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Original Article

Diagnostic performance of microRNA-34a, let-7f and microRNA-31 in epithelial ovarian cancer prediction

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ABSTRACT

Objective: To correlate the genome-wide methylation signature of microRNA genes with dysregulated expression of selected candidate microRNA in tissue and serum samples of epithelial ovarian cancer (EOC) and control using quantitative reverse transcription polymerase chain reaction (qRT-PCR), and evaluation of EOC predictive value of candidate microRNA at an early stage.
Methods: We performed Methylated DNA Immunoprecipitation coupled with NGS (MeDIP-NGS) sequencing of 6 EOC and 2 normal tissue samples of the ovary. Expression of selected microRNA from tissue (EOC=85, normal=30) and serum (EOC=50, normal=15) samples was evaluated using qRT-PCR. We conducted bioinformatics analysis to identify the candidate miRNA's potential target and functional role.
Results: MeDIP-NGS sequencing revealed hypermethylation of several microRNAs gene promoters. Three candidate microRNAs were selected (microRNA-34a, let-7f, and microRNA-31) from MeDIP-NGS data analysis based on log2FC and P-value. The relative expression level of microRNA-34a, let-7f, and microRNA-31 was found to be significantly reduced in early-stage EOC tissues and serum samples ($p<0.0001$). The receiver operating characteristic analysis of microRNA-34a, let-7f and miR-31 showed improved diagnostic value with area under curve(AUC) of 92.0 ($p<0.0001$), 87.9 ($p<0.0001$), and 85.6 ($p<0.0001$) and AUC of 82.7 ($p<0.0001$), 82.0 ($p<0.0001$), and 81.0 ($p<0.0001$) in stage III-IV and stage I-II EOC serum samples respectively. The integrated diagnostic performance of microRNA panel (microRNA-34a+let-7f+microRNA-31) in late-stage and early-stage serum samples was 95.5 and 96.9 respectively.
Conclusion: Our data correlated hypermethylation-associated downregulation of microRNA in EOC. In addition, a combined microRNA panel from serum could predict the risk of EOC with greater AUC, sensitivity, and specificity.

Keywords: microRNA; Ovarian Cancer; Biomarker; Epigenetics; Diagnosis

Funding

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Conflict of Interest

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

Availability of Data and Materials

The datasets used and/or analysed during the current study are included in this manuscript and can also be made available from corresponding author on reasonable request.

Author Contributions

Conceptualization: G.S., V.K., S.M.; Formal analysis: G.S., C.A.; Investigation: K.V., C.A.; Methodology: K.V.; Project administration: S.M.; Resources: G.S., V.K., C.A., S.M.; Software: K.V., G.S.; Supervision: S.M.; Validation: K.V., V.K., S.M.; Writing - original draft: K.V.; Writing - review & editing: S.M.

Synopsis

miR-34a, let-7f, and miR-31 promoters were significantly methylated in EOC samples.
Significant reduced level of miR-34a, miR-31 and let-7f was observed in EOC samples.
Individual and combined miRNA panel have higher diagnostic value for EOC prediction.
miR-34a, let-7f and miR-31 can discriminate metastatic over non-metastatic samples.

INTRODUCTION

According to the recent report of the International Agency for Research on Cancer (IARC-2020), around 3,13,959 new ovarian cancer (OC) cases were recorded globally, with an incident rate of 6.6 and a mortality rate of 4.4 [1]. Due to the asymptomatic nature of the disease, around 70% of women are presented at a late stage, causing higher morbidity. About 90% of ovarian cancer cases are of epithelial subtype. Currently, the higher level of cancer antigen 125 (CA-125) is a hallmark for OC prediction. However, the higher level of CA-125 is often associated with the advanced stage of the disease, and only 10% of early-stage OC patients are correlated with higher expression of CA-125 [2]. Moreover, the routinely used physical screening method has lower sensitivity and specificity towards detecting ovarian cancer at an early stage [3]. Therefore, a minimally invasive, cost-effective diagnostic assay with advanced diagnostic significance needs to be developed to assess ovarian cancer risk at the early stage of disease progression.

