe Y, Yoshino M, Matsuishi T, Takanashi J, Shotelersuk V, Tekin M, Ochi N, Kubota M, Ito N, Ihara K, Hara T, Tonoki H, Ohta T, Saito K, Matsuo M, Urano M, Enokizono T, Sato A, Tanaka H, Ogawa A, Fujita T, Hiraki Y, Kitanaka S, Matsubara Y, Makita T, Taguri M, Nakashima M, Tsurusaki Y, Saitsu H, Yoshiura K, Matsumoto N, Niikawa N.	MLL2 and KDM6A mutations in patients with Kabuki syndrome.	Am J Med Genet Part A	61(9): 2234-2243, 2013.
Tanaka K, Sekijima Y, Yoshida K, Tamai M, Kosho T, Sakurai A, Wakui K, Ikeda S, Fukushima Y.	Follow-up nationwide survey on predictive genetic testing for late-onset hereditary neurological diseases in Japan.	J Hum Genet.	58(8): 560-563, 2013.

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
Kosho T (corresponding author), Mizumoto S, Sugahara K.	Carbohydrate (N-acetylgalactosamine 4-O) sulfotransferase 14 (CHST14).	Taniguchi N, Honke K, Fukuda M, Narimatsu H, Yamaguchi Y, Angata T	Handbook of glycosyltransferases and related genes	Springer	Germany	In press	
Kosho T (corresponding author).	Discovery and delineation of dermatan 4-*O*-sulfotransferase-1 (D4ST1)-deficient Ehlers-Danlos syndrome.	Oiso N, Kawada A	Current Genetics in Dermatology	InTech	Croatia	2013	73-86

IV. 研究成果の刊行物・別刷り

Journal of Human Genetics (2014), 1–5

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ORIGINAL ARTICLE

Single human papillomavirus 16 or 52 infection and later cytological findings in Japanese women with NILM or ASC-US

Shuhei Abe[1], Kiyonori Miura[1], Akira Kinoshita[2], Hiroyuki Mishima[2], Shoko Miura[1], Kentaro Yamasaki[1], Yuri Hasegawa[1], Ai Higashijima[1], Ozora Jo[1], Atsushi Yoshida[1], Masanori Kaneuchi[1], Koh-ichiro Yoshiura[2] and Hideaki Masuzaki[1]

The relationship between oncogenic human papillomavirus (HPV) infection and later cytological findings in the uterine cervix is unknown in women who were negative for intraepithelial lesion and malignancy (NILM) or atypical squamous cells of undetermined significance (ASC-US). This was investigated in this study in a Japanese population to determine the clinical utility of oncogenic (HPV) genotyping. The relative risk of progressive cytological findings 2 years after identification of oncogenic HPV infection was higher than in cases of non-oncogenic HPV infection (relative risk 3.827; 95% confidence interval (CI): 1.282–11.422), as well as in cases of negative HPV infection (relative risk 2.124; 95% CI: 1.451–3.110). Moreover, the relative risk of progression of cytological findings 2 years later in cases of HPV-16 infection was higher than in cases of HPV-52 infection (relative risk 2.094; 95% CI: 1.005–3.935). Therefore, the initial HPV-DNA genotype may be a potential predictive marker of later progression of cytological findings in the uterine cervix in cases of NILM or ASC-US.
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Keywords: cytological findings; genotype; human papillomavirus; oncogenic HPV-16; oncogenic HPV-52; uterine cervical cancer

INTRODUCTION

Persistent infections with oncogenic human papillomaviruses (HPVs), including 16 different HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82), are recognized as a major risk factor for the development of cervical cancer.[1] HPV-16 and HPV-18 infection accounts for approximately 70% of cancers and 50% of high-grade cervical intraepithelial neoplasia.[2] Although the risk of cervical cancer may be an order of magnitude higher for HPV-16 infection than other oncogenic HPV types, our previous studies showed that HPV-52 was a more common genotype in Nagasaki, Japan, compared with the distribution of high-risk HPV genotypes in other countries.[3,4] In addition, HPV-52 was the most common genotype among HPV-infected pregnant Japanese women. The second most common genotype was HPV-16, and these two genotypes collectively accounted for around 60% of HPV-positive pregnant women.[4] Hence, geographic variations in HPV-type distributions should be an important consideration.

The screening of high-grade squamous intraepithelial lesions (HSILs) and cervical cancer currently involves the detection of oncogenic HPV by cervicovaginal Pap smears,[5] and it is both important and necessary to clarify the association between the individual oncogenic HPV genotypes in each region and the progression of later cytopathological findings in the uterine cervix. When women are screened using cervicovaginal Pap smears, most are diagnosed with negative for intraepithelial lesion or malignancy (NILM) or atypical squamous cells of undetermined significance (ASC-US). ASC-US consists of various cytological abnormalities, which may contain low-grade squamous intraepithelial lesions or HSILs of the uterine cervix. Therefore, testing for oncogenic HPV is now routinely carried out in both Japan and the United States for managing women with ASC-US. However, the clinical usefulness of oncogenic HPV testing is limited by the fact that oncogenic HPV infections are relatively common among women without cervical intraepithelial neoplasia (CIN) or cancer. Also, in the United States, the clinical usefulness of HPV-16 and/or HPV-18 genotyping to triage oncogenic HPV-positive women with NILM aged 30 years and above has been recently recognized in clinical management guidelines; HPV-16-positive or HPV-18-positive women with NILM are referred for colposcopy, whereas other oncogenic HPV-positive women with NILM are recommended to repeat the cervical cytology and oncogenic HPV testing in 12 months.[6] Nevertheless, the data supporting the use of HPV genotyping in this manner are relatively

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limited. Moreover in clinical management, it is not obvious how oncogenic HPV-positive women with NILM or ASC-US cytology should be managed in primary cervical cancer screening, or how the presence of oncogenic HPV infection affects later cytological findings in Japanese women with a cytological diagnosis of NILM or ASC-US at first admission.

Therefore, this study aimed to understand the clinical utility of oncogenic HPV genotyping in Japanese women by investigating the relationship between oncogenic HPV infection in a Japanese population and cytological changes in the uterine cervix. Subsequently, we investigated how a single HPV-16 or HPV-52 infection affects the cytological findings in Japanese women with NILM or ASC-US 2 years later.

MATERIALS AND METHODS

Patients

Women admitted to five hospitals in Nagasaki Prefecture, Japan, between 2007 and 2011 as a pre-vaccination population for the HPV vaccine[3,4] were informed of the purpose of the study and gave their consent for participation. We identified 319 cases with a cytological diagnosis of NILM or ASC-US at first admission, and followed them up by cervicovaginal Pap smears and HPV genotyping over the next 2 years. The study protocol was approved by the ethical review board of Nagasaki University and the other hospitals involved.

Sample collection and cytological diagnoses

Specimens were collected using a Cervex Brush (Rovers Medical Devices, Oss, The Netherlands) and suspended in 10 ml of SurePath preservative fluid (Becton Dickinson, Franklin Lakes, NJ, USA). Samples from the same vial were used for cytological testing with the Bethesda III system (2001) and for HPV genotype testing.[3,4] Cervical specimens for cytology and HPV genotyping were obtained at each visit from participants who received regular follow-up examinations. To minimize the possibility of diagnostic variations, cytologic diagnoses of the specimens were performed by the same experienced cytoscreener and a qualified medical doctor in a commercial laboratory (SRL, Tokyo, Japan), who were blinded to the results of the HPV genotyping test. Cervical cytological findings were reported as NILM, ASC-US, low-grade squamous intraepithelial lesion, atypical cells—cannot exclude HSILs (ASC-H) and HSIL. Regarding cervical cytological findings determined 2 years after first admission, cases showing progression (changing from 'NILM' to 'ASC-US or worse cytological findings', or from 'ASC-US' to 'low-grade squamous intraepithelial lesion or worse cytological findings') were defined as the progression group, and cases showing no change or regression (from 'NILM' to 'NILM', or from 'ASC-US' to 'ASC-US or NILM') were defined as the non-progression group. Colposcopy was performed in the cases diagnosed as ASC-US with oncogenic HPV infections (data not shown).

HPV genotyping test

Genotyping of HPV DNA in SurePath preservative fluid was carried out after preparing glass slides using the Linear Array HPV Genotyping Test Kit (Roche Molecular Systems, Indianapolis, IN, USA). PGMY09/PGMY11 primers[7] amplified the L1 conserved region by polymerase chain reaction, and hybridization of the HPV amplicon was performed using an array of oligonucleotide probes that allowed the independent identification of individual HPV genotypes. This kit can detect the following 37 HPV genotypes: 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108 (89). For consistency with previous studies, 16 HPV genotypes (16, 18, 31, 33, 35, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73 and 82) were considered to be high risk as they had previously been related to cervical cancer.[1,8,9]

Statistical analysis

Patient backgrounds were compared between the progression and non-progression groups using Student's *t*-tests and χ^2 tests for continuous and discrete variables, respectively. Differences in oncogenic HPV genotyping between the two groups were evaluated using relative risk. Statistical analyses were performed with SPSS software version 19 (IBM Japan, Tokyo, Japan). Significant differences were defined as $P<0.05$.

