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Original Article

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Preoperative serum microRNAs as potential prognostic biomarkers in ovarian clear cell carcinoma

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ABSTRACT

Objective: Ovarian clear cell carcinoma (OCCC) is a subtype of epithelial ovarian carcinoma with poor prognosis. However, no effective biomarkers have been established for predicting unfavorable events, including recurrence and poor prognoses. Serum microRNAs (miRNAs) have been increasingly reported to be useful in predicting a patient's condition and have been recognized as a potentially less-invasive source for liquid biopsy in cancer. Therefore, this study aimed to evaluate serum miRNA profiles from patients with OCCC and to establish biomarker for predicting the prognoses.
Methods: The GSE106817, which included preoperative serum miRNA profiles of patients with ovarian tumors, was used, and clinical information was investigated. In all, 66 patients with OCCC were included, excluding those with other histological subtypes or insufficient prognostic information. Moreover, miRNA profiles of OCCC tissues were also examined.
Results: The median follow-up period was 64.3 (8.0–153.3) months. Based on multivariable Cox regression analyses and the expression of miRNAs in OCCC tissues, miR-150-3p, miR-3195, and miR-7704 were selected as miRNA candidates associated with both progression-free survival (PFS) and overall survival (OS). Then, the prognostic index was calculated based on expression values of 3 serum miRNAs. Kaplan-Meier survival analysis indicated that the prognostic index was significantly predictive of PFS and OS (p=0.004 and p=0.012, respectively).
Conclusion: Preoperative serum miRNA profiles of miR-150-3p, miR-3195, and miR-7704 can be used to potentially predict the prognosis of patients with OCCC.

Keywords: MicroRNAs; Biomarkers; Prognosis; Ovarian Neoplasms; Serum; Disease Progression

Synopsis

Serum, ovarian clear cell carcinoma (OCCC) tissue, and extracellular vesicles miRNA profiles were analyzed. The combination of serum miR-150-3p, -3195, and -7704 were significantly associated with both overall survival and progression-free survival. Serum microRNAs can be useful clinical tools for the prognostic prediction in OCCC.

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Conflict of Interest
No potential conflict of interest relevant to this article was reported.

Author Contributions
Conceptualization: Y.A.; Data curation: S.K., Y.K., K.T.; Formal analysis: S.K., Y.K.; Funding acquisition: Y.K., O.T., Y.A; Investigation: S.K., K.T.; Project administration: Y.A.; Resources: Y.K., O.T., Y.Y., K.H., Y.A.; Software: Y.Y.; Supervision: Y.K., K.H., Y.A.; Validation: Y.Y.; Visualization: S.K., Y.K.; Writing - original draft: S.K.; Writing - review & editing: Y.K., K.H., Y.A.

INTRODUCTION

Ovarian clear cell carcinoma (OCCC) is one of the major subtypes of epithelial ovarian carcinoma (EOC). The incidence rate of OCCC markedly differs among ethnic groups, particularly high in Asia, specifically 3.1% in blacks, 4.8% in whites, and 11.1% in Asians [1]. It is likely to be detected at an early stage and resulted in favorable outcome [2,3]. However, the prognosis of patients with recurrent OCCC was markedly worse than those with high-grade serous carcinoma (HGSOC) because of resistance to platinum-based chemotherapy [4]. Sensitivity to initial platinum chemotherapy depends on the pathological subtype in about 80% of HGSOC patients compared to only about 15% of OCCC patients [2,5]. Accurate prediction of prognosis can help physicians make decisions on types of therapies that benefit the patients.

MicroRNAs (miRNAs) are single-strand, non-coding endogenous small RNAs which regulate the expression of target genes and have important functions in cancer progression [6,7]. Moreover, miRNAs are secreted from cells and exist stably in body fluids by escaping RNase degradation. Some miRNAs were wrapped up by extracellular vesicles (EVs) which intervene cell-to-cell communication in the original and distant microenvironments [8-10]. Previous studies reported various functions of miRNAs in EOC development. For instance, as an intracellular function, miR-193a-3p has tumor suppressor role by modulating the MAPK/ERK signaling pathway, whereas miR-506 inhibits proliferation by targeting cyclin-dependent CDK4 and CDK6 in EOC cells [11,12]. Moreover, as extracellular function, EOC-derived miR-99a-5p induced vitronectin and fibronectin upregulation in peritoneal mesothelial cells, allowing easy cancer cell invasion [13]. Furthermore, due to miRNA stability in body fluids, circulating miRNAs have received more attention as noninvasive biomarkers [14]. We have demonstrated that serum miRNAs were potentially noninvasive diagnosis biomarkers of EOCs with high accuracy and prognostic biomarkers of HGSOC [15,16].