The microRNA, a class of small single-stranded, non-coding RNA molecules of 18-22 nucleotide in size, plays a vital role in cell physiology and disease progression by regulating ~60% of human genes. Dysregulated miRNAs expression is frequently correlated with aberrant microRNAs gene hypomethylation or hypermethylation and has been experimentally discovered to portray a crucial function in human cancer progression [4-6]. Therefore, assessment of microRNA expression could aid in early diagnosis and better disease management.

In this study, we investigated the genome-wide methylation signature of microRNA genes using MeDIP-NGS sequencing and correlated microRNA gene methylation pattern with dysregulated expression of selected microRNA in tissue and serum samples of EOC and control using quantitative reverse transcription polymerase chain reaction (qRT-PCR). Moreover, we discovered the EOC predictive value of candidate microRNA at an early stage and correlated microRNA expression with clinical features.

MATERIALS AND METHODS

1. Study layout, and clinicopathological Samples

Informed consent was obtained from OC patients at the Department of Surgical Oncology, King's George Medical University, Lucknow. The study was approved by the Institute Ethical Committee (Ref. No: IEC/2019-20/01). Total 115 fresh frozen tissues (85 histologically verified EOC and 30 normal) were collected in RNAlater® solution (Sigma-Aldrich, Cat no: R0901). Total sixty-five matched preoperative serum (50 EOC and 15 normal) samples were also taken in BD Vacutainer® tubes, processed straightway for serum separation, and stored at -80 ºC. Clinicopathological characteristics of patients were evaluated to establish tumor stage, presence or absence of metastases, cancer type, and subtype at the source