RESULTS

Study population backgrounds

Of the 319 Japanese women (253 cases of NILM and 66 cases of ASC-US) originally studied, HPV genotyping showed that 178 (159 cases of NILM and 19 cases of ASC-US) had no HPV infection, 26 (17 cases of NILM and 9 cases of ASC-US) had only a non-oncogenic HPV infection, 38 (25 cases of NILM and 13 cases of ASC-US) had multiple oncogenic HPV infections, and 77 (52 cases of NILM and 25 cases of ASC-US) had a single oncogenic HPV infection (Figure 1). The 77 cases with a single oncogenic HPV infection included HPV-16 ($n=20$; 13 cases of NILM and 7 cases of ASC-US), HPV-18 (1 case of NILM), HPV-31 ($n=7$; 6 cases of NILM and 1 case of ASC-US), HPV-35 (2 cases of ASC-US), HPV-39 (1 case of ASC-US), HPV-51 ($n=3$; 1 case of NILM and 2 cases of ASC-US), HPV-52 ($n=29$; 22 cases of NILM and 7 cases of ASC-US), HPV-58 ($n=11$; 6 cases of NILM and 5 cases of ASC-US), HPV-59 (2 cases of NILM) and HPV-68 (1 case of NILM) (Figure 1). Of the 25 cases of ASC-US with a single oncogenic HPV infection, 12 were followed up with HPV genotyping. Of the 12 cases of ASC-US with a single oncogenic HPV infection at first admission, persistent infection of oncogenic HPV was detected 2 years later in 4 (33.3%; 3 cases showing a cytological 'progression', and 1 case showing a cytological 'non-progression'), while oncogenic HPV had disappeared in the remaining 8 cases (66.7%, all 8 cases showing a cytological 'non-progression' 2 years later; $P=0.005$, χ^2 test).

There was no significant difference in patient background between the single oncogenic HPV infection and HPV-negative groups (Table 1), the single oncogenic HPV infection and single non-oncogenic HPV infection groups (Table 1), or between the HPV-16 infection and HPV-52 infection groups (Table 2). There was no significant difference in age distributions between the progression and the non-progression groups (Table 3).

Relationship between oncogenic HPV infection and the progression of cytological findings in Japanese women with NILM or ASC-US

Of the 77 cases with a single oncogenic HPV infection, 30 (38.96%) were classified in the progression group, whereas 47 (61.04%) were in the non-progression group. This compares with 37 (20.79%) and 141 (79.21%), respectively, for the 178 cases of negative HPV infection, and 3 (11.54%) and 23 (88.46%), respectively, for the 26 cases of non-oncogenic HPV infection. The relative risk of progression of cytological findings 2 years later in cases of oncogenic HPV infection was higher than in cases of non-oncogenic HPV infection, as well as in cases of negative HPV infection (Table 4). However, the relative risk of progression of cytological findings 2 years later in cases of non-oncogenic HPV infection was not higher than in cases of negative HPV infection (Table 4).

Association between single HPV-16 or HPV-52 infection and later cytological findings in Japanese women with NILM or ASC-US

In the 20 cases of single HPV-16 infection (HPV-16 group), 13 (65%) were classified in the progression group, whereas 7 (35%) were in the

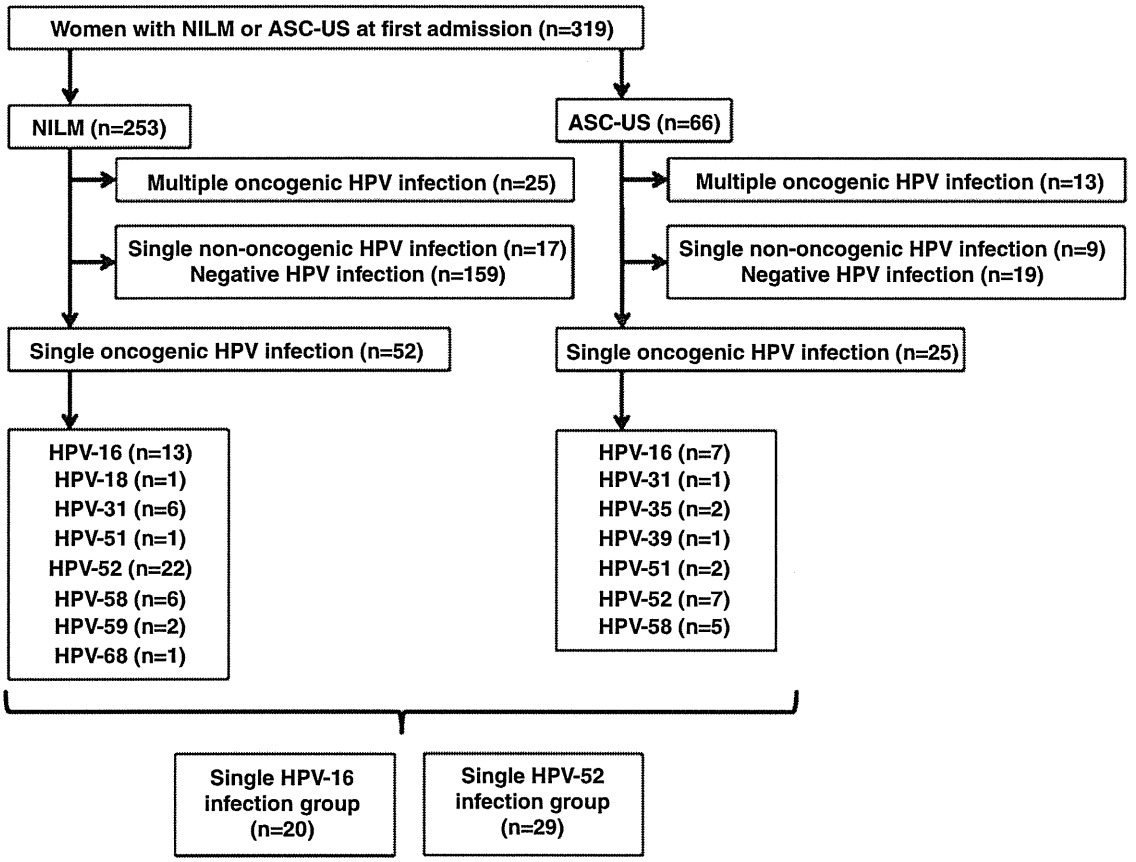

Figure 1 Results of HPV genotyping in women with NILM or ASC-US at first admission. ASC-US, atypical squamous cells of undetermined significance; HPV, human papillomavirus; NILM, negative for intraepithelial lesion and malignancy.

Table 1 Patient background in cases of single oncogenic HPV, non-oncogenic HPV or negative HPV infections

	Results of HPV test			P-*value*	
	A	*B*	*C*		
	Oncogenic HPV group (n = *77*)	*Non-oncogenic HPV group* (n = *26*)	*Negative HPV group* (n = *178*)	*A* vs *B*	*A* vs *C*
Age at first sampling (years)[a]	42.58 (11.78)	40.27 (14.18)	41.95 (11.03)	0.232	0.876
Smoking[b]	12 (15.6%)	6 (23.1%)	21 (11.8%)	0.384	0.408
Use of oral contraceptive[b]	3 (3.9%)	0 (0.0%)	6 (3.4%)	0.307	0.835
Parity[b]					
Nulliparous	24 (31.2%)	5 (19.2%)	60 (33.7%)	0.495	0.924
Primiparous	14 (18.2%)	6 (23.1%)	31 (17.4%)		
Multiparous	39 (50.6%)	15 (57.7%)	87 (48.9%)		
Body mass index ($kg\,m^{-2}$)[a]	21.32 (2.47)	21.17 (2.34)	21.27 (2.76)	0.963	0.727

Abbreviation: HPV, human papillomavirus.
[a]Mean (s.d.). Student's *t*-test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.
[b]Number of cases (percentage). χ^2 test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.

non-progression group. This compares with 9 (31.03%) and 20 (68.97%), respectively, for the 29 cases of single HPV-52 infection (HPV-52 group). The relative risk of progression of cytological findings 2 years later in cases of HPV-16 infection was higher than in cases of HPV-52 infection (Table 5).

DISCUSSION

In this study, we investigated the association between common oncogenic HPV infection in a Japanese population and the progression of cytological findings 2 years later in Japanese women with NILM or ASC-US.

Matsumoto *et al.*[10] previously reported that in Japanese women with cytological low-grade squamous intraepithelial lesion and histological CIN grade 1–2 lesions, the oncogenic HPV infection group showed a greater tendency to develop CIN3 within 5 years than the non-oncogenic HPV infection group.[1] Consistent with these results, we confirmed that infection with common oncogenic HPV in Japan poses a higher risk for the progression of cytological findings

in the uterine cervix 2 years later than non-oncogenic HPV infection or no HPV infection.

As our study cohort was a pre-vaccination population; there is no influence of the HPV vaccine on our observations. Although more spontaneous regression of HPV infection usually occurs among young women, there was no significant difference in age distributions between the progression and the non-progression groups in this study. Therefore, we were able to investigate how the HPV genotype affects later cytological findings in Japanese women with NILM or ASC-US. Our investigation suggests that Japanese women with oncogenic HPV infections should undergo periodic checkups for uterine cervical cancer over the course of 2 years from initial identification of infection. In a previous study by Dufresne *et al.*,[11] persistent infection of oncogenic HPV was detected in 33 (67.6%) of 34 cases of ASC-US with oncogenic HPV infections, while in the remaining 11 cases (32.4%), 6 months later oncogenic HPV had disappeared and there was no sign of CIN2/3. In our study, among the 12 single oncogenic HPV-positive women diagnosed with ASC-US at first admission, persistent infection of oncogenic HPV was detected in four (33.3%), while oncogenic HPV had disappeared in the remaining eight cases (66.7%) 2 years later and there was no sign of cytological progression in these eight cases 2 years later. These results confirm that, as time passes, oncogenic HPV can disappear spontaneously in women with ASC-US, and that the negative predictive value of the HPV test may help to reduce the number of unnecessary colposcopies carried out in women with ASC-US.[11]

Khan *et al.*[12] reported that among the cases of NILM with HPV-16, the 10-year cumulative rate of CIN3 or cancer was 17% and the 2-year cumulative incidence rate of CIN3 or cancer was 9.8%. This is similar to our results, which showed that 3 (15%) of 20 single HPV-16-positive cases of 'NILM or ASC-US' at first admission were diagnosed with ASC-H or HSIL by cytological testing 2 years later (data not shown). Recently, the ATHENA (Addressing the Need for Advanced HPV Diagnostics) study reported that, among cases of NILM or ASC-US, the estimated absolute risk of CIN2 or worse in cases of HPV-16 and/or HPV-18 was higher than in cases of other oncogenic HPV.[13–15] Our result is consistent with these studies that showed HPV-16 is associated with a higher risk of cervical cancer onset than oncogenic HPV genotypes in other regions.[16] We clarified that a single HPV-16 infection of women with 'NILM or ASC-US'

Table 2 Patient backgrounds in cases of single HPV 16 or single HPV 52 infections

	Single HPV-16 infection group (n = 20)	*Single HPV-52 infection group (n = 29)*	P-*value*
Age at first sampling (years)[a]	43.60 (15.99)	40.83 (10.01)	0.768
Smoking[a]	5 (25%)	4 (13.8%)	0.319
Use of oral contraceptive[b]	1 (5%)	0 (0%)	0.224
Parity[b]			
Nulliparous	7 (35%)	11 (37.9%)	0.381
Primiparous	5 (25%)	3 (10.3%)	
Multiparous	8 (40%)	15 (51.7%)	
Body mass index ($kg\,m^{-2}$)[a]	21.84 (3.27)	21.42 (2.15)	0.935

Abbreviation: HPV, human papillomavirus.
[a]Mean (standard deviation). Student's *t*-test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.
[b]Number of cases (percentage). χ^2 Test was used to analyze differences between groups. Statistical significance was defined as $P<0.05$.

Table 3 Age distributions between the progression and the non-progression groups

Age (years)	*Progression group*	*Non-progression group*	P-*value*[a]
20–29	4	35	
30–39	24	72	0.06
⩾40	42	104	
Total cases	70	211	

[a]Pearson's χ^2 test was used to analyze differences in age distributions between groups. Statistical significance was defined as $P<0.05$.

Table 5 Association between common oncogenic HPV genotype in Japan and progression of cytological findings 2 years later

	Results of HPV test			
	A Single HPV-16 infection	*B Single HPV-52 infection*	*Total cases*	*Relative risk (95% confidence interval) A* vs *B*
Progression group	13	9	22	2.094 (1.005–3.935)
Non-progression group	7	20	27	
Total cases	20	29	49	

Abbreviation: HPV, human papillomavirus.

Table 4 Association between HPV infection and progression of cytological findings 2 years later

	Results of HPV test				*Relative risk (95% confidence interval)*		
	A Single oncogenic HPV infection	*B Single non-oncogenic HPV infection*	*C Negative HPV*	*Total cases*	*A* vs *B*	*A* vs *C*	*B* vs *C*
Progression group	30	3	37	70	3.376 (1.282–11.422)	1.874 (1.451–3.110)	2.012 (0.573–7.067)
Non-progression group	47	23	141	211			
Total cases	77	26	178	281			

Abbreviation: HPV, human papillomavirus.

cytology is associated with a higher risk of progression of cytological findings 2 years later compared with a single HPV-52 infection, supporting the recommendation of colposcopy for HPV-16 genotyping of women with NILM or ASC-US cytology.[6]

According to the Cochrane Database of Systematic Reviews, HPV-triage with hybrid capture 2 is recommended to triage women with ASC-US because of its greater accuracy (significantly higher sensitivity, and similar specificity) compared with repeat cytology.[17] Regarding uterine cervical cancer in Japanese women, the genetics of HPV infection (oncogenic HPV genotype and oncogenic HPV viral loads) is not sufficient to cause CIN3/cervical cancer, and host genetic factors are also likely to contribute to cervical cancer pathogenesis.[2,4,5,8,18–22] Further study focused on cancer genetics in both the HPV virus and host may enable the development of a comprehensive system of early detection and prevention of uterine cervical cancer.

In conclusion, for the first time, we confirmed that common oncogenic HPV infection is associated with a higher risk of progression of cytological findings 2 years later in Japanese women with NILM or ASC-US; of these infections, HPV-16 was found to have a higher risk of progressive cytological findings than HPV-52. Therefore, the initial HPV-DNA genotype may be a potential predictive marker of later progression of cytological findings in the uterine cervix in Japanese women with NILM or ASC-US.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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1 Muñoz, N., Bosch, F. X., de Sanjosé, S., Herrero, R., Castellsagué, X., Shah, K. V. *et al.* The International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. *New Engl. J. Med.* **348,** 518–527 (2003).
2 Yamasaki, K., Miura, K., Shimada, T., Ikemoto, R., Miura, S., Murakami, M. *et al.* Pre-vaccination epidemiology of human papillomavirus infections in Japanese women with abnormal cytology. *J. Obstet. Gynaecol. Res.* **37,** 1666–1670 (2011).
3 Yamasaki, K., Miura, K., Shimada, T., Miura, S., Abe, S., Murakami, M. *et al.* Epidemiology of human papillomavirus genotypes in pregnant Japanese women. *J. Hum. Genet.* **56,** 313–315 (2011).
4 Inoue, M., Okamura, M., Hashimoto, S., Tango, M. & Ukita, T. Adoption of HPV testing as an adjunct to conventional cytology in cervical cancer screening in Japan. *Int. J. Gynaecol. Obstet.* **111,** 110–114 (2010).
5 Smith, J. S., Lindsay, L., Hoots, B., Keys, J., Franceschi, S., Winer, R. *et al.* Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *Int. J. Cancer* **121,** 621–632 (2007).
6 Saslow, D., Solomon, D., Lawson, H. W., Killackey, M., Kulasingam, S. L., Cain, J. *et al.* American Cancer Society; American Society for Colposcopy and Cervical Pathology; American Society for Clinical Pathology. American Cancer Society, American Society for Colposcopy and Cervical Pathology, and American Society for Clinical Pathology screening guidelines for the prevention and early detection of cervical cancer. *Am. J. Clin. Pathol.* **137,** 516–542 (2012).
7 Gravitt, P. E., Peyton, C. L., Alessi, T. Q., Wheeler, C. M., Coutlée, F., Hildesheim, A. *et al.* Improved amplification of genital human papillomaviruses. *J. Clin. Microbiol.* **38,** 357–361 (2000).
8 Asato, T., Maehama, T., Nagai, Y., Kanazawa, K., Uezato, H. & Kariya, K. A large case-control study of cervical cancer risk associated with human papillomavirus infection in Japan, by nucleotide sequencing-based genotyping. *J. Infect. Dis.* **189,** 1829–1832 (2004).
9 Walboomers, J. M., Jacobs, M. V., Manos, M. M., Bosch, F. X., Kummer, J. A., Shah, K. V. *et al.* Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J. Pathol.* **189,** 12–19 (1999).
10 Matsumoto, K., Oki, A., Furuta, R., Maeda, H., Yasugi, T., Takatsuka, N. *et al.* Japan HPV and Cervical Cancer Study Group. Predicting the progression of cervical precursor lesions by human papillomavirus genotyping: a prospective cohort study. *Int. J. Cancer* **128,** 2898–2910 (2011).
11 Dufresne, S., Sauthier, P., Mayrand, M. H., Petignat, P., Provencher, D., Drouin, P. *et al.* Human papillomavirus (HPV) DNA triage of women with atypical squamous cells of undetermined significance with amplicor HPV and hybrid capture 2 assays for detection of high-grade lesions of the uterine cervix. *J. Clin. Microbiol.* **49,** 48–53 (2011).
12 Khan, M. J., Castle, P. E., Lorincz, A. T., Wacholder, S., Sherman, M., Scott, D. R. *et al.* The elevated 10-year risk of cervical precancer and cancer in women with human papillomavirus (HPV) type 16 or 18 and the possible utility of type-specific HPV testing in clinical practice. *J. Natl Cancer Inst.* **97,** 1072–1079 (2005).
13 Stoler, M. H., Wright, T. C. Jr, Sharma, A., Apple, R., Gutekunst, K. & Wright, T. L. ATHENA (Addressing the Need for Advanced HPV Diagnostics) HPV Study Group. High-risk human papillomavirus testing in women with ASC-US cytology: results from the ATHENA HPV study. *Am. J. Clin. Pathol.* **135,** 468–475 (2011).
14 Wright, T. C. Jr, Stoler, M. H., Sharma, A., Zhang, G., Behrens, C. & Wright, T. L. ATHENA (Addressing the Need for Advanced HPV Diagnostics) Study Group. Evaluation of HPV-16 and HPV-18 genotyping for the triage of women with high-risk HPV+ cytology-negative results. *Am. J. Clin. Pathol.* **136,** 578–586 (2011).
15 Stoler, M. H., Wright, T. C. Jr, Sharma, A., Zhang, G., Apple, R., Wright, T. L. *et al.* ATHENA (Addressing the Need for Advanced HPV Diagnostics) Study Group. The interplay of age stratification and HPV testing on the predictive value of ASC-US cytology. Results from the ATHENA HPV study. *Am. J. Clin. Pathol.* **137,** 295–303 (2012).
16 Bouvard, V., Baan, R., Straif, K., Grosse, Y., Secretan, B., El Ghissassi, F. *et al.* A review of human carcinogens—part B: biological agents. *Lancet Oncol.* **10,** 321–322 (2009).
17 Arbyn, M., Roelens, J., Simoens, C., Buntinx, F., Paraskevaidis, E., Martin-Hirsch, P. P. *et al.* Human papillomavirus testing versus repeat cytology for triage of minor cytological cervical lesions. *Cochrane Database Syst. Rev.* **3,** CD008054 (2013).
18 Magnusson, P. K., Lichtenstein, P. & Gyllensten, U. B. Heritability of cervical tumours. *Int. J. Cancer* **88,** 698–701 (2000).
19 Yoshida, T., Sano, T., Kanuma, T., Owada, N., Sakurai, S., Fukuda, T. *et al.* Quantitative real-time polymerase chain reaction analysis of the type distribution, viral load, and physical status of human papillomavirus in liquid-based cytology samples from cervical lesions. *Int. J. Gynecol. Cancer* **18,** 121–127 (2008).
20 Hamaguchi, Y., Miura, K., Abe, S., Kinoshita, A., Miura, S., Yamasaki, Y. *et al.* Initial viral load in cases of single human papillomavirus 16 or 52 persistent infection is associated with progression of later cytopathological findings in the uterine cervix. *J. Med. Virol.* **85,** 2093–2100 (2013).
21 Matsumoto, K., Maeda, H., Oki, A., Takatsuka, N., Yasugi, T., Furuta, R. *et al.* HLA class II DRB1*1302 allele protects against progression to cervical intraepithelial neoplasia grade 3: a multicenter prospective cohort study. *Int. J. Gynecol. Cancer* **22,** 471–478 (2012).
22 Abe, S., Miura, K., Kinoshita, A., Mishima, H., Miura, S., Yamasaki, K. *et al.* Copy number variation of the antimicrobial-gene, *defensin beta 4,* is associated with susceptibility to cervical cancer. *J. Hum. Genet.* **58,** 250–253 (2013).

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Identification of endometrioid endometrial carcinoma-associated microRNAs in tissue and plasma

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HIGHLIGHTS

- A set of endometrioid endometrial carcinoma (EEC)-associated miRNAs in tissue and plasma was identified by next-generation sequencing approach.
- EEC-associated miRNAs in tissues and plasma samples could distinguish EEC sample from NE sample with high accuracy.
- EEC-associated miRNA levels in EEC tissues and plasma samples were associated with pathological characteristics.

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ABSTRACT

Objective. This study aimed to identify a set of endometrioid endometrial carcinoma EEC-associated microRNAs (miRNAs) in tissue and plasma, and evaluate their clinical significance.

Methods. A set of EEC-associated miRNAs in tissue and plasma was identified by next-generation sequencing (NGS), which could enable in-depth characterization of the global repertoire of miRNAs.

Results. NGS identified 11 candidate EEC-associated miRNAs. Quantitative reverse-transcriptase PCR identified 8 EEC-associated miRNAs in tissue (upregulated: miR-499, miR-135b, miR-205, downregulated: miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21). Expression of hsa-miR-499 in International Federation of Gynecology and Obstetrics (FIGO) Stage IA and Grade 1 tissues was significantly lower than in others (FIGO Stage IB or more advanced, and Grade 2 or 3). By receiver operating characteristic (ROC) curve analysis, compared with single EEC-associated miRNA, two miRNA signatures (miR135b/miR195 and miR135b/miR30a-3p) could distinguish between EEC and normal endometrial tissue samples yielding a high area under the curve (AUC) of 0.9835 [95% confidence interval (CI): 0.9677–1.0], and 0.9898 (95% CI: 0.9677–1.0), respectively. As possible non-invasive markers for EEC, four EEC-associated miRNAs (increased level: miR-135b and miR-205, decreased-level: miR-30a-3p and miR-21) in plasma were identified. Circulating levels of three EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) in plasma were significantly decreased after hysterectomy. ROC curves analysis revealed that miR-135b and miR-205 levels in plasma yielded AUCs of 0.9722 (95% CI: 0.913–1.0) and 1.0 (95% CI: 1.0–1.0), respectively.

Conclusion. Measurement of tissue and plasma EEC-associated miRNAs may be useful for early detection, diagnostic, and follow-up tests for EEC.

Introduction

Endometrial cancer is a common malignancy of the female reproductive tract. The most dominant subtype, endometrioid endometrial carcinoma (EEC) accounts for ~80% of cases [1]. Accumulation of several genetic and epigenetic alternations in oncogenes and tumor suppressor genes is involved in the development of endometrial carcinoma [2]. However, such alterations are not uniformly found in all EEC cases, and information regarding the molecular mechanisms of EEC etiology is still limited. The search for novel molecular markers for early detection and predicting outcomes has been ongoing in most cancers with a view to identifying molecular targets for therapeutic agents.

MicroRNAs (miRNAs) are non-protein-coding small RNAs (21–25 nucleotides) that function as regulators of gene expression by antisense complimentarily to specific mRNAs [3,4]. As miRNAs are expressed in tissue-specific patterns [3], miRNAs predominantly expressed in EEC tissues are probably involved in cell proliferation, differentiation, apoptosis, and carcinogenesis of the endometrium [5,6]. Recently, by searching a panel of microarray assays, miRNA signatures in tissue and

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plasma could be used to distinguish EEC from normal endometrium (NE) [7,8]. This suggests that EEC-associated miRNAs have the potential to be developed as novel diagnostic and therapeutic molecules. However, the data regarding EEC-associated miRNAs in tissue and plasma are limited; therefore, investigation of EEC-associated miRNAs is likely to shed light on the molecular mechanisms of EEC etiology.

Microarray technology is high throughput, but can only detect a limited number of miRNAs because of the nature of probe hybridization [9]. Next-generation sequencing (NGS) technology using Illumina technology generates short reads (35 bp) but more than 1 million bp of sequence data per run, and can be used to measure the abundance of small-RNA sequences in a sample. miRNAs are only 21–25 bp in length; therefore, this technology can enable in-depth characterization of the global repertoire of miRNAs [10].

In this study, to get a clue regarding novel diagnostic and therapeutic molecules of EEC, we tried to identify EEC-associated miRNAs in tissue and plasma. First, by comparative analysis of NGS-generated miRNA expression profiles of EEC tissue, NE tissue and blood cells from the same patient, we selected candidate EEC-associated miRNAs, whose expression level was negative in the blood of patients, and in EEC tissues was >2 times up- or downregulated compared with that in NE tissue. Second, by comparative analysis of EEC and NE tissues using real-time quantitative RT-PCR (qRT-PCR), we identified EEC-associated miRNAs in tissue. Subsequently, to identify and characterize EEC-associated miRNAs in plasma, the circulating levels of EEC-associated miRNA in plasma in women with EEC or NE. Finally, the relationship between EEC-associated miRNA expression and clinicopathological characteristics, and the diagnostic value of EEC-associated miRNA expression in tissue and plasma were analyzed tentatively.

Materials and methods

Sample collection

Study subjects were recruited at the Department of Obstetrics and Gynecology, Nagasaki University Hospital, Japan. All samples were obtained after receiving written informed consent, and the study protocol was approved by the Institutional Review Board for Ethical, Legal and Social Issues of Nagasaki University.

For NGS analysis, EEC tissue, NE tissue, and blood cells were obtained from an identical patient with International Federation of Obstetricians and Gynecologists (FIGO) Stage IA (Grade 1) EEC. EEC and NE tissue samples were obtained immediately after total hysterectomy with bilateral salpingo-oophorectomy. EEC was diagnosed by endometrial biopsy prior to the operation. Diagnosis of EEC and NE tissue was confirmed by pathological analysis. EEC and NE tissue samples were placed in RNAlater (Ambion, Austin, TX, USA). The blood samples (7 mL) were collected before the operation and placed in tubes containing EDTA. Using a mirVana miRNA Isolation Kit (Ambion), total RNA containing small RNA molecules was extracted from each sample immediately after sampling. Quality assessment and concentration measurements of total RNA, including small RNAs, were performed using a Bioanalyzer (Agilent Technologies, South Queensferry, UK) and a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), respectively.

For subsequent expression analysis by qRT-PCR, EEC tissues were obtained from 28 cases of EEC (EEC group) and NE tissues from 14 cases of non-EEC (NE group). In addition to total hysterectomy with bilateral salpingo-oophorectomy, lymphadenectomy was performed in 25 cases. Tumor stage was determined according to 2009 revised FIGO classification [11]. In cases of NE, total hysterectomy was performed because of uterine myoma. Final diagnosis of EEC or NE was confirmed by pathological analysis. None of the EEC patients had a history of other malignant disease or had received neoadjuvant therapy. After the operation, patients were submitted to radiotherapy and/or chemotherapy according to FIGO guidelines. Between the EEC and NE groups, there were no significant differences in body mass index (BMI), history of parity, smoking, diabetes, or family history of endometrial cancer (data not shown). The mean (SD) patient age was 60.6 (10.8) years in the EEC group and 42.8 (5.1) years in the NE group (Student's t test, $P < 0.001$). Clinicopathological characteristics in the EEC group are listed in Supplementary Table 1.

To obtain cell-free plasma miRNAs, blood samples (7 mL) were collected from 12 cases of EEC and 12 of NE. Blood sampling was performed 1 day before the operation and 7 days after. Between the EEC and NE groups, there were no significant differences in BMI, history of parity, smoking, diabetes, or family history of endometrial cancer (data not shown). The mean (SD) patient age was 50.8 (8.3) years in the EEC group and 36.5 (8.3) years in the NE group (Student's t test, $P = 0.001$). Pathological characteristics in the EEC group are listed in Supplementary Table 2. Cell-free plasma samples were prepared from the blood by a double centrifugation method as described previously [12]. Total RNA containing small RNA molecules was extracted from 1.2 mL cell-free plasma samples as described previously [12]. Extracted total RNAs were stored at −80 °C. Although there were differences in age between the EEC and NE groups in both tissue and plasma, there was no significant correlation between expression of studied miRNAs and age of patients (data not shown).

In miRNA expression analysis in endometrial tissue, there is no consensus on universal endogenous normalization controls because small RNAs, including RNU48 and RNU6B, have been suggested as reference RNAs, but exhibit high variability [13]. In addition, it is recommended that the quantitative mRNA measurements in plasma are expressed as an absolute concentration [14]. Therefore, we considered that the quantitative miRNA measurements may be the same as quantitative mRNA measurements in plasma. In this study, absolute real-time qRT-PCR analysis was performed.

Small RNA library construction and NGS analysis

To screen for EEC-associated miRNAs, NGS was applied to a set of EEC tissues, NE tissues and blood from the same EEC patient. Isolation of total RNA including small RNAs, their quality assessment, concentration measurements, small RNA library construction, NGS and miRNA mapping were performed as described previously [15–18].

To compare miRNA levels across data sets, the sequencing read count for each miRNA was normalized to the total read count of 1,000,000 in each sample, and expressed as reads per million (RPM) [15–17]. For mapped data, when the normalized miRNA read count was negative in patient's blood, and was >2 times up- or downregulated in EEC tissues than in NE tissue, these miRNAs were selected as candidate EEC-associated miRNAs. These miRNAs were then analyzed by RT-PCR in tissue and plasma from the EEC and NE group.

Real-time qRT-PCR analysis of miRNAs

All specific primers and TaqMan probes were purchased from TaqMan MicroRNA Assays (Applied Biosystems). Real-time qRT-PCR of miRNAs in tissues and plasma samples was performed as described previously [12,15]. For each miRNA assay, we prepared a calibration curve by 10-fold serial dilution of single-stranded cDNA oligonucleotides corresponding to each miRNA sequence from 1.0×10^2 to 1.0×10^8 copies/mL. Each sample and each calibration dilution were analyzed in triplicate. Each assay could detect down to 100 RNA copies/mL. Every batch of amplifications included three water blanks as negative controls for each of the reverse transcription and PCR steps. All data were collected and analyzed using an ABI Prism 7900 Sequence Detector (Applied Biosystems).

Statistical analysis

Patient backgrounds were compared by Student's t test and Pearson's χ^2 test for continuous and discrete variables, respectively, of

EEC and NE cases. Absolute quantification data were analyzed with SDS 2.3 software (Applied Biosystems). The expression levels of EEC-associated miRNAs in tissues and the cell-free plasma concentrations of EEC-associated miRNAs in cases of EEC and NE were converted into multiples of the median (MoM) of concentration in the cases of NE. Differences between the two groups were evaluated with Mann–Whitney's *U* test or Kruskal–Wallis test. Changes in the cell-free plasma concentration of EEC-associated miRNAs before and after the operation were compared by the Wilcoxon signed-rank test. Statistical analyses were performed with SPSS version 19 (IBM Japan, Tokyo, Japan). To determine the ability of miRNAs to classify EEC and NE samples, receiver operating characteristic (ROC) curves were plotted with an R package, pROC [19]. To develop miRNA signatures featuring the best accuracy in distinguishing between EEC and NE samples a multivariate logistic regression model was utilized. Evaluation of obtained regression models was performed with the Wald test. Statistical analyses were performed using R (R Core Team, Vienna, Austria). Significant differences were defined as $P < 0.05$.

Results

Screening of candidate EEC-associated miRNAs by NGS

NGS analysis yielded 20,674,015 reads from EEC tissue, 19,107,722 reads from blood cells, and 20,375,081 reads from NE tissue. All the above sequence data were deposited in DDBJ Sequence Read Archive (DRA) (accession ID: DRA001166). High-throughput sequencing assays can be susceptible to noise and variability; therefore, measurement of miRNA expression was normalized using the library size (1,000,000 reads). Eleven candidate EEC-associated miRNAs were identified (Table 1). Candidate EEC-associated miRNAs identified were located on various chromosomal regions. Out of 11 candidate EEC-associated miRNAs, 5 (miR-10b, miR-499, miR-184, miR-195 and miR-135b) were upregulated in EEC tissue, while 6 (miR-203, miR-10a, miR-30a-5p, miR-205, miR-30a-3p and miR-21) were downregulated in EEC tissue than NE (Table 1).

Confirmation of EEC-associated miRNAs in tissue by qRT-PCR

Expression levels of the 11 candidate EEC-associated miRNAs in 28 EEC and 14 NE tissues were measured by qRT-PCR. Eight miRNAs showed significantly different expression between EEC and NE tissues, and were identified as EEC-associated miRNAs in tissue. The expression levels of 3 EEC-associated miRNAs (miR-499, miR-135b and miR-205) were significantly higher in EEC than NE tissues (Mann–Whitney *U* test, $P = 0.003$, $P < 0.001$ and $P = 0.002$, respectively), while those of 5 EEC-associated miRNAs (miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21) were significantly downregulated in EEC tissue (Mann–Whitney *U* test, $P = 0.006$, $P < 0.001$, $P = 0.019$, $P = 0.001$, and $P = 0.011$, respectively; Table 2). Meanwhile, there was no significant difference in the levels of 3 candidate EEC-associated miRNAs (miR-184, miR-203 and miR-10a) between EEC and NE tissues (Table 2). Using a database search of predicted miRNA targets in mammals (www.targetscan.org), we searched the candidate target mRNAs of EEC-associated miRNAs in tissue. Three mRNAs (MutS homolog 2: MSH2, Leukotriene B4 12-hydroxydehydrogenase: LTB4DH and IκB kinase α: IKKα) were selected as common target mRNAs of 3 upregulated EEC-associated miRNAs in EEC tissue, while there was no common target mRNAs of 5 downregulated EEC-associated miRNAs.

Identification of EEC-associated miRNAs in plasma

Regarding the 8 EEC-associated miRNAs in tissue, circulating levels of each miRNA in plasma from 12 women with EEC and 12 with NE tissue were measured by qRT-PCR. Four miRNAs showed significantly different circulating levels between the EEC and NE groups, and were identified as EEC-associated miRNAs in plasma. The expression levels of 2 EEC-associated miRNAs (miR-135b and miR-205) were significantly higher in plasma samples from the EEC group than the NE group (Mann–Whitney *U* test, $P < 0.001$), while those of 2 EEC-associated miRNAs (miR-30a-3p and miR-21) were significantly lower in plasma samples from the EEC group than the NE group (Mann–Whitney *U* test, $P = 0.009$ and $P = 0.033$, respectively). Meanwhile, there was no significant difference in the levels of 4 EEC-associated miRNAs (miR-10b, miR-30a-5p, miR-195 and miR-499) between plasma samples from the EEC and NE groups (Table 3).

Identification of EEC-associated miRNAs that showed significantly decreased concentrations in plasma after hysterectomy

The 4 EEC-associated miRNAs that showed significantly increased or decreased levels in plasma in the EEC group compared with the NE group (Table 3, increased level: miR-135b and miR-205, decreased level: miR-30a-3p and miR-21) were selected for analysis before and after hysterectomy. The plasma concentrations of 3 miRNAs (Table 4, miR-135b, miR-205 and miR-30a-3p) were significantly decreased after hysterectomy (Wilcoxon signed-rank tests, $P = 0.003$, Table 3), and were considered as possible molecular markers in plasma. Meanwhile, there was no significant difference in the plasma level of hsa-miR-21 before and after hysterectomy (Table 3).

Relationship between EEC-associated miRNA expression and clinicopathological characteristics

To investigate the clinical significance of EEC-associated miRNAs in tissue and plasma, we compared EEC-associated miRNA expression in groups distinguished based on FIGO stage, histopathological grade, or relapse. Significant relationships were found between expression of miR-499 and FIGO stage, and between expression of miR-205 and histological grade. The expression level of miR-499 in 14 cases of FIGO Stage

Table 1
Candidate EEC-associated miRNAs detected by next-generation sequencing analysis.

miRNA	Chromosome localization	Blood cell (reads per million)	EEC tissue (reads per million)	NE tissue (reads per million)	EEC/NE
hsa-miR-10b	2q31.1	3	2220	770	2.88
hsa-miR-499	20q11.22	0	2010	705	2.85
hsa-miR-184	15q25.1	11	2006	851	2.36
hsa-miR-195	17p13.1	0	12,901	6320	2.04
hsa-miR-135b	1q32.1	0	133	66	2.02
hsa-miR-203	14q32.33	0	1712	3514	0.48
hsa-miR-10b	17q21.32	2	263	602	0.44
hsa-miR-30a-5p	6q13	50	15,732	45,694	0.34
hsa-miR-205	1q32.2	0	285	1356	0.21
hsa-miR-30a-3p	6q13	29	1610	9476	0.17
hsa-miR-21	17q23.1	30	219	1369	0.16

Normalized read counts are described as reads per million.

Table 2
Expression of candidate EEC-associated miRNAs in carcinoma tissues from patients with EEC group and NE tissues from patients without carcinoma.

miRNA	NE group ($n = 14$)	EEC group ($n = 28$)	P value
miR-10b	1.0 (0.68–1.62)	0.79 (0.17–2.81)	0.006
miR-499	1.0 (0.28–1.99)	2.49 (0.22–40.08)	0.003
miR-184	1.0 (0.1–18.2)	0.82 (0.06–169.3)	NS
miR-195	1.0 (0.024–1.96)	0.32 (0.10–0.87)	<0.001
miR-135b	1.0 (0.57–3.40)	5.13 (0.49–15.13)	<0.001
miR-203	1.0 (0.38–1.49)	1.13 (0.18–3.92)	NS
miR-10b	1.0 (0.29–1.55)	0.79 (0.17–2.81)	NS
miR-30a-5p	1.0 (0.23–2.0)	0.61 (0.27–1.62)	0.019
miR-205	1.0 (0.06–4.07)	2.47 (0.0–6.19)	0.002
miR-30a-3p	1.0 (0.54–2.04)	0.53 (0.1–2.45)	0.001
miR-21	1.0 (0.39–2.76)	0.64 (0.27–1.22)	0.011

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between groups were analyzed by Mann–Whitney U test. $P < 0.05$ was considered significant.
NS, not significant.

II or more advanced was significantly higher than that in 4 cases of FIGO Stage IA and IB (Mann–Whitney U test, $P = 0.019$, Supplementary Table 1). The expression level of miR-205 in EEC cases with Grade 3 tumor ($n = 2$) was significantly higher than that in cases with Grade 1 ($n = 15$) and 2 ($n = 11$) tumors (Kruskal–Wallis test, $P = 0.024$, Supplementary Table 1). The expression level of miR-499 in 7 cases of FIGO Stage IA and Grade 1 tumor was significantly lower than in 21 cases of other tumors (FIGO Stage IB or more advanced, and Grade 2 or 3) (Mann–Whitney U test, $P = 0.047$, Table 4). Meanwhile, there was no significant difference in the tissue levels of all EEC-associated miRNAs between groups distinguished according to the presence of lymph node metastasis or occurrence of relapse (Table 4).

Circulating miRNA levels of EEC-associated miRNAs in plasma were compared in groups distinguished according to FIGO stage and histopathological grade. We compared FIGO Stage IA Grade 1 tumors with others (more advanced FIGO stage and/or histopathological grade). The plasma concentration of miR-21 in 4 cases of FIGO Stage IA and Grade 1 tumors was significantly higher than that in 8 cases of more advanced tumors (Mann–Whitney U test, $P = 0.017$, Table 5). Meanwhile, there was no significant difference in the plasma concentrations of other EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) and carbohydrate antigen (CA)125 before and after hysterectomy (Table 5).

Diagnostic value of EEC-associated miRNA expression in tissue and plasma

ROC curves for discriminating EEC samples from NE were constructed based on EEC-associated miRNA expression in tissues (EEC, $n = 28$; NE, $n = 14$). Analysis of the ROCs revealed high area under curve (AUC) values for each EEC-associated miRNA in tissues (Fig. 1): miR-499, miR-30a-5p, miR-21, miR-10b, miR-205, miR-30a-3p, miR-195 and miR-135b yielded AUC of 0.7143 [95% confidence interval (CI): 0.5537–0.8749], 0.7245 (95% CI: 0.5445–0.9045), 0.7423 (95% CI: 0.5744–0.9103), 0.7602 (95% CI: 0.6132–0.9072), 0.8112 (95% CI: 0.666–0.9565), 0.8265 (95% CI: 0.6953–0.9578), 0.8736 (95% CI: 0.7145–1.0) and 0.9184 (95% CI: 0.8285–1.0), respectively (Fig. 1A). The miRNA signatures consisting of 2 miRNAs yielded elevated AUCs in comparison to single miRNAs. miR135b/miR195 and miR135b/miR30a-3p yielded AUCs of 0.9835 (95% CI: 0.9677–1.0, $P < 0.048$, Wald test, Fig. 1A), and 0.9898 (95% CI: 0.9677–1.0, $P < 0.038$, Wald test, Fig. 1A), respectively.

ROC curves for discriminating women with EEC from those with NE were constructed based on EEC-associated miRNA levels in plasma samples (EEC, $n = 12$; NE, $n = 12$). Analysis of the ROCs revealed high AUC values for each EEC-associated miRNA in plasma (Fig. 1B); miR-21, miR-30a-3p, miR-135b and miR-205 yielded AUC of 0.7569 (95% CI: 0.5611–0.9528), 0.8125 (95% CI: 0.6381–0.9869), 0.9722 (95% CI: 0.913–1.0) and 1.0 (95% CI: 1.0–1.0), respectively.

Discussion

In this study, we identified EEC-associated miRNAs in tissue and plasma, and evaluated their clinical significance.

NGS can be used to investigate all known and unknown miRNAs, while oligonucleotide microarray methods can only be used to examine a limited number of known miRNAs present on each array. Therefore, using NGS allows whole genome analysis to be performed to identify candidate miRNAs that are differentially expressed. In addition, the miRNA expression in each case of EEC depends on the heterogeneity of cancer. Our NGS analyses identified 11 candidate EEC-associated miRNAs (upregulated: miR-10b, miR-499, miR-184, miR-19 and miR-135b, downregulated: miR-203, miR-10a, miR-30a-5p, miR-205, miR-30a-3p and miR-21) at various chromosomal regions. Although all miRNAs were previously known, nine of the 11 were newly identified as candidate EEC-associated miRNAs (except for miR-203 and miR-205) that had not been identified in previous microarray studies [5–7,20]. Therefore, this indicates that NGS enables a more in-depth

Table 3
Circulating levels of EEC-associated miRNAs in plasma samples from patients without carcinoma (NE plasma) and patients with EEC (EEC plasma) before and after surgery.

miRNA	A	B	C	P value	
	NE plasma ($n = 12$)	EEC plasma before operation ($n = 12$)	EEC plasma after operation ($n = 11$)	A vs B	B vs C
miR-135b	1.0 (0.57–3.40)	5.13 (0.49–15.13)	0.0062 (0–0.96)	<0.001	0.003
miR-205	1.0 (0.06–4.07)	2.34 (0.0–6.19)	0.56 (0.34–0.94)	<0.001	0.003
miR-30a-3p	1.0 (0.54–2.04)	0.53 (0.096–2.25)	0.062 (0–0.12)	0.009	0.003
miR-21	1.0 (0.39–2.76)	0.64 (0.27–1.22)	0.59 (0.023–3.11)	0.033	NS
miR-10b	1.0 (0.34–2.49)	0.745 (0.05–1.55)	–	NS	–
miR-30a-5p	1.0 (0.21–2.74)	0.48 (0.05–1.3)	–	NS	–
miR-195	1.0 (0.24–2.1)	0.615 (0.05–1.73)	–	NS	–
miR-499	ND	ND	–	–	–

Expression levels are described as MoM values [median (minimum – maximum)]. Significant differences between control and EEC plasma before surgery were analyzed by Mann–Whitney U test, and significant differences between EEC plasma before and after operation were analyzed by Wilcoxon singed-rank test. $P < 0.05$ was considered significant.
–, not analyzed; ND, not detected; NS, not significant.

Table 4
Association between clinicopathological characteristics and EEC-associated miRNA levels in EEC tissues.

	FIGO stage and grade			Lymph node metastasis			Relapse		
	IA G1 ($n = 7$)	Others[a] ($n = 21$)	*P* value	– ($n = 21$)	+ ($n = 4$)	*P* value	– ($n = 25$)	+ ($n = 3$)	*P* value
miR-135b	5.64 (3.51–12.13)	4.39 (0.49–15.13)	NS	5.55 (0.49–12.13)	6.03 (1.49–15.13)	NS	5.67 (0.86–15.13)	4.09 (0.49–4.68)	NS
miR-205	2.92 (1.14–6.09)	2.24 (0–6.19)	NS	2.14 (0–6.19)	3.03 (1.43–6.12)	NS	2.74 (0–6.19)	1.43 (1.4–2.34)	NS
miR-21	0.89 (0.43–1.22)	0.58 (0.27–0.96)	NS	0.7 (0.32–1.22)	0.55 (0.46–0.96)	NS	0.7 (0.27–1.22)	0.55 (0.32–0.81)	NS
miR-30a-3p	0.595 (0.22–2.45)	0.43 (0.1–2.15)	NS	0.28 (0.24–0.45)	0.57 (0.13–2.45)	NS	0.55 (0.1–2.45)	0.45 (0.2–0.72)	NS
miR-499	1.09 (0.41–2.54)	2.92 (0.22–40.08)	0.047	2.09 (0.22–40.48)	5.24 (2.48–26.26)	NS	2.48 (0.21–40.48)	3 (2.73–7.21)	NS
miR-10b	0.8 (0.49–2.59)	0.66 (0.17–2.81)	NS	0.79 (0.21–2.81)	0.88 (0.3–1.15)	NS	0.79 (0.17–2.81)	0.85 (0.56–1.15)	NS
miR-30a-5p	0.69 (0.39–1.26)	0.61 (0.27–1.62)	NS	0.62 (0.27–1.62)	0.58 (0.46–0.68)	NS	0.61 (0.28–1.62)	0.61 (0.27–1.39)	NS
miR-195	0.28 (0.13–0.87)	0.35 (0.1–0.74)	NS	0.26 (0.13–0.87)	0.35 (0.1–0.54)	NS	0.33 (0.13–0.87)	0.18 (0.1–0.74)	NS

Expression levels of miRNAs are described as MoM values [median (minimum — maximum)]. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P < 0.05$ was considered significant.
NS, not significant.
[a] Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1.

characterization of the global repertoire of miRNAs compared with oligonucleotide microarray analysis and/or the heterogeneity of cancer because the discovery set used for NGS analysis was obtained from a single cancer patient, a limitation of this study. Therefore, it is critical to explore additional studies of miRNAs based on the heterogeneity of EEC. Consistent with a previous study, several dysregulated miRNAs in EEC tissues were identified in our analyses [5–7,20]. However, in previous studies of EEC tissues, miR-200 family, miR-9, miR-203, miR-205 and miR-210 were upregulated, while miR-410, miR-17-5p, miR-214, miR-99a,b, miR-199b, miR-100, miR-20a, miR-221, miR-222 and miR-424 were downregulated [5,7,20–26]. The discrepancy between our study and the previous studies reflects the difference in the way to select the candidate EEC-associated miRNAs at the beginning of each study. Previous studies have selected EEC-associated miRNAs with predominantly dysregulated expression in the EEC tissues at the beginning of their study [7]. In contrast, we selected the miRNAs that had predominantly dysregulated expression in EEC tissues compared with NE tissues, but negative expression (<100 read counts) in blood cells as candidate EEC-associated miRNAs. This was because one of our goals was to identify the EEC-associated miRNAs in plasma as non-invasive diagnostic markers for EEC. Another reason for the discrepancy between the present and previous studies may be related to the method of obtaining samples for high-throughput analysis. Previous studies obtained EEC and NE samples from different individuals. However, each case had a heterogeneous background and each miRNA expression pattern in EEC and NE was affected by various factors, for example, the phase during the menstrual cycle, and the background affecting the molecular pathways of EEC and NE differed among individuals. Therefore, in the present study, to make uniform the influence of backgrounds affecting miRNA expression in EEC and NE, we compared EEC and NE tissue from the same EEC patient (FIGO Stage IA, Grade 1) at the same time.

Table 5
Association between pathological characteristics, EEC-associated miRNA levels and CA 125 levels in plasma from patients with EEC.

	FIGO stage and grade		
	IA G1 ($n = 4$)	Others[a] ($n = 8$)	*P* value
miR-135b	3.71 (3.61–7.67)	6.52 (1.47–9.22)	NS
miR-205	4.95 (3.95–5.79)	5.38 (2.96–7.36)	NS
miR-21	0.24 (0.013–0.45)	0.82 (0.31–1.73)	0.017
miR-30a-3p	0.52 (0.34–0.65)	0.69 (0.52–1.08)	NS
CA125	22.5 (17.6–51.2)	17.5 (7.7–127.4)	NS

Expression levels of miRNAs are described as MoM values [median (minimum — maximum)], and CA 125 levels as U/mL. Significant differences between groups were analyzed by Mann–Whitney *U* test. $P < 0.05$ was considered significant.
NS, not significant.
[a] Includes tumors with more advanced FIGO stage and/or histopathological grade than Stage IA, Grade 1.

Subsequent confirmation analysis using qRT-PCR identified 8 EEC-associated miRNAs in tissue (upregulated: miR-499, miR-135b and miR-205, downregulated: miR-10b, miR-195, miR-30a-5p, miR-30a-3p and miR-21). miR-205 was upregulated in the qRT-PCR study, although it was downregulated in the NGS experiment. Additionally, miR-10b and miR-195 were downregulated in the qRT-PCR study, although they were upregulated in the NGS experiment. This discrepancy was also found in a previous study [7], and might have been because single cases were analyzed by NGS but multiple cases by qRT-PCR.

We identified novel and already known EEC-associated miRNAs [5,7,20–26]. miR-205 is frequently dysregulated in many human cancers, suggesting its important roles in initiation and progression of cancer. Previous studies identified significantly overexpressed hsa-miR-205 in endometrial cancer compared with NE tissue, and JPH4, ESRRG and PTEN were the candidate tumor suppressor genes in EEC [5,27,28]. In contrast, miRNA-205 was significantly suppressed in renal cancer cell lines and tumors when compared with normal tissues and a non-malignant cell line [29]. The expression of miRNA-205 is significantly high in some malignancies but significantly low in other malignancies, depending on the organs from which the malignancy comes. These observations suggest that a miRNA has more than one target mRNA.

By using the database search, 3 mRNAs (MSH2, LTB4DH and IKKα) were selected as common targets of 3 up-regulated EEC-associated miRNAs in EEC tissue. An oncogenic miRNA acts as an oncogene and has increased expression in tumor cells, while a tumor suppressor miRNA acts as a tumor suppressor gene and has decreased expression in tumor cells. All 3 candidate target mRNAs are tumor suppressor genes [30–34], thus, it is compatible that they are candidate target mRNAs of upregulated EEC-associated miRNAs (oncogenic miRNAs) in EEC tissue.

The relationship between EEC-associated miRNA expression in EEC tissue and clinicopathological characteristics was investigated. The expression level of has-miR-499 in tissues of FIGO Stage II or more

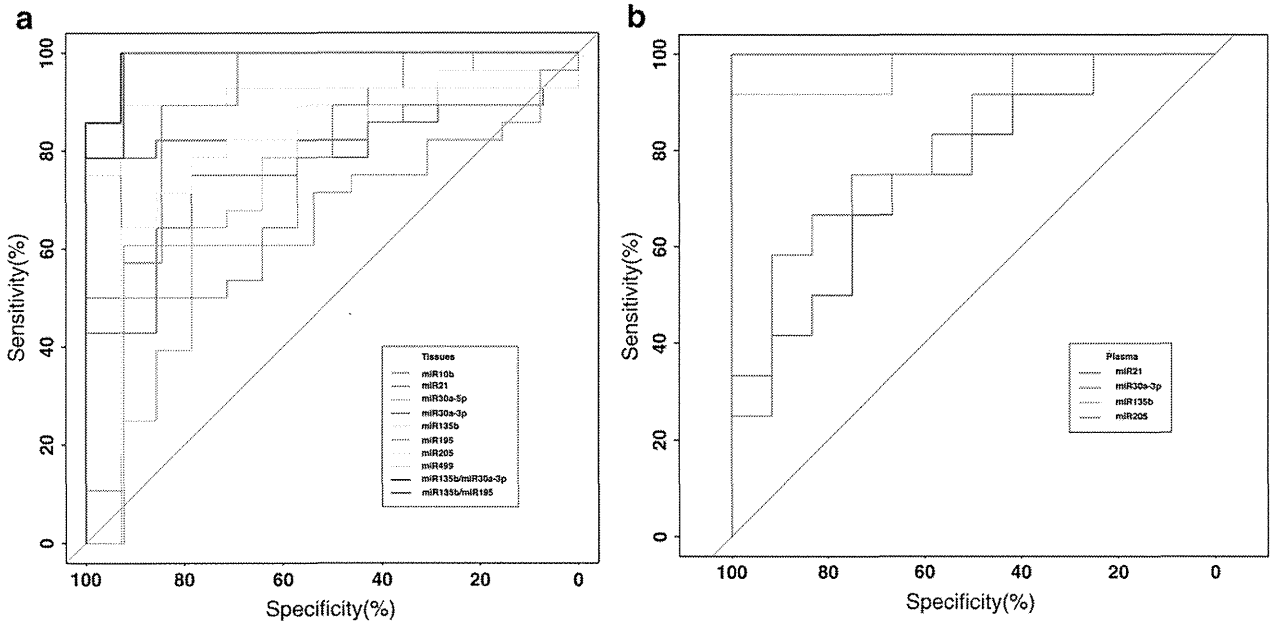

Fig. 1. ROC curve analysis using tissue and plasma miRNA profiles for discriminating EEC samples from NE samples. (A) Tissue miRNA profiles (EEC, $n = 28$; control, $n = 14$); miR-10b, miR-499, miR-195, miR-135b, miR-30a-5p, miR-205, miR-30a-3p and miR-21 yielded AUC of 0.7602 (95% CI: 0.6132–0.9072), 0.7143 (95% CI: 0.5537–0.8749), 0.8736 (95% CI: 0.7145–1.000), 0.9184 (95% CI: 0.8285–1.0), 0.7245 (95% CI: 0.5445–0.9045), 0.8112 (95% CI: 0.666–0.9565), 0.8265 (95% CI: 0.6953–0.9578) and 0.7423 (95% CI: 0.5744–0.9103), respectively. The miR signatures consisting of 2 miRNAs yielded elevated AUC values in comparison to single miRNAs. The miR135b/miR30a-3p yielded an AUC of 0.9898 (95% CI: 0.9677–1.0, $P < 0.038$, Wald test), and miR135b/miR195 yielded AUC of 0.9835 (95% CI: 0.9677–1.0, $P < 0.048$, Wald test). (B) Plasma miRNA profiles (EEC, $n = 12$; control, $n = 12$); miR-135b, miR-205, miR-30a-3p and miR-21 yielded AUC values of 0.9722 (95% CI: 0.913–1.0), 1.0 (95% CI: 1.0–1.0), 0.8125 (95% CI: 0.6381–0.9869), and 0.7569 (95% CI: 0.5611–0.9528), respectively.

advanced tumors was significantly higher than that in tissues of Stages IA and IB tumors. The expression level of miR-205 in EEC of FIGO Grade 3 tumor was significantly higher than that in Grade 1 and 2 tumors. The expression level of has-miR-499 in tissues of FIGO Stage IA and Grade 1 tumors was significantly lower than in tissues of other tumors (FIGO Stage IB or more advanced, and Grade 2 or 3). ROC curve analysis revealed that single regulated EEC-associated miRNAs in tissues could distinguish between EEC and NE tissue samples yielding high AUCs. In addition, 2 miRNA signatures, miR135b/miR195 and miR135b/miR30a-3p, classified EEC tumor tissues with higher accuracy than single miRNAs. These observations suggest that EEC-associated miRNA signatures in tissue could be a diagnostic marker, a supportive marker to estimate the pathological stage and grade of EEC, and potential markers to decide treatment strategies for each EEC case [7].

Finally, as non-invasive markers for EEC, four EEC-associated miRNAs (increased level: miR-135b, miR-205, decreased-level: miR-30a-3p and miR-21) in plasma were identified. Increased levels of EEC-associated miRNAs in plasma also showed higher expression level in EEC tissue, and decreased levels of EEC-associated miRNAs in EEC plasma showed lower expression level in EEC tissue. This suggests that circulating levels of EEC-associated miRNA in plasma reflect the expression status of EEC-associated miRNA in tissue. Torres et al. evaluated miRNA profiles in matched tissue and plasma samples from EEC patients, and showed diagnostic and prognostic significance of plasma miRNA signatures in EEC [7]. Although invasive procedures including biopsies or surgery were performed in the current clinical diagnosis, plasma-based biomarkers may lead to development of a non-invasive test of EEC. To date, miRNA expression pattern is known to be aberrant in cancer, and tumor-cell-derived miRNAs in circulation may be stored in microvesicles that are secreted by various cell types. Additionally, cell-free miRNAs are remarkably stable molecules in plasma [35]. Although the source of plasma EEC-associated miRNAs has not been determined so far, they might derive from exosomes shed from apoptotic or broken cells in EEC and NE [35–37]. In this study, circulating levels of 3 EEC-associated miRNAs (miR-135b, miR-205 and miR-30a-3p) in plasma were significantly decreased after surgery, suggesting that these miRNAs in plasma were mainly from EEC and NE, and may serve as a non-invasive biomarker for diagnosis of EEC, for example, early detection of early-stage EEC or relapse.

As for the clinical significance of plasma EEC-associated miRNAs, circulating levels of EEC-associated miRNAs in plasma were compared in groups distinguished according to FIGO stage and histopathological grade. In comparison of FIGO Stage IA (Grade 1) tumors with others (more advanced FIGO stage and/or histopathological grade), the plasma concentration of miR-21 in cases of FIGO Stage IA (Grade 1) tumors was significantly higher than in more advanced tumors, suggesting that this miRNA may have the potential to detect early-stage EEC. ROC curve analysis revealed that 4 single regulated EEC-associated miRNAs in plasma could distinguish between EEC and NE cases yielding high AUCs (Fig. 1B). Two single miRNAs, miR-135b and miR-205, yielded 0.9722 (95% CI: 0.913–1.0) and 1.0 (95% CI: 1.0–1.0), respectively. CA125 is a current tumor marker for EEC, and can be measured simply and non-invasively, and provide a useful indicator of tumor status. However, the sensitivity and positive predictive value of CA125 is relatively low in detecting EEC [38]. In contrast, EEC-associated miRNAs have different expression profiles in NE and EEC, suggesting that EEC-associated miRNAs in plasma may be used as additional biomarkers for EEC diagnosis.

In conclusion, a set of EEC-associated miRNAs in tissue and plasma of EEC patients was identified by NGS, which could enable in-depth characterization of the global repertoire of miRNAs. EEC-associated miRNA levels in tissue and plasma were associated with pathological characteristics, and could distinguish EEC from NE samples with high accuracy. Although our data are still preliminary because of the small sample size, the measurement of EEC-associated miRNAs in the tissue and plasma may be used as a diagnostic, prognostic, and follow-up test for EEC. Future studies regarding the biological pathway of EEC-associated miRNAs in tissue and plasma may contribute to the elucidation of molecular pathogenesis of EEC, endometrium development, and discovery of novel therapeutic targets of EEC.

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Conflict of interest

The authors declare no conflict of interest.

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References

[1] Amant F, Moerman P, Neven P, Timmerman D, Van Limbergen E, Vergote I. Endometrial cancer. Lancet 2005;366:491–505.
[2] Matias-Guiu X, Catasus L, Bussaglia E, Lagarda H, Garcia A, Pons C, et al. Molecular pathology of endometrial hyperplasia and carcinoma. Hum Pathol 2001;32:569–77.
[3] Mendell JT. MicroRNAs: critical regulators of development, cellular physiology and malignancy. Cell Cycle 2005;4:1179–84.
[4] Plasterk RH. Micro RNAs in animal development. Cell 2006;124:877–81.
[5] Chung TK, Cheung TH, Huen NY, Wong KW, Lo KW, Yim SF, et al. Dysregulated microRNAs and their predicted targets associated with endometrioid endometrial adenocarcinoma in Hong Kong women. Int J Cancer 2009;124:1358–65.
[6] Ramón LA, Braza-Boïls A, Gilabert J, Chirivella M, España F, Estellés A, et al. MicroRNAs related to angiogenesis are dysregulated in endometrioid endometrial cancer. Hum Reprod 2012;27:3036–45.
[7] Torres A, Torres K, Pesci A, Ceccaroni M, Paszkowski T, Cassandrini P, et al. Diagnostic and prognostic significance of miRNA signatures in tissues and plasma of endometrioid endometrial carcinoma patients. Int J Cancer 2013;132:1633–45.
[8] Jia W, Wu Y, Zhang Q, Gao G, Zhang C, Xiang Y. Identification of four serum microRNAs from a genome-wide serum microRNA expression profile as potential non-invasive biomarkers for endometrioid endometrial cancer. Oncol Lett 2013;6:261–7.
[9] Coppée JY. Do DNA, microarrays have their future behind them? Microbes Infect 2008;10:1067–71.
[10] Yang Q, Lu J, Wang S, Li H, Ge Q, Lu Z. Application of next-generation sequencing technology to profile the circulating microRNAs in the serum of preeclampsia versus normal pregnant women. Clin Chim Acta 2011;412:2167–73.
[11] Pecorelli S. Revised FIGO staging for carcinoma of the vulva, cervix, and endometrium. Int J Gynaecol Obstet 2009;105:103–4 [Erratum in: Int J Gynaecol Obstet 2010;108:176.].
[12] Miura K, Miura S, Yamasaki K, Higashijima A, Kinoshita A, Yoshiura K, et al. Identification of pregnancy-associated microRNAs in maternal plasma. Clin Chem 2010;56:1767–71.
[13] Meyer SU, Pfaffl MW, Ulbrich SE. Normalization strategies for microRNA profiling experiments: a 'normal' way to a hidden layer of complexity? Biotechnol Lett 2010;32:1777–88.
[14] Ng EK, Tsui NB, Lam NY, Chiu RW, Yu SC, Wong SC, et al. Presence of filterable and nonfilterable mRNA in the plasma of cancer patients and healthy individuals. Clin Chem 2002;48:1212–7.
[15] Higashijima A, Miura K, Mishima H, Kinoshita A, Jo O, Abe S, et al. Characterization of placenta-specific microRNAs in fetal growth restriction pregnancy. Prenat Diagn 2013;33:214–22.
[16] Hansen KD, Irizarry RA, Wu Z. Removing technical variability in RNA-seq data using conditional quantile normalization. Biostatistics 2012;13:204–16.
[17] Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat Methods 2008;5:621–8.
[18] Li R, Li Y, Kristiansen K, et al. SOAP: short oligonucleotide alignment program. Bioinformatics 2008;24:713–4.
[19] Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez J-C, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. BMC Bioinformatics 2011;12:77.
[20] Banno K, Kisu I, Yanokura M, Masuda K, Ueki A, Kobayashi Y, et al. Epigenetics and genetics in endometrial cancer: new carcinogenic mechanisms and relationship with clinical practice. Epigenomics 2012;4:147–62.
[21] Cohn DE, Fabbri M, Valeri N, Alder H, Ivanov I, Liu CG, et al. Comprehensive miRNA profiling of surgically staged endometrial cancer. Am J Obstet Gynecol 2010;202:656 [e1–8].
[22] Chung TK, Lau TS, Cheung TH, Yim SF, Lo KW, Siu NS, et al. Dysregulation of microRNA-204 mediates migration and invasion of endometrial cancer by regulating FOXC1. Int J Cancer 2012;130:1036–45.
[23] Lee JW, Park YA, Choi JJ, Lee YY, Kim CJ, Choi C, et al. The expression of the miRNA-200 family in endometrial endometrioid carcinoma. Gynecol Oncol 2011;12:56–62.
[24] Boren T, Xiong Y, Hakam A, Wenham R, Apte S, Wei Z, et al. MicroRNAs and their target messenger RNAs associated with endometrial carcinogenesis. Gynecol Oncol 2008;110:206–15.
[25] Wu W, Lin Z, Zhuang Z, Liang X. Expression profile of mammalian microRNAs in endometrioid adenocarcinoma. Eur J Cancer Prev 2009;18:50–5.
[26] Torres A, Torres K, Pesci A, Ceccaroni M, Paszkowski T, Cassandrini P, et al. Deregulation of miR-100, miR-99a and miR-199b in tissues and plasma coexists with increased expression of mTOR kinase in endometrioid endometrial carcinoma. BMC Cancer 2012;12:369.
[27] Su N, Qiu H, Chen Y, Yang T, Yan Q, Wan X. miR-205 promotes tumor proliferation and invasion through targeting ESRRG in endometrial carcinoma. Oncol Rep 2013;29:2297–302.
[28] Lee H, Choi HJ, Kang CS, Lee HJ, Lee WS, Park CS. Expression of miRNAs and PTEN in endometrial specimens ranging from histologically normal to hyperplasia and endometrial adenocarcinoma. Mod Pathol 2012;25:1508–15.
[29] Majid S, Saini S, Dar AA, Hirata H, Shahryari V, Tanaka Y, et al. MicroRNA-205 inhibits Src-mediated oncogenic pathways in renal cancer. Cancer Res 2011;71:2611–21.
[30] Nystrom-Lahti M, Parsons R, Sistonen P, Pylkkanen L, Aaltonen LA, Leach FS, et al. Mismatch repair genes on chromosomes 2p and 3p account for a major share of hereditary nonpolyposis colorectal cancer families evaluable by linkage. Am J Hum Genet 1994;55:659–65.
[31] Okuda T, Sekizawa A, Purwosunu Y, Nagatsuka M, Morioka M, Hayashi M, et al. Genetics of endometrial cancers. Obstet Gynecol Int 2010. http://dx.doi.org/10.1155/2010/984013.
[32] Backes FJ, Leon ME, Ivanov I, Suarez A, Frankel WL, Hampel H, et al. Prospective evaluation of DNA mismatch repair protein expression in primary endometrial cancer. Gynecol Oncol 2009;114:486–90.
[33] Tong WG, Ding XZ, Talamonti MS, Bell RH, Adrian TE. LTB4 stimulates growth of human pancreatic cancer cells via MAPK and PI-3 kinase pathways. Biochem Biophys Res Commun 2005;335:949–56.
[34] Zhu F, Park E, Liu B, Xia X, Fischer SM, Hu Y. Critical role of IkappaB kinase alpha in embryonic skin development and skin carcinogenesis. Histol Histopathol 2009;24:265–71.
[35] Kosaka N, Iguchi H, Ochiya T. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 2010;101:2087–92.
[36] Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 2007;9:654–9.
[37] Chen X, Liang H, Zhang J, Zen K, Zhang CY. Secreted microRNAs: a new form of intercellular communication. Trends Cell Biol 2012;22:125–32.
[38] Sebastianelli A, Renaud MC, Grégoire J, Roy M, Plante M. Preoperative CA 125 tumour marker in endometrial cancer: correlation with advanced stage disease. J Obstet Gynaecol Can 2010;32:856–60.