In this study, we evaluated the association between preoperative serum miRNA profile and the prognostic information of patient with OCCC. Moreover, miRNA profiles of OCCC tissues were used to select OCCC-associated miRNAs [17]. Then, several prognostic circulating miRNAs were identified, and prognostic indices were calculated to improve the accuracy. Therefore, this study provides novel evidence of the usefulness of circulating miRNAs as noninvasive biomarkers.

MATERIALS AND METHODS

1. Preoperative serum miRNA expression profiles

GSE106817 was used in this study. Data included miRNA profiles of 4,046 serum samples from patients with ovarian tumors and healthy controls who were admitted or referred to the National Cancer Center Hospital between 2008 and 2016. All serum miRNA profiles obtained using by 3D-Gene® Human miRNA Oligo Chip and 3D-Gene® miRNA Labeling kit (Toray Industries, Inc., Tokyo, Japan) [15]. A total of 442 patients with ovarian tumors with preoperative serum miRNA profiles were selected [15]. In addition, medical records, such as histological subtype, age, the International Federation of Gynecology and Obstetrics (FIGO) stage, residual tumor volume, adjuvant therapy, and recurrence or death events, were retrospectively reviewed. We confirmed that serum samples were collected prior to starting initial treatment including primary debulking surgery or neoadjuvant chemotherapy

(NAC). We also confirmed that full staging surgery was performed. Six patients received NAC including 3 in stage IV and 3 in stage III. Some patients received NAC without enlarged regional lymph nodes before initial treatment and a lymphadenectomy was not performed in the interval debulking surgery. This study was approved by the institutional review board of National Cancer Center Hospital Institutional (2015-376 and 2016-29), and each patient provided written informed consent. Furthermore, 210 miRNAs, detected in EVs derived from EOC cell lines, were selected using the criteria in our previous study [15].

2. Tissue miRNA expression profiles

The GSE200852 dataset was used to select OCCC tissue-associated miRNAs. The data were sequencing-based miRNA profiles of 25 OCCC tissues (samples 1–25) [17]. We retrospectively reviewed the medical records of patients treated at Nagoya University Hospital (Nagoya, Japan) between 2005 and 2016, and identified 25 patients with OCCC. We used 20 formalin-fixed paraffin-embedded tissue samples and 5 fresh-frozen surgical tissue samples. The tissue samples and serum samples were collected from different patients and not paired. The data analysis was performed using the CLC Genomics Workbench version 9.5.3 program (Qiagen, Hilden, Germany). After the adaptor trimming, the data were mapped to the miRbase version 21, allowing maximum of 2 mismatches.

3. Cell lines

RMG-2, TOV21G, OVTOKO, OVMANA, and KOC7C cells kindly gifted by Department of Obstetrics and Gynecology, School of Medicine, Fujita Health University. OVISE cells were purchased the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). These cell lines were maintained in RPMI (Nacalai Tesque, Kyoto, Japan) containing 10% fetal bovine serum (Cosmo Bio, Tokyo, Japan), penicillin, and streptomycin (Meiji Seika Pharma, Tokyo, Japan). These cell lines were confirmed to be negative for mycoplasma contamination, and cells were used in 5–10 passages for experiments. Comprehensive miRNA sequencing was performed according to the method described in our previous report [18].

4. Statistical analyses

Statistical analyses were performed with SPSS version 28 (IBM Corp., Armonk, NY, USA). Progression-free survival (PFS) was defined as the date from initial treatment to the time of tumor progression or last contact, while overall survival (OS) was defined as the date from initial treatment to the time of death from any cause. The log-rank test was used for risk analysis of PFS and OS, as measured using the Kaplan-Meier (KM) method. Univariate and multivariate Cox proportional hazards model analysis (Cox analysis) were used to calculate hazard ratios (HRs) and 95% confidence intervals. A multivariate Cox analysis for PFS and OS was performed with each miRNA, age, FIGO stage, and residual tumor volume. The prognostic index was calculated based on a multivariate Cox analysis for OS using expression levels of miRNA candidates.