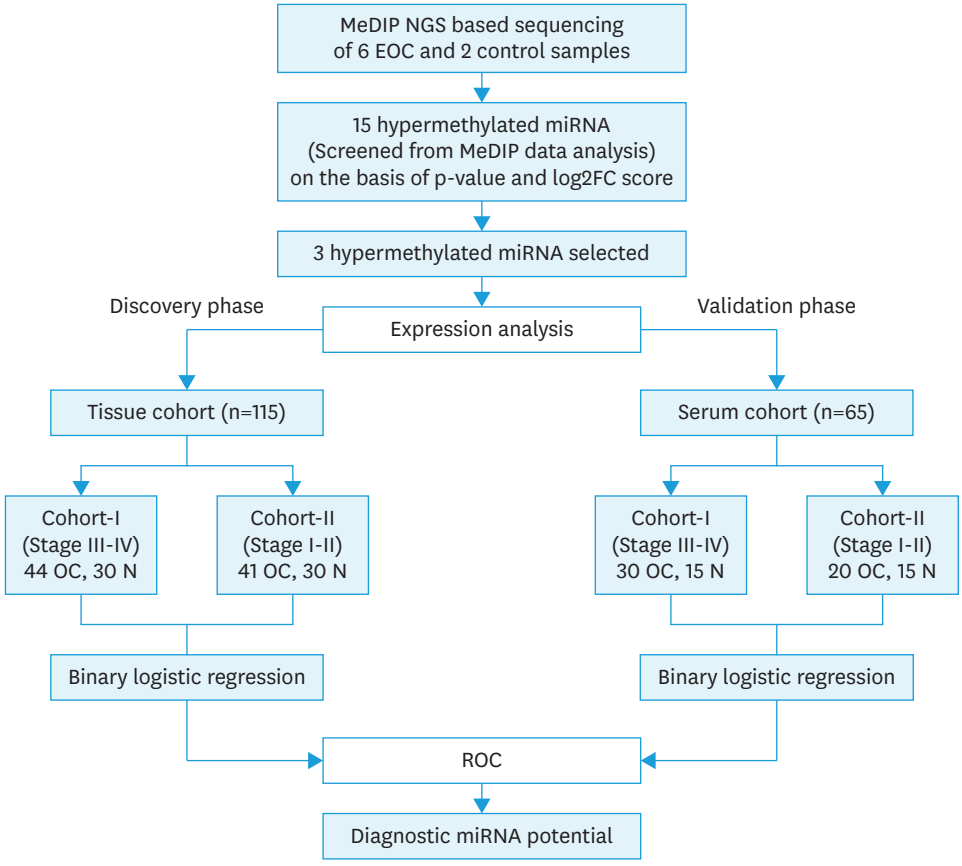

Fig. 1. Represents layout of study design.
EOC, epithelial ovarian cancer; MeDIP, methylated DNA immunoprecipitation; OC, ovarian cancer; ROC, receiver operating characteristics curve.

hospital. microRNA expression was evaluated in tissue and serum cohorts. The tissue cohort (discovery phase) was separated into cohort-I {advanced-stage EOC (stage III-IV) (n=44) and control samples (n=30) and cohort-II (early-stage EOC [stage I-II] [n=41])} and control (n=30). Similarly, in the validation phase (serum cohort), was separated into cohort-I {advanced-stage EOC (stage III-IV) (n=30), and control (n=15) and cohort-II (early-stage EOC [n=20], and control [n=15])} (**Fig. 1**).

2. MeDIP-NGS sequencing and microRNA-Target enrichment analysis

MeDIP-NGS sequencing and bioinformatics analysis of isolated gDNA from six EOC and two control samples were performed. To avoid any sample biasness during MeDIP-NGS, we included two early-stage (stage I-II) and four advanced stage samples (stage III-IV). Moreover, sample included in this study was of Indian origin and they were histologically confirmed serous subtypes of EOC. In addition, microRNA-target enrichment analysis, GO and KEGG analysis were performed. The detailed methodology is given in **Data S1**.

3. microRNA isolation from tissue and serum samples

All tissue and serum samples were processed for miRNA extraction using miRNeasy Mini Kit, Cat No: 217004, Qiagen and miRNeasy Serum/Plasma Kit, Cat. No: 217184; Qiagen, respectively, following the manufacturer's instruction. For PCR efficiency analysis and normalization of serum microRNA, 3.5 µL of miRNeasy Serum/Plasma spike-In Control

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(Qiagen, Hilden, Germany) (1.6×108 working solution) was added and eluted in 20 µL of pre-heated (at 95°C) DEPC treated Milli-Q water. Tissue and serum microRNAs concentration and purity were determined through a micro-spectrophotometer (DeNovix, Wilmington, DE, USA). The total microRNA yield of individual samples ranged between 0.3 µg to 2.5 µg for tissue and 0.2 µg to 1 µg for serum samples.

4. Quantification of microRNA and normalization

Quantitative expression analysis of selected microRNA in tissue and serum samples was performed on StepOneTM Plus RT-PCR (Applied Biosystems). Total one microgram of tissue microRNA and total serum microRNA samples were converted into cDNA using miScript® II RT Kit, Cat No.: 218160, Qiagen, India, following the manufacturer's instruction and diluted in RNase free water to make up the final concentration of 8 ng/µL and stored in −80 ºC. Further, the expression level of candidate microRNA was assessed using miScript SYBR® Green PCR Kit (Qiagen) and amplified by microRNA specific forward primer (miScript Primer Assay, Cat No: 218300, Qiagen). Tissue samples derived microRNA expression was normalized using microRNA-191 as an endogenous control while the combined value of cel-miR-39 and miR-191 was used to normalize serum microRNA (CtmicroRNA-0.5*(Ctcel-microRNA-39 +CtmicroRNA-191). The qRT-PCR reaction of each sample was executed in triplicates, and the relative expression of selected microRNAs was evaluated using the 2-ΔΔCT method.

5. Statistical analysis

The statistical analysis was accomplished in SPSS® (V26, IBM Corp., Armonk, NY,USA), and graphs were drawn in GraphPad Prism (V9.0). The data were presented as mean±SD, and categorical variables were presented as percentages or counts. The microRNAs expression data were evaluated using Mann-Whitney U tests, ANOVA test, and Spearman's rank-order correlations test. The binary logistic regression analysis (univariate and multivariate) was performed to estimate the predictive probability of individual and combined-microRNA panel; probability value was further used to assess individual and combined microRNA panel's diagnostic potential by Receiver Operating Characteristics curve analysis. All tests were two-tailed, and $p<0.05$ was termed as statistically significant.

6. Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of Motilal Nehru National Institute of Technology Allahabad (Ref. No: IEC/2019-20/01). An informed consent was obtained from all patients involved in the study.

RESULTS

1. Patient's clinicopathological data

A total of 115 tissue samples (EOC=85 and control=30) and 65 serum samples (EOC=50, and controls=15) were enrolled in this study. Based on pathological analysis reports, samples were divided into histological subtype (includes: serous [n=50; 58.82%], mucinous [n=14; 16.5%], clear cell [n=10; 11.5%], and endometrioid [n=11; 12.9%]), FIGO stages (includes: stage-I [n=25; 29.47%], stage-II [n=16; 18.83%], and stage III-IV [n=44; 51.78%]), Metastases (includes: 45 metastatic [52.90%] and 40 non-metastatic [47.0%]), and Menopausal status (includes: 52 present [61.11%] and 33 not present [38.8%]) (**Table 1**: A=tissue cohort; B=serum cohort).

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Table 1. Represents clinical characteristics of the patients recruited in this study

Variables	(A) Tissue cohort (n=115)					(B) Serum cohort (n=65)			
	Case (n=85)	Control (n=30)	Relative expression of miR-34a ($2^{-\Delta\Delta CT}$)	Relative expression of let-7f ($2^{-\Delta\Delta CT}$)	Relative expression of miR-31 ($2^{-\Delta\Delta CT}$)	Case (n=50)	Relative expression of miR-34a ($2^{-\Delta\Delta CT}$)	Relative expression of let-7f ($2^{-\Delta\Delta CT}$)	Relative expression of miR-31 ($2^{-\Delta\Delta CT}$)
Age, No. (%)									
<45	26 (37.1)	16 (53.3)	−6.39±3.66	−5.29±3.60	−5.87±2.54	14 (28.0)	−5.63±2.57	−3.01±2.92	−2.76±2.08
≥45	44 (62.8)	14 (46.6)	−6.30±3.91	−5.40±4.85	−6.65±3.63	36 (72.0)	−5.88±2.80	−6.94±4.15	−4.97±3.62
p-value			ns	ns	ns		ns	<0.001	ns
Histological type, No. (%)									
Mucinous	14 (16.5)	-	−6.15±3.12	−3.71±3.31	−7.03±3.40	8 (22.8)	−4.88±1.28	−7.05±4.77	−3.71±3.25
Serous	50 (58.8)	-	−6.52±4.34	−6.30±4.93	−6.36±3.04	15 (42.8)	−6.74±2.80	−6.35±4.65	−4.28±3.64
Clear cell	10 (11.5)	-	−5.58±3.38	−4.10±3.12	−8.34±4.57	6 (17.1)	−5.32±2.82	−5.71±3.88	−7.38±1.86
Endometrioid	11 (12.9)	-	−6.36±2.42	−5.03±4.34	−4.28±2.18	6 (17.1)	−3.84±2.99	−3.63±1.81	−2.82±2.13
Other	-	-	-	-	-	-	-	-	-
p-value			ns	<0.05	<0.05		<0.05	<0.05	<0.05
Distant metastasis, No. (%)									
Absent	40 (47.0)	-	−5.19±3.36	−3.56±2.66	−5.35±2.35	14 (40.0)	−4.42±1.66	−5.55±3.88	−3.00±3.08
Present	45 (52.9)	-	−7.22±3.89	−7.11±5.14	−7.40±3.76	21 (60.0)	−6.74±2.86	−6.46±4.68	−5.25±3.34
p-value			<0.005	<0.005	<0.005		<0.005	ns	<0.05
FIGO stage, No. (%)									
Stage I-II	41 (48.2)	-	−5.19±3.36	−3.56±2.66	−5.35±2.35	20 (57.1)	−4.24±1.72	−8.37±4.92	−6.43±4.14
Stage III-IV	44 (51.7)	-	−7.22±3.89	−7.11±5.14	−7.40±3.76	15 (42.8)	−6.85±2.78	−4.15±2.60	−2.96±1.80
p-value			<0.005	<0.005	<0.005		<0.005	<0.005	0.0071
Menopause status, No. (%)									
Yes	52 (61.1)	-	−7.25±4.17	−5.89±5.07	−7.22±3.64	22 (62.8)	−6.26±3.02	−6.83±4.19	−4.82±3.36
No	33 (38.8)	-	−4.87±2.63	−4.52±3.30	−5.11±2.26	13 (37.1)	−5.07±1.99	−4.35±3.90	−3.58±3.40
p-value			<0.005	ns	<0.05		ns	<0.05	ns
Serum CA125 (U/mL)	184±329.1	33.8±61.4				172±352.4			

ns, not significant.

2. MeDIP-sequencing data analysis

The MeDIP-Seq was performed for all eight samples on Illumina NextSeq 500 platform. The mean size of library fragment generated during MeDIP-Seq was ranging between 290–300 bp, and after quality assessment, around 9.8 GB of the mean bases were sequenced per sample. Next, raw sequence data were analyzed, and around 60.1–80.1 million clean reads per sample were obtained. Further, clean reads were mapped against the latest build reference genome (GRCh37), revealing an 80.0%–92.7% mapping percentage for each sample (**Fig. 2A**). Detailed sequencing data analysis statistics are given in supplementary materials (**Table S1**). Furthermore, diffRep software was employed to identify potential differentially methylated regions (DMRs) in cancerous and control samples. Total 2,25,868 DMRs ($p<0.05$; FC≥2) were found in all EOC samples, which includes 1,01,246 (44.82%) hypomethylated and 1,24,622 (55.17%) hypermethylated DMRs (**Fig. 2C**). Moreover, the distribution of DMRs on the chromosome was assessed, which revealed the highest amount of DMRs present in chromosome one (**Fig. 2B**). Further, genome-wide dispersal of DMRs was assessed, and most of the hypomethylated and hypermethylated DMRs were located in gene-body and other-intergenic regions on the genome (**Figs. 2D and E**). Further, the region-analysis (v1.0) tool was used to annotate hypermethylated genomic intervals. The ensemble database was used to retrieve the gene information while performing annotation. All raw data and processed data generated from MeDIP-Sequencing were uploaded on the public repository database Gene Expression Omnibus (GEO) with GEO accession number GSE180292.

3. Identification of candidate hypermethylated microRNA

The putative promoter location of microRNA was identified by online tools (microTSS, miRstart, and TransmiR-v2.0). Only overlapping putative promoter regions predicted in all

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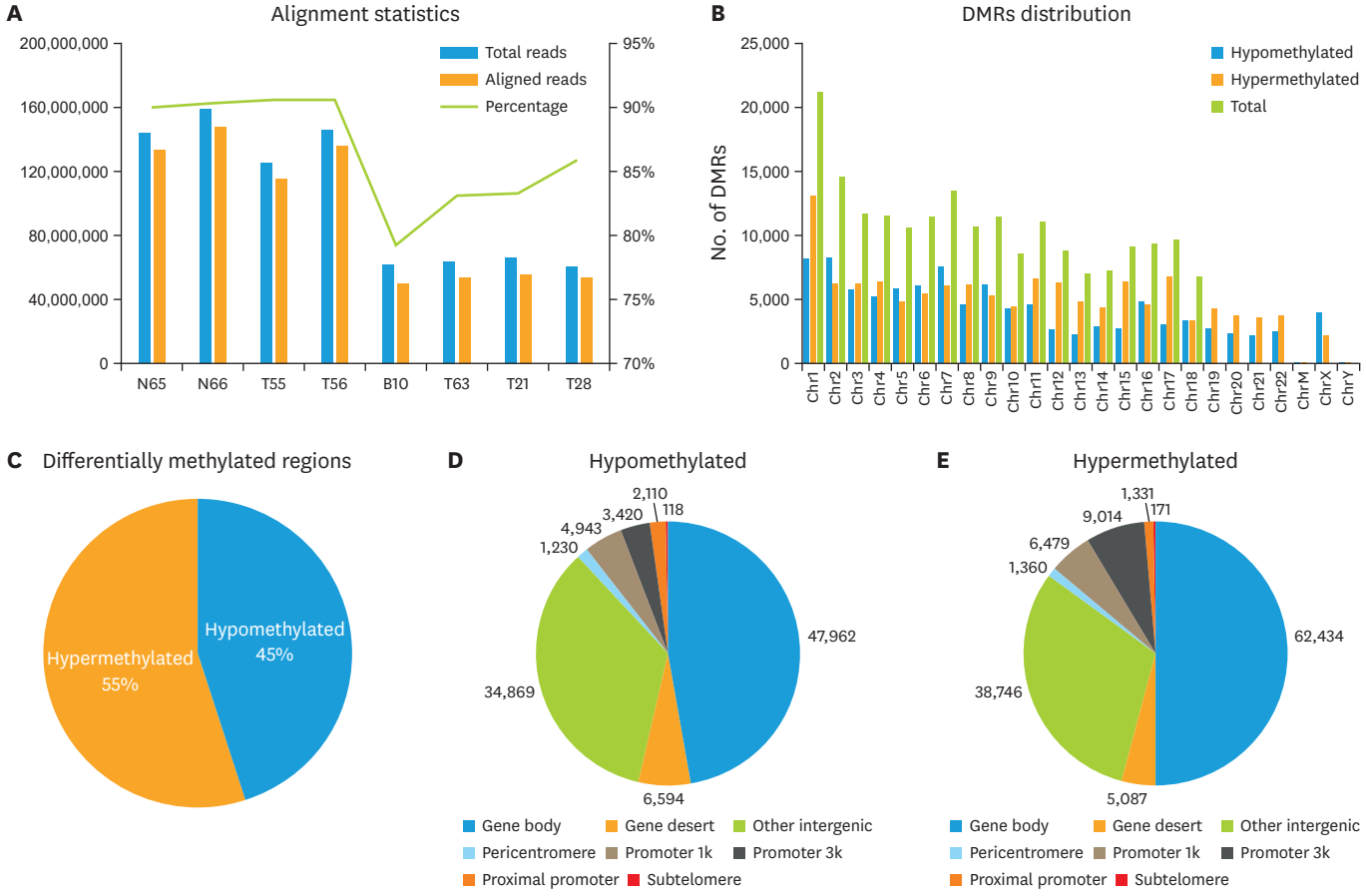

Fig. 2. MeDIP-Seq data analysis results 8 tissue samples. (A) Alignment percentage of clean reads on genome. (B) Chromosome-wise distribution of hypomethylated and hypermethylated DMRs in genome. (C) Percentage of hypermethylated and hypomethylated DMRs. (D) Hypomethylated DMRs scattered in different location of genome. (E) Hypermethylated DMRs scattered in different genomic location.
DMR, differentially methylated region.

three online tools were used for further analysis. Further, predicted promoters regions were manually investigated in DMRs (obