miR-146a-5p and miR-191-5p as novel diagnostic marker candidates for ovarian clear cell carcinoma

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Abstract. Ovarian cancer is a malignant gynecologic disease rarely diagnosed in the early stages. Among the various types of ovarian cancer, clear cell carcinoma has a poor prognosis due to its malignant potential. MicroRNAs (miRNAs/miRs) regulate gene expression in cells by suppressing the translation of target genes or by degrading the target mRNA. miRNAs are also secreted from the cells in the blood, binding to proteins or lipids and assisting in cell-cell communication. Therefore, serum miRNAs may be considered potential diagnostic biomarkers for ovarian cancer. The present study investigated and identified specific miRNAs associated with ovarian clear cell carcinoma and compared them to those in ovarian endometrioma samples and healthy controls. CA125, an ovarian tumor marker, did not differ between patients with ovarian clear cell carcinoma, endometriosis or healthy controls. Subsequently, four miRNAs (miR-146a-5p, miR-191-5p, miR-484 and miR-574-3p) were analyzed. The expression levels of miR-146a-5p and miR-191-5p were significantly increased in the serum samples from patients with ovarian clear cell carcinoma compared with those in the healthy controls, but there was no significant difference compared with in patients with endometriosis. Furthermore, the bioinformatics analysis showed that *CCND2* and *NOTCH2* were the candidate target genes of miR-146a-5p and miR-191-5p. In conclusion, the results of the present study demonstrated that miR-146a-5p and miR-191-5p may be useful as early and non-invasive diagnostic tools in ovarian clear cell carcinoma. These miRNAs can help in distinguishing between ovarian clear cell carcinoma and ovarian endometrioma. To the best of our knowledge, no previous studies have screened any candidates specifically for ovarian clear cell carcinoma.

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Abbreviations: miRNA, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; ROC, receiver operating characteristic

Key words: ovarian cancer, malignant, endometrioma, endometriosis, non-invasive, sensitivity, specificity, ROC curve, CA125

Introduction

Ovarian cancer accounts for the highest fatality among gynecological malignancies, with an increasing number of patients worldwide (1). Ninety percent of ovarian cancers are epithelial cell types, encompassing various histologic types with diverse molecular alterations, clinical behaviors, and therapeutic outcomes. The remaining 10% comprises non-epithelial ovarian cancers, such as exceedingly rare tumors, primarily germ cell tumors, sex cord stromal tumors, and small cell carcinomas (2). Ovarian cancer accounts for 2.5% of all malignancies in women but 5% of all cancer deaths because four out of five patients are diagnosed at an advanced stage (3). Patients with advanced-stage ovarian cancer have achieved the best outcomes via complete resection of the diseased tissues and combination chemotherapy (4). DNA damage repair (DDR) defects are prevalent in various cancer types, and these alterations can be strategically utilized for therapeutic purposes. Epithelial ovarian cancer (EOC) stands out as one of the tumor types with the highest percentage of hereditary cases. Mutations occurring within DNA repair pathways elevate the risk of developing resistance to chemotherapy. Considering the substantial occurrence of homologous recombination deficiency in ovarian clear cell carcinoma, it becomes susceptible to PARP inhibitor therapy. Notably, the U.S. Food and Drug Administration (FDA) and/or the European Medicines Agency (EMA) have approved olaparib, rucaparib, and niraparib, among the PARP inhibitors, for use in EOC across different treatment contexts (5).

However, the 5-year survival rate of advanced ovarian cancer (stages III and IV) was only approximately 20% and was considered to have the poorest prognosis among female genital malignancies (6).

There are two recognized types of EOCs. Type I EOC is believed to be relatively slow-growing and genetically stable, often originating from identifiable precursor lesions like

endometriosis or borderline tumors with low malignant potential. In contrast, type II EOC are proposed to be biologically aggressive tumors from their outset, with a tendency for metastasis even from small primary lesions (7). Histopathologically, ovarian cancer comprises five major subgroups: clear cell, endometrioid, mucinous, high-grade serous, and low-grade serous. High-grade serous is the predominant subtype of EOC, comprising approximately 75% of all cases; it follows the type II pathway of development and is characterized by the presence of p53 and BRCA mutations (7). Among them, clear cell carcinoma is common in Japanese patients, but the etiology is still unclear. This group of ovarian cancer is more resistant to the standard platinum and paclitaxel chemotherapy than the other advanced serous ones (8). Hence, the prognosis for advanced ovarian clear cell cancer is poor compared to that of early-stage ovarian cancer (6). This necessitates the early diagnosis of ovarian clear cell carcinoma. To date, there has been no specific test for the diagnosis of early-stage ovarian cancer. The patients suspected of ovarian cancer are conventionally tested using transvaginal sonography and tumor markers, such as CA125. CA125 assessment is the standard method for diagnosis, following response to treatment, for predicting the prognosis of ovarian cancer like clear cell carcinoma (9). The level of this marker does not increase at an early stage and is not increased in ovarian clear cell carcinoma, as reported previously (10). To date, no studies have screened any candidates specifically for clear cell carcinoma. This economic aspect is also evident in the research conducted on cost-effective approaches for the early detection and prevention of ovarian cancer in the past decade. Clearly, the cost of treatment per patient with ovarian cancer remains the highest among all cancer types. For instance, the average initial cost in the first year can reach approximately USD 80,000, with the final year cost potentially escalating to USD 100,000 (11).

Extracellular RNA, including serum microRNAs (miRNAs), has received much attention recently. miRNAs comprise small non-coding RNAs of 20-25 nucleotides that regulate gene expression in cells by suppressing the translation of the target gene or by degrading the target mRNA (12). The miRNAs secreted from cells are stably present in body fluids in extracellular vesicles containing exosomes or bind to the proteins or lipids (13) playing an important role in cell-cell communication (14). Many studies have reported serum miRNAs as promising biomarkers for various diseases because they reflect physiological and pathological states (15-17).

There are some ovarian clear cell carcinoma-specific miRNAs. Recently, Yokoi *et al* reported some miRNAs to be specific to ovarian cancer (18). On the other hand, histopathological examination revealed endometriosis prevalent in middle-aged women to be associated with the risk of ovarian cancer. Similarly, ovarian endometrioma is associated with the risk of endometriosis-associated ovarian cancer, especially clear cell carcinoma (19,20).

Clinically, it is difficult to distinguish between ovarian endometriosis and clear cell carcinoma because of the evident similarities on ultrasound and increasing CA125 levels in both endometrioma and clear cell carcinoma. In this report, we independently investigated and explored the specific miRNAs in clear cell carcinoma compared to ovarian endometrioma and healthy patients.

Materials and methods

Study design. The present study was approved by the internal review boards of Tokyo Medical University (Tokyo, Japan; approval no. 3769). Written informed consent was obtained from all the patients before the collection of specimens, according to the Declaration of Helsinki. The patient backgrounds were obtained through interviews. The blood samples were collected before operations, chemotherapy, and radiation therapy. The ovarian clear cell carcinoma and endometriosis were diagnosed based on the histological examinations.

A total of 64 patients participated in the research conducted at the Tokyo Medical University Hospital from February 2010 to January 2019 and at the Jikei University School of Medicine between August 2008 and November 2011. Twenty-nine patients had ovarian clear cell carcinoma, 17 had endometriosis, and 18 were healthy. The patients were diagnosed with ovarian clear cell carcinoma, 6 with stage Ia, 5 with stage Ic1, 5 with stage IC2, 2 with stage IC3, 1 with stage IIa, 2 with stage IIb, 1 with stage IIIa1, 4 with stage IIIb, 2 with stage IIIc, and 1 with stage IVb.

Serum preparation and total RNA extraction. The blood samples were collected from patients with ovarian clear cell carcinoma and endometriosis, as well as from the healthy controls. We measured the CA125 levels in patients with endometriosis and ovarian cancer before surgery. The blood serum was separated by centrifugation at 1,800 rpm for 10 min and stored at -80˚C. The total RNA was extracted from the serum using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Search for candidate miRNAs with TaqMan Array Human microRNA Cards. We used TaqMan™ Array Human MicroRNA A+B Cards Set v3.0 (Thermo Fisher Scientific, Inc.) to search for candidate miRNAs in 20 samples (16 samples of ovarian clear cell carcinoma and four healthy control samples). Using a volcano plot, we identified target miRNAs with significantly different expression levels in control and ovarian clear cell carcinoma patients. Then, using an amplification plot, we narrowed down the miRNAs that were amplified in almost all targets. Finally, we determined the target miRNAs based on relative gene expression.

miRNA expression analysis by quantitative polymerase chain reaction (qPCR) and receiver operating characteristic curves. Four miRNAs (miR-146a-5p, miR-191-5p, miR-484, and miR-574-3p) were analyzed by the TaqMan miRNA expression analysis (Thermo Fisher Scientific, Inc.) and reverse transcription-qPCR (RT-qPCR). The expression analyses were performed using the TaqMan Advanced miRNA assays (Thermo Fisher Scientific, Inc.) for human miR-146-5p (478399_mir), miR-191-5p (477952_mir), miR-484 (478308_mir), miR-574-3p (478163_mir), and miR-16 (477860_mir) as an endogenous control (21). The cDNA was synthesized using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.).

qPCR was performed with RT primers using the Universal Master Mix and specific miRNAs using the Applied Biosystems StepOnePlus™ real-time PCR system (Thermo

Fisher Scientific, Inc.). The sequence detection was performed according to the manufacturer's protocol.

The reaction mixtures were incubated at 95˚C for 2 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. The miRNA expression levels in the participants with ovarian clear cell carcinoma and endometriosis compared to healthy controls were calculated using the comparative $2^{-\Delta\Delta Cq}$ method (22). Receiver operating characteristic (ROC) curves were generated using the miR-146a miR-191 expression profile. The graphical plots of the true and false positive rates are shown. The area under the ROC curve represents the identification accuracy.

Statistical analysis. The statistical analyses of the causal association between the clinical background, the expression level of the miRNAs, and the ROC curve analysis were performed using SPSS-27 software. The statistical significance was determined by the Kruskal-Wallis test (between healthy controls, endometriosis, and ovarian clear cell carcinoma) followed by Dunn's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

MiRNA 146a-5p and miRNA 191-5p analyzed using MiRTarBase. Subsequent to the identification of differentially expressed miRNAs, the predicted target genes for these altered miRNAs were subjected to experimental validation using the miRNA-target interaction database MiRTarBase (http://mirtarbase.cuhk.edu.cn/php/index.php) (23).

Results

Characteristics of the participants. Of the 64 participants, 18 were healthy (control), 17 had endometriosis, and 29 had ovarian cancer. The median age of healthy patients was 47.5 years (range 31-82 years), median age for patients with endometriosis was 35 years (range 22-56 years), and median age for patients with ovarian clear cell carcinoma was 53 years (range 31-81 years). Table I shows the clinical characteristics and the values of CA125 in patients with ovarian clear cell carcinoma (One patient did not check CA125 before the operation).

Table II shows the clinical characteristics and the value of CA125 in patients with endometriosis. CA125 varied differently in each endometriosis and ovarian clear cell carcinoma patient.

Identifying the candidate miRNAs. Based on the volcano plot, 18 miRNAs were identified (Fig. 1). In the amplification plot, 7 miRNAs (mir-146a-5p, mir-191-5p, mir-223-3p, mir-24-3p, mir-320a-3p, mir-484, 574-3p) were confirmed as amplified. The results of gene expression analysis showed that hsa-miR-191-5p and hsa-miR-574-3p were more than 100-fold differentially expressed in patients with carcinoma compared to the controls. Differential expression was also observed for hsa-miR-146a-5p and hsa-miR-24-3p (Fig. 2). Among the 16 samples of ovarian clear cell carcinoma, 12 samples with similar miRNA amplification were again analyzed using a volcano plot, and four miRNAs (mir-146a-5p, mir-191-5p, mir-484, 574-3p) were listed.

miRNA expression status in ovarian clear cell carcinoma. The expression of miR-484 and miR-574-3p were not different among the three groups. However, the miR-146a-5p and miR-191-5p expression levels were significantly increased in the serum samples from the participants with ovarian clear cell carcinoma compared to the healthy controls but not in the participants with endometriosis ($P<0.05$).

The median serum miR-146a-5p expression level was 0.72 in the healthy patients, 0.57 in the patients with endometriosis, and 4.42 in patients with ovarian clear cell carcinoma, respectively ($P<0.01$, Fig. 3; Kruskal-Wallis test).

Table I. Characteristics of patients with ovarian clear cell carcinoma.

Characteristics	Ovarian clear cell carcinoma (n=29)
Age, years	
Median	53
Range	31-81
Clinical stage	
IA	6
IC1	5
IC2	5
IC3	2
IIA	1
IIB	2
IIIA1	1
IIIB	4
IIIC	2
IVB	1
Serum CA125 antigen, ng/ml	
Median	407
Range	13-5,877

Carbohydrate antigen 125, CA125.

Table II. Characteristics of the patients with ovarian endometriosis.

Characteristics	Endometriosis (n=17)
Age, years	
Median	35
Range	22-56
BMI, kg/m^2	
Median	21.6
Range	17.7-34.7
Tumor size, cm	
Median	62
Range	30-150
Serum CA125 antigen, ng/ml	
Median	55.1
Range	10.7-555.6

BMI, body mass index; carbohydrate antigen 125, CA125.

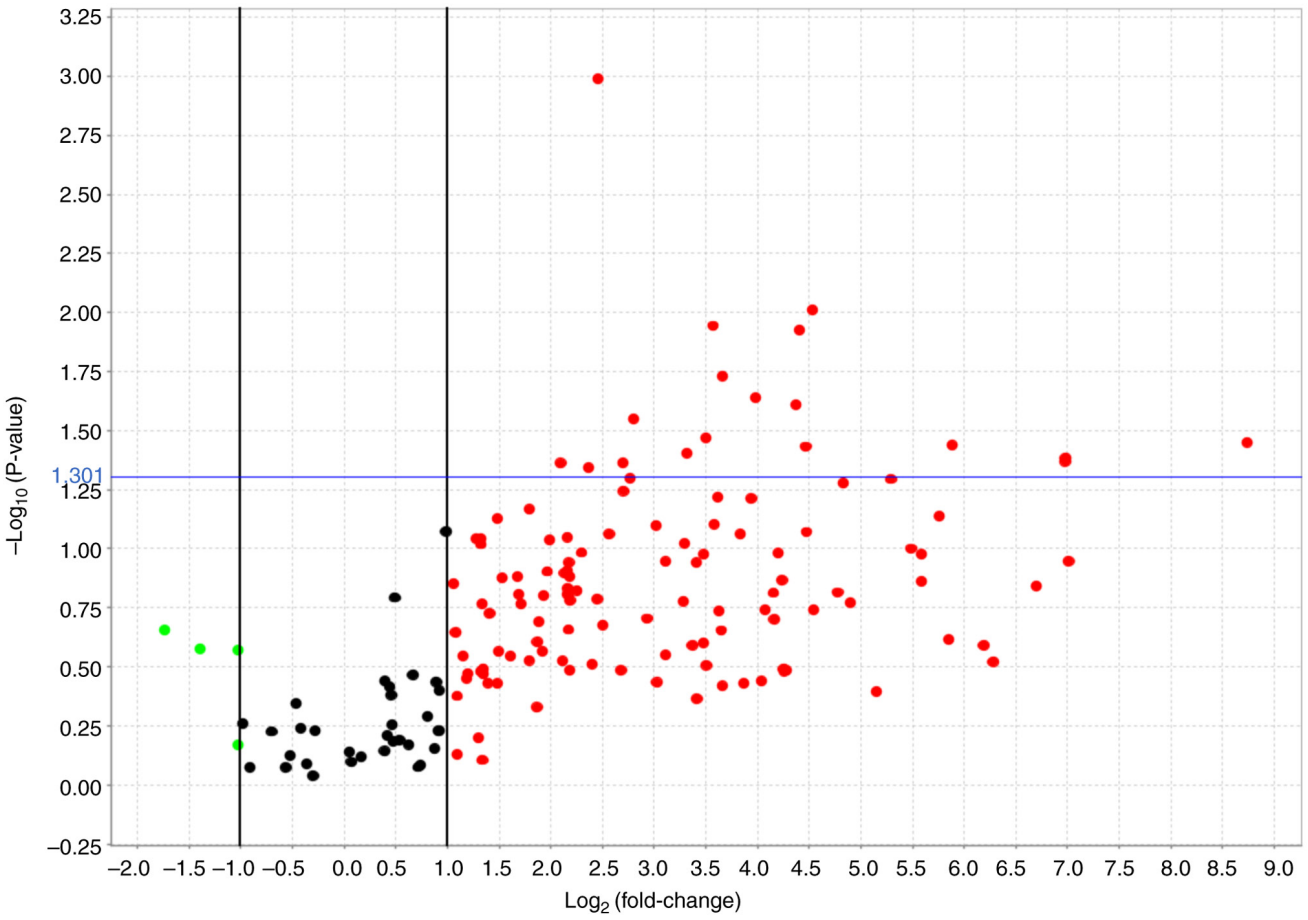

Figure 1. Volcano plot. Target miRNAs with significantly different expression levels in healthy and ovarian clear cell carcinoma patients were listed. As a result, 18 miRNAs were identified as candidates. miRNA, microRNA.

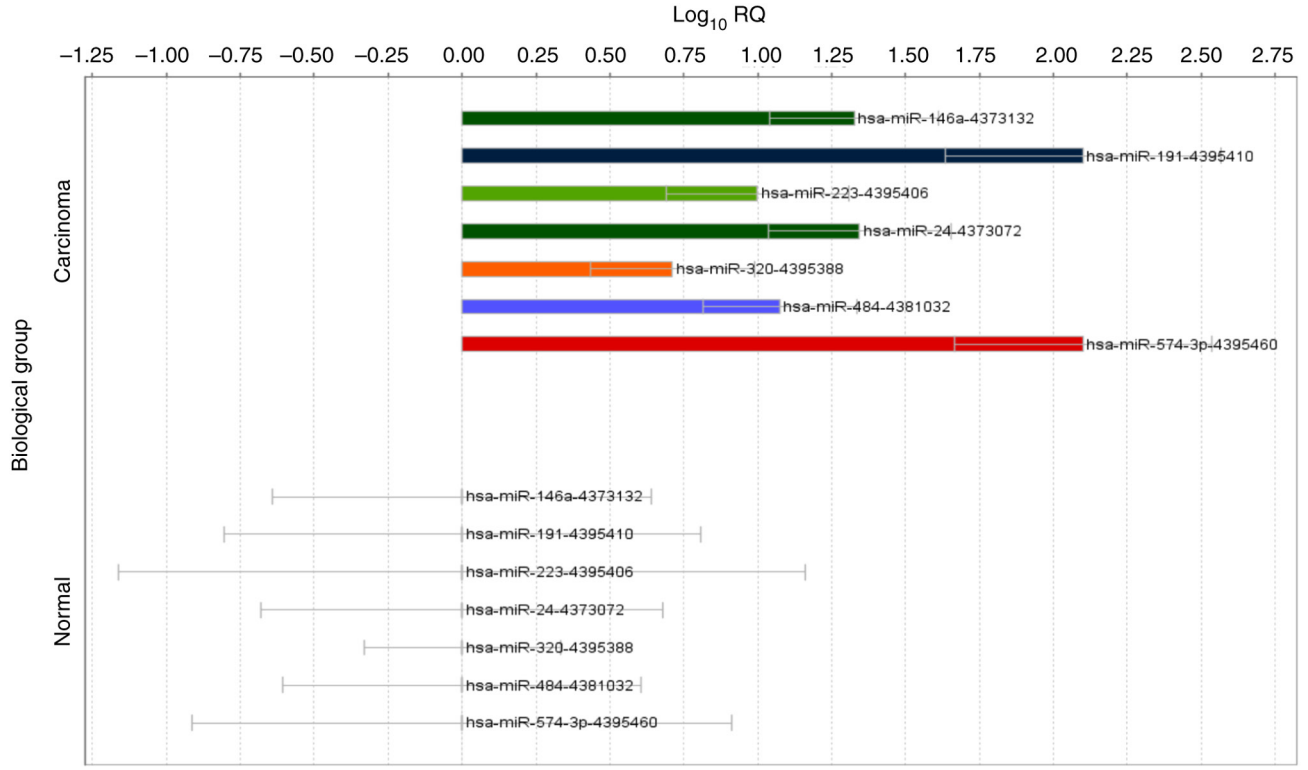

Figure 2. Gene expression. Hsa-miR-191-5p and hsa-miR-574-3p were differentially expressed more than 100-fold compared to the control. Differential expression was also observed for hsa-miR-146a-5p and hsa-miR-24-3p. miR, microRNA.

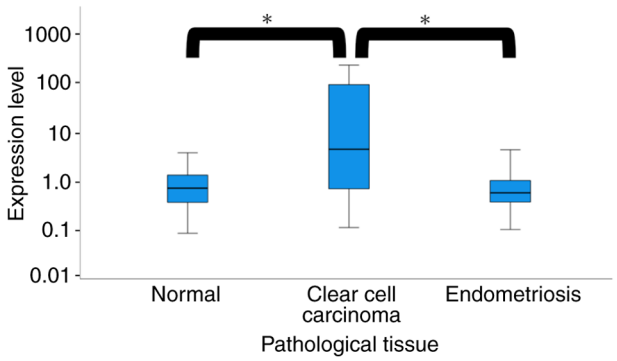

Figure 3. The serum miR-146a-5p expression level in the participants. The serum miR-146a-5p expression levels in the patients with ovarian clear cell carcinoma and ovarian endometriosis at various stages by a quantitative polymerase chain reaction. *P<0.05. miR, microRNA.

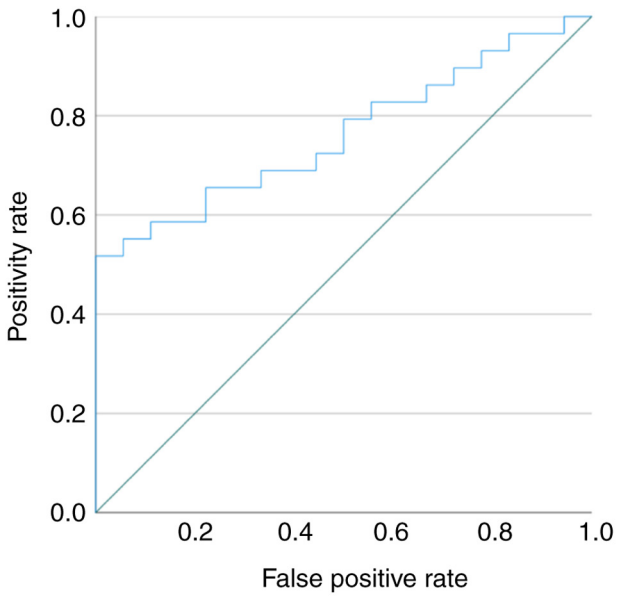

Figure 4. ROC for miR-146a-5p in the normal and ovarian clear cell carcinoma. The area under the receiver operating characteristic curve for miR-146a-5p based on quantitative polymerase chain reaction data. The cut-off level of plasma miR-146a-5p was 4.42, the sensitivity was 79.3%, and the specificity was 50.0%. miR, microRNA.

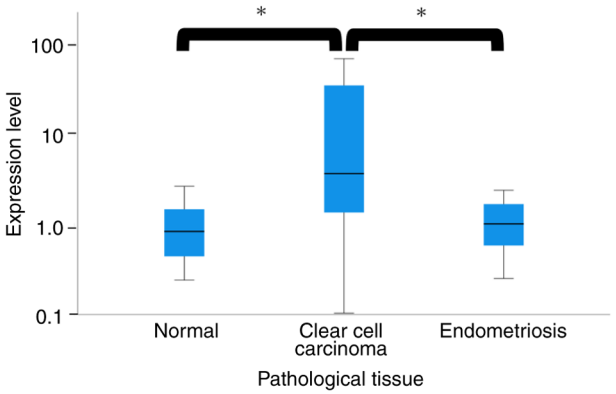

Figure 5. The serum miR-191-5p expression level in participants. The serum miR-191-5p expression levels in the participants with ovarian clear cell carcinoma and ovarian endometriosis at various stages by a quantitative polymerase chain reaction. *P<0.05. miR, microRNA.

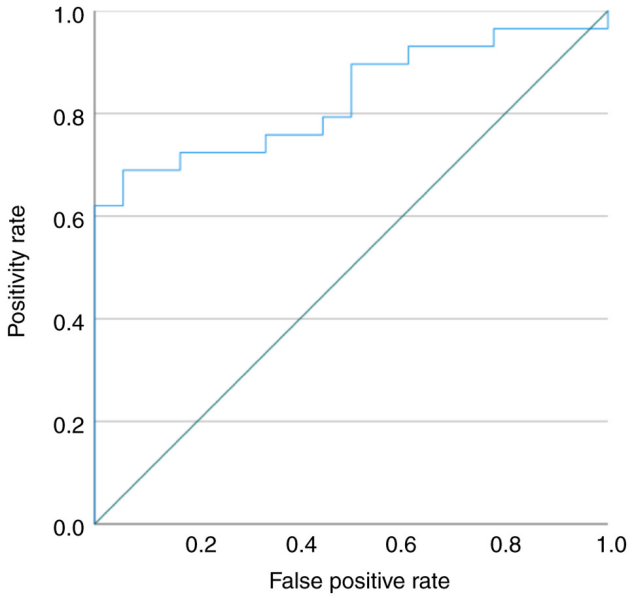

Figure 6. ROC for miR-191-5p in the normal and ovarian clear cell carcinoma. The area under the receiver operating characteristic curve for miR-191-5p based on quantitative polymerase chain reaction data. The cut-off level of plasma miR-191-5p was 3.58, the sensitivity was 89.7%, and the specificity was 50.0%. miR, microRNA.

The ROC curve showed that the miR-146a-5p serum levels may differentiate patients with ovarian clear cell carcinoma from the healthy controls, and the ROC curve area was 0.762 (95% confidence interval: 0.629-0.896; Fig. 4).

When the cut-off value was 0.652 (relative expression value), miR-146a-5p was 79.3% sensitive to ovarian clear cell carcinoma and 50.0% specific compared to the healthy controls. In contrast, the median serum miR-191-5p expression level was 0.833 in the healthy participants, 1.00 in the patients with endometriosis, and 3.58 in patients with ovarian clear cell carcinoma (P<0.01, Fig. 5; Kruskal-Wallis test). The ROC curve showed that the miR-191-5p serum levels may differentiate patients with ovarian clear cell carcinoma from healthy controls, and the ROC curve area was 0.830 (95% confidence interval: 0.714-0.945) (Fig. 6). When the cut-off value was 0.723 (relative expression value), the miR-191-5p was 89.7% sensitive to ovarian clear cell carcinoma and 50.0% specific compared to the healthy controls. Compared to the cancer stage, no difference was observed in the expression of miR-146a-5p and miR-191-5p.

The MiRTarBase was used to identify the predicted target genes of miR-146a-5p and miR-191-5p to determine their biological significance. More than 50 target genes were extracted by the MiRTarBase, and the target genes that showed strong evidence are summarized in Fig. S1. The *CCND2* and *NOTCH2* genes were the candidate targets of miR-146a-5p and miR-191-5p.

Discussion

The early detection of cancer may contribute to improved patient survival rates. BRCA1/2 germline mutations represent

the most potent identified genetic risk factors for EOC and are detected in 6-15% of women diagnosed with EOC. Determining the BRCA1/2 status can aid in providing patients with counseling regarding their anticipated survival outcomes. It is noteworthy that BRCA1/2 carriers with EOC tend to exhibit more favorable responses to platinum-based chemotherapies compared to non-carriers (24).

Biomarkers that can be used to detect cancer at an early stage are important for the diagnosis and prognosis of cancer. Targeted proteomics serves as a crucial technique for validating and confirming discovered biomarkers. It works in conjunction with untargeted proteomics to complete the biomarker discovery and validation cycle. Additionally, peptidomics, a newly established subdivision of proteomics, can provide insights into novel biomarkers. Peptidomics focuses on studying peptides to determine their specific forms, and like proteomics, it aids in identifying new peptides present in tissues. Lastly, exosomes play a vital role in intercellular communication and have emerged as promising diagnostic and prognostic biomarkers for ovarian clear cell carcinoma. They have the potential to transport certain tumor-associated proteins (25).

This study aimed to investigate the novel miRNAs in ovarian clear cell carcinoma. As previously reported, CA125 levels were not distinguishable between endometriosis and clear cell carcinoma (26). The expression levels of miR-146a-5p and miR-191-5p were significantly elevated in patients with ovarian clear cell carcinoma in the three groups. In the ROC analysis, miR-146-5p and miR-191-5p revealed around 0.8 sensitivity for ovarian clear cell carcinoma. This indicated that miR-146-5p and miR-191-5p were useful for exclusion diagnosis.

Using bioinformatics analysis, MiRTarBase showed that the *CCND2* and *NOTCH2* genes were the candidate targets of miR-146a-5p and miR-191-5p (Fig. S1). CCND2 belongs to the cyclin family, which functions in cell cycle progression (27). CCND2 forms a complex with the cyclin-dependent kinase CDK4 or CDK6 and functions as the regulatory subunit of the complex, whose activity is required for the cell cycle G1/S transition (28). As CCND2 shortens the G1 phase and participates in cell progression, the CCND2 gene is suspected to be involved in cancer cell growth (29).

Several studies have demonstrated that CCND2 is associated with tumorigenesis (30). Chang *et al* (31) revealed that CCND2 is involved in stimulating the proliferation, cell cycle progression, migration, and invasion of ovarian cancer cells. NOTCH2 promotes cell proliferation and epithelial-mesenchymal transition in the EOC cell lines (32). MiR-146a-5p and MiR-191-5p may be upregulated in patients with ovarian cancer to inhibit the function of NOTCH2 and prevent the progression of ovarian cancer.

Our results showed that CCND2 and NOTCH2 are the candidates for both miRNAs. Thus, it was hypothesized that in patients with ovarian clear cell carcinoma, miR-146a-5p and miR-191-5p were upregulated to inhibit the function of CCND2 and NOTCH2. A recent report has revealed that ovarian clear cell carcinoma exhibits a unique genetic profile characterized by a lower p53 mutation rate (25%) and a lower BRCA1/2 mutation rate (6.3%) compared to high-grade serous ovarian cancer. However, it demonstrates higher mutation rates in genes such as *ARID1A*, *PIK3CA*, and *PTEN*. This highlights the genetic differences between ovarian clear cell carcinoma and high-grade serous ovarian cancer (33). A major limitation of this study was the small sample size.

Our results showed that miR-146a-5p and miR-191-5p may be useful as early and non-invasive diagnostic tools in the search for ovarian clear cell cancer. These miRNAs can also distinguish between ovarian clear cell carcinoma and ovarian endometrioma.

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Availability of data and materials

The microarray datasets generated and/or analyzed during the current study are available in the Gene Expression Omnibus repository, (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE239685). All other data generated or analyzed during this study are included in this published article.

Authors' contributions

ST, AO and HN confirm the authenticity of all the raw data. ST, JK, HN, TU, SH, MK, OA and TM performed data analysis. ST, JK, HN, AO and SH explained the present study to patients and obtained informed consent. ST performed the experiments. ST and JK wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The patient data were used according to the ethical principles of The Declaration of Helsinki. The study protocol was approved by the internal review boards of Tokyo Medical University (Tokyo, Japan; (approval no. 3769), and all patients provided written informed consent before participation.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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