RESULTS

1. Patient characteristics

We identified 68 patients with OCCC after excluding 354 patients with benign tumors, other malignancies, other epithelial ovarian cancers, or borderline ovarian malignancies (**Fig. S1**). Then, 2 patients were further excluded due to insufficient clinical information. Finally, the association between preoperative serum miRNA profiles and prognosis was assessed in

Table 1. Clinical characteristics of 66 patients with clear cell ovarian carcinoma

Characteristic	Case (n=66)
Age (yr)	
Median (range)	55.5 (27–76)
Stage	
I	36 (54.5)
II	9 (13.636)
III	18 (27.272)
IV	3 (4.545)
Cytoreductive surgery	
Complete	65 (98.5)
Optimal	1 (1.5)
Adjuvant chemotherapy	
Yes	60 (90.9)
No	6 (9.1)

Data are shown as number (%) unless otherwise noted.

66 patients with OCCC, and their characteristics are shown in **Table 1**. The median age of patients with OCCC at the time of diagnosis was 55.5 (range, 27–76) years, and 36 patients (54.5%) were diagnosed with FIGO stage I disease. All patients, except one patient, received complete cytoreductive surgery. Furthermore, 60 (90.9%) patients received adjuvant chemotherapy, typically consisting of carboplatin plus paclitaxel combination chemotherapy. The median follow-up period for surviving patients was 64.3 (range, 8.0–153.3) months.

2. Univariate Cox regression analysis for miRNA profile and prognostic information

The flowchart for selecting miRNA candidates is shown in **Fig. 1**. First, univariate Cox analysis was performed for PFS and OS using serum miRNA profiles as a continuous variable. Univariate Cox analysis for OS identified that 2 and 22 types of miRNAs were significantly associated with shorter and longer OS, respectively (**Fig. 2A**). Similarly, univariate Cox analysis for PFS identified that 1 and 16 miRNAs were significantly associated with shorter and longer PFS, respectively (**Fig. 2B**). Fourteen miRNAs were significantly associated with both better OS and PFS (miR-150-3p, -365a-5p, -718, -762, -939-5p, -1227-5p, -1915-3p, -3195, -3621, -4674, -6727-5p, -6805-5p, -6845-5p, and -7704). Seven miRNAs were significantly associated with better OS (miR-486-3p, -671-5p, -3180, -4516, -4667-5p, -4730, and -4741), whereas 2 miRNAs were significantly associated with better PFS (miR-486-3p and -1237-5p). Conversely, miR-6802-5p was associated with poor OS and PFS, and miR-6752-5p was a poor prognostic miRNA for OS. We then assessed the expression of each miRNA derived from the OCCC tissues (**Fig. 2**). Twelve miRNAs (miR-150-3p, -365a-5p, -486-3p, -671-5p, -939-5p, -3180, -3195, -4516, -4667-5p, -4730, -6805-5p, and -7704) were associated with OS and detected in the OCCC tissues (**Fig. 2A**). Similarly, 7 miRNAs (miR-150-3p, -365a-5p, -486-3p, -939-5p, -3195, -6805-5p, and -7704) were associated with PFS and detected in the OCCC tissues (**Fig. 2B**).

3. Multivariate Cox regression analysis for miRNA profile and prognostic information

Then, whether the 12 OS-related miRNAs were independent prognostic factors was evaluated. The multivariate Cox analysis for OS revealed that 6 of 12 miRNAs were associated with significantly favorable OS (miR-150-3p, HR=0.682, p=0.007; miR-365a-5p, HR=0.454, p=0.023; miR-671-5p, HR=0.578, p=0.005; miR-3195, HR=0.365, p=0.017; miR-4730, HR=0.358, p=0.015; miR-7704, HR=0.246, p=0.028, **Table S1**). Similarly, whether the 7 PFS-related miRNAs were independent prognostic factors was also evaluated, and the multivariate Cox analysis for PFS revealed that 4 of 7 miRNAs were associated with significantly favorable

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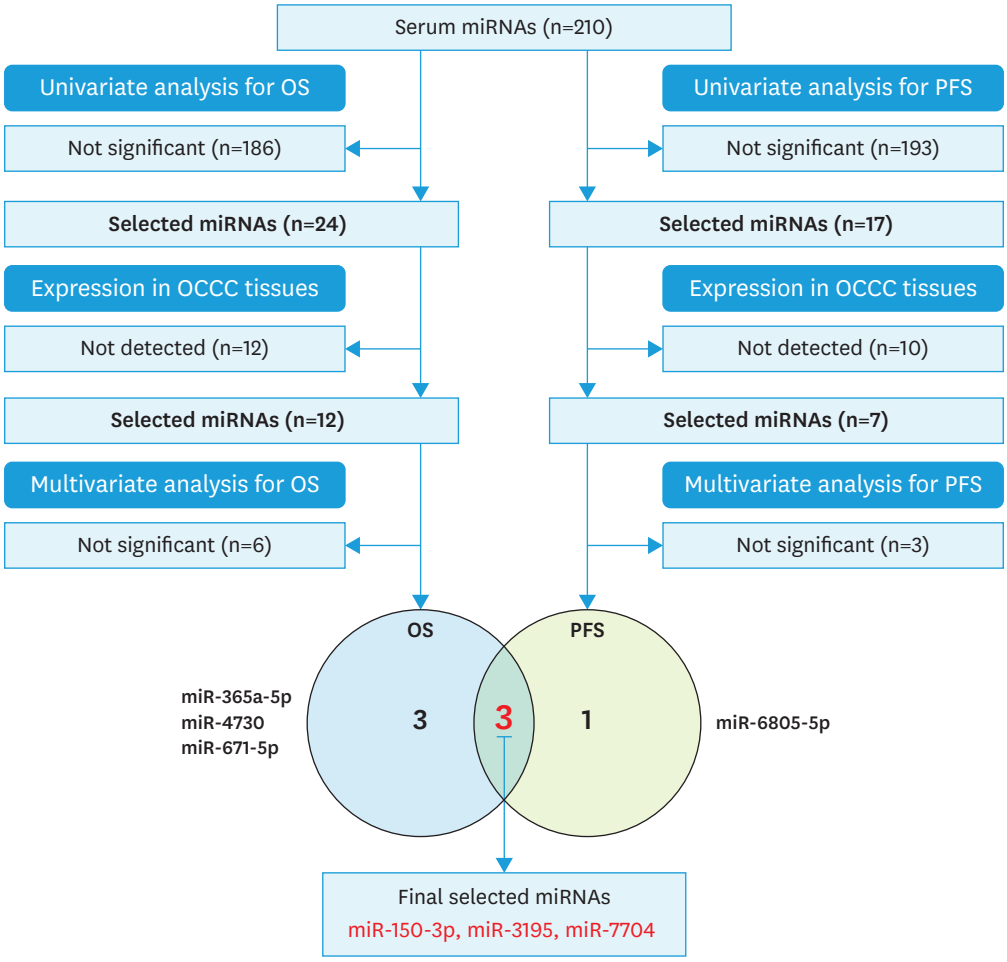

Fig. 1. Flowchart of miRNA selection. miRNA candidates (1) were significantly different in univariate Cox regression analysis based on serum miRNA levels, (2) were detected in OCCC tissue, (3) were significantly different in multivariate Cox regression analysis based on serum miRNA levels, (4) and were common in both OS and PFS. OCCC, ovarian clear cell carcinoma. Serum miRNA profiles were obtained from the GSE106817 dataset and tissue miRNA profiles were obtained from the GSE200852 dataset.
miRNA, microRNA; OCCC, ovarian clear cell carcinoma; OS, overall survival; PFS, progression-free survival.

PFS (miR-150-3p, HR=0.731, p=0.018; miR-3195, HR=0.283, p=0.003; miR-6805-5p, HR=0.167, p=0.011; miR-7704, HR=0.278, p=0.020, **Table S1**). The Venn diagram showed that miR-150-3p, -3195, and -7704 were independent prognostic factors for both OS and PFS (**Fig. 1**).

4. The prognostic impact of 7 miRNAs on PFS and OS

KM curves were generated for the 7 independent prognostic miRNAs (**Fig. 1**). Patients were stratified into 2 subgroups based on the median expression level of each miRNA. Among 6 independent prognostic miRNAs (miR-150-3p, -365a-5p, -671-5p, -3195, -4730, and -7704) for OS, only miR-4730 was associated with significantly favorable OS (p=0.011, **Fig. S2**). Similarly, of 4 miRNAs (miR-150-3p, -3195, -6805-5p, and -7704) for PFS, miR-3195 and -7704 were associated with significantly favorable PFS (p=0.033, and p=0.044, **Fig. S2**).

5. Establishment of the prognostic index model based on 3 serum miRNAs

Based on a multivariate Cox analysis, the prognostic index was calculated using 3 miRNAs to improve the accuracy of prognostic miRNAs: "(Prognostic Index)=(0.463×miR-150-3p)+(1.323×miR-3195)+(0.636×miR-7704)." Patient characteristics stratified into 2 subgroups

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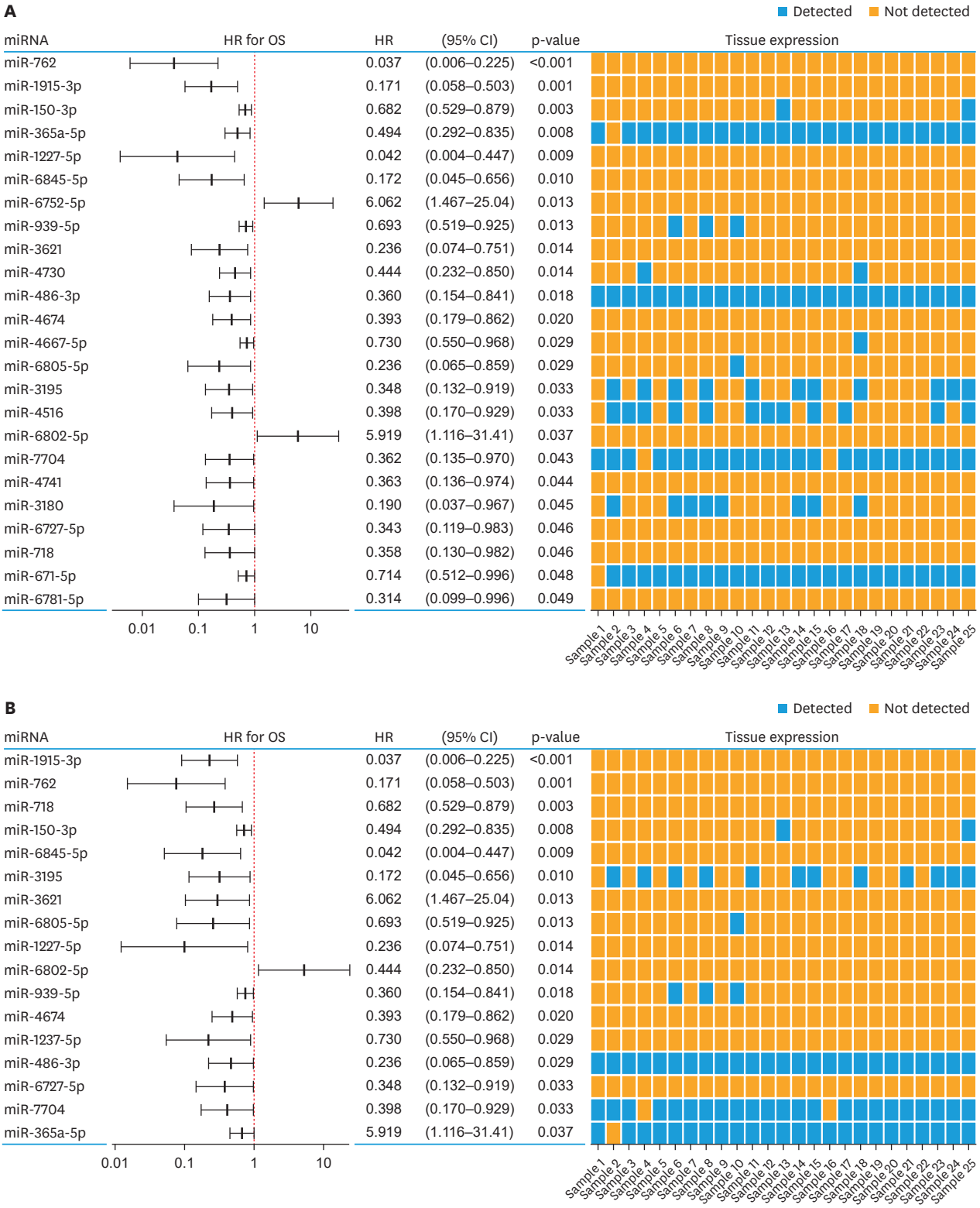

Fig. 2. Univariate Cox regression analysis for serum miRNAs and the expression of miRNAs in OCCC tissues. (A) Twenty-four significantly OS-associated serum miRNAs. (B) Seventeen significantly OS-associated serum miRNAs. Univariate Cox regression analysis for OS or PFS was calculated using serum miRNA levels as a continuous variable. The heatmaps show miRNA expression in OCCC tissues. Serum miRNA profiles were obtained from GSE106817 dataset. Tissue miRNA profiles were obtained from GSE200852 dataset.
CI, confidence interval; HR, hazard ratio; miRNA, microRNA; OCCC, ovarian clear cell carcinoma; OS, overall survival; PFS, progression-free survival.

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Table 2. Characteristics of patients with clear cell ovarian carcinoma, stratified by prognostic index

Characteristic	Prognostic index		
	High (n=33)	Low (n=33)	p-value
Age (yr)			0.379
Median (range)	55 (27–73)	55.5 (34–75)	
FIGO stage			0.806
I	18 (54.5)	18 (54.5)	
II	5 (15.2)	4 (12.1)	
III	7 (21.2)	11 (33.3)	
IV	3 (9.1)	0 (0)	
Cytoreductive surgery			0.321
Complete	32 (97.0)	33 (100)	
Optimal	1 (3.0)	0 (0)	
Adjuvant chemotherapy			0.400
Yes	29 (87.9)	31 (93.9)	
No	4 (12.2)	2 (6.1)	
miRNA levels			
Median (range)			
miR-150-3p	6.216 (4.856–7.898)	5.547 (0.082–6.918)	<0.001
miR-3195	8.757 (8.040–9.232)	8.377 (6.784–8.890)	<0.001
miR-7704	13.455 (12.641–14.379)	12.696 (11.703–14.140)	<0.001
Prognostic index*	22.964 (23.784–19.309)	21.667 (22.192–19.309)	<0.001

*Prognostic Index=(0.463×miR-150-3p)+(1.323×miR-3195)+(0.636×miR-7704).
FIGO, International Federation of Gynecology and Obstetrics; miRNA, microRNA.

based on the median level of prognostic index are shown in **Table 2**. The expression of 3 miRNAs was bifurcated based on the prognostic index (**Fig. S3**). The KM curves for OS revealed that patients with a high prognostic index had significantly longer OS than those with a low prognostic index (p=0.012, **Fig. 3A**). Similarly, KM curves for PFS showed that patients with a high prognostic index had significantly longer PFS than those with low (p=0.004, **Fig. 3B**). Similarly for subgroup of stage, the prognostic index was significantly associated with PFS in patients with stage II–IV disease (p=0.006); however, no significant differences were observed in OS. Moreover, patients with stage I disease and high prognostic index tended to have a favorable OS and PFS (p=0.080 and p=0.070).

6. Expression of 3 selected miRNAs in OCCC cell lines and in tissues from a previous study

To investigate whether selected miRNAs are involved in OCCC, we analyzed the miRNA profiles of 6 OCCC cell lines; that is, RMG-2, TOV21G, OVISE, OVTOKO, OVMANA, and KOC7C, on the Illumina MiSeq platform. Based on the GSE106817 dataset and miRNA sequencing, miR-150-3p, miR-3195, and miR-7704 expressions were confirmed in the OCCC cell lines (**Fig. S4**) [15]. Second, regarding tissue miRNA, we found 2 previous studies that were acceptable for analysis [19,20]. In 2 studies, miR-150-3p expression was confirmed in 12 samples of OCCC tissue, and miR-3195 expression was confirmed in 6 samples (**Table S2**).

DISCUSSION

OCCC recurrence is the clinical turning point associated with therapeutic resistance and poor prognosis. Thus, the prognostic biomarkers might provide useful information to decide treatment strategies. Previous research about OCCC demonstrated that FIGO stages and residual tumor volume were independent predictors of survival; however, they were insufficiently used as predictors in the clinical setting [21]. In this study, the combination of serum miR-150-3p, -3195, and -7704 profiles was identified to be significantly associated with

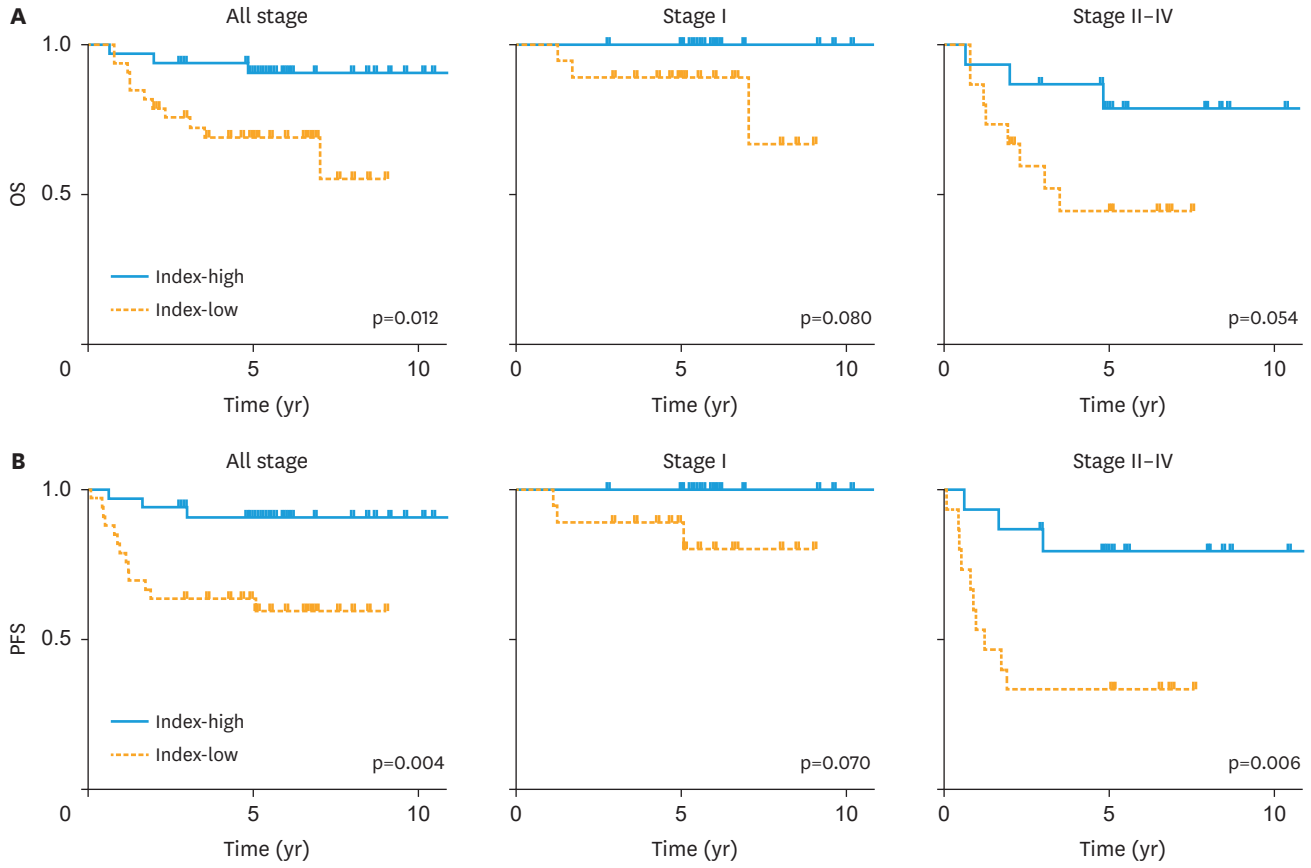

Fig. 3. Prognostic impacts of the prognostic index. Kaplan-Meier curves show (A) OS and (B) PFS stratified by the median prognostic index as follows: "(Prognostic Index)=(0.463×miR-150-3p)+(1.323×miR-3195)+(0.636×miR-7704)." Blue and red lines represent low- and high-level groups, respectively.
OS, overall survival; PFS, progression-free survival.

PFS and OS. The selected 3 serum miRNA profiles, as potential prognostic biomarkers, could provide novel therapeutic strategies, according to prognostic stratification within stage I and advanced stage groups. Based on these biomarkers of high index, if the prognosis was known to be good, it could be considered that postoperative chemotherapy was unnecessary in patients with stage I. The same applies to patients with advanced stages and this biomarker of high index; it could be suggested that the completion of cytoreducted surgery and chemotherapy may be important for a good prognosis.

The expression level of miRNAs has been considered to have a role in the pathogenesis of various types of human cancers, and clinical application of circulating miRNAs as noninvasive biomarkers is under investigation. Recent research of EOC has revealed circulating miRNAs as the diagnostic biomarker in body fluids, such as plasma, serum, and urine [15,22,23]. Furthermore, several studies investigated that expression levels of circulating miRNAs might be associated with treatment response and prognosis [16,22,24,25]. Certain miRNAs have been reported to might contribute to treatment resistance, such as platinum chemotherapy [17,25,26]. However, these reports were based on the majority of HGSOC patients among those eligible for EOC, and only a few patients with OCCC were enrolled. Therefore, the validity of miRNAs as a biomarker and their functions in OCCC are still unknown. In the present study, data of serum and tissue miRNA profiles of OCCC and EOC cell-derived EVs miRNAs were used [15]. Thus, the 3 prognostic miRNAs would be OCCC-derived miRNAs.

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Then, the prognostic index was calculated from 3 serum miRNAs: miR-150-3p, -3195, and -7704. However, our previous study on HGSOC identified miR-187-5p, -6870-5p, -1908-5p, and -6727-5p as prognostic miRNAs [16]. The prognostic index model from the current study was applied to patients with HGSOC in GSE106817 dataset. The results in OS and PFS were not unpredictable. Similarly, applying the previous HGSOC index model to patients with OCCC did not predict prognosis (**Fig. S5**). Therefore, the prognostic biomarkers depend on histologic subtypes and should be independently evaluated.

OCCC is characterized by PIK3CA, ARID1A point mutation driver events, and PI3K/AKT/mTOR dysregulation [27-30]. However, the association between specific driver mutations and the prognosis of patients with OCCC remains still controversial [31]. Reportedly, miR-150 inhibits PI3K/AKT/mTOR signaling by directly targeting IGF1R and IRS1 [32,33]. Moreover, low expression of miR-150-5p/3p was associated with poor prognosis, and miR-150-5p/3p was dysregulated in the EOC development and progression [32]. Similarly, previous studies reported that higher serum and tissue miR-3195 expressions were associated with longer OS in patients with lung cancer [34,35]. miR-3195 had a tumor suppressor function, as enhanced miR-3195 expression restrained cell proliferation, migration, and invasion in several cancer cells [35-37]. In breast cancer cells, miR-7704 has been shown to also act as a tumor suppressor function [38,39]. Although miR-7704 has been previously shown to be highly expressed in CD44-positive ovarian cancer stem cells, the function of miR-7704 in EOC remains unclear [40].

This study had several limitations. First, our results were based on dataset reanalysis and, thus, validation experiments cannot be performed using the same samples. This study population was specific in the sense that almost all patients were optimally cytoreducted, so this might not be suitable for patients with residual tumors either less than or more than 1 cm. Although the number of patients is limited in this study, the serum sample size of 66 is relatively large in OCCC patients. However, further studies with a larger or independent cohort are essential to determine the usefulness of the biomarker. Second, expression of miRNAs in OCCC tissues has been considered, but it was unclear where the serum miRNAs originated. Furthermore, the function of miRNAs in OCCC should be evaluated.

In conclusion, we revealed that 3 circulating miRNAs, miR-150-3p, -3195, and -7704, profiles were useful as predicting prognostic biomarkers in patients with OCCC that can help clinicians decide the types of surgery and adjuvant therapies based on individual risks. Further studies of circulating miRNA analysis are needed for early practical clinical application.

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SUPPLEMENTARY MATERIALS

Table S1

Multivariate Cox regression analysis for OS and PFS

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Table S2
Studies that investigated tissue microRNAs in ovarian clear cell carcinoma

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Fig. S1
Flowchart of patients' selection. We identified a total of 442 patients with ovarian tumors with preoperative serum microRNA profiles [15]. Among them, 68 patients were diagnosed with ovarian clear cell carcinoma. Finally, 66 patients were assessed in this study, after excluding 2 patients with insufficient clinical information.

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Fig. S2
Prognostic impacts of serum miRNA levels. Kaplan-Meier curves for (A) OS according to 6 significantly independent miRNAs and (B) PFS according to 4 significantly independent miRNAs. Patients were stratified based on the median level of each miRNA; blue and red lines represent low- and high-level groups, respectively.

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Fig. S3
Distributions of miRNAs. Serum miRNA levels were stratified by the median prognostic index.

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Fig. S4
The expression of miRNAs in OCCC cell lines. The heatmaps show miRNA expression in the OCCC cell lines. Data was based on the GSE106817 dataset and miRNA-sequence [15].

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Fig. S5
Prognostic impacts of the prognostic index. Kaplan-Meier curves show patients with (A) OCCC and (B) HGSOC stratified by the median prognostic index as follows: "HGSOC-index in OS=(0.148×miR-187-5p)+(0.273×miR-6870-5p)+(0.186×miR-1908-5p)," "HGSOC-index in PFS=(0.031×miR-187-5p)+(0.231×miR-6870-5p)+(0.351×miR-6727-5p)," "(OCCCC-index)=(0.463×miR-150-3p)+(1.323×miR-3195)+(0.636×miR-7704)." Blue and red lines represent low- and high-level groups, respectively.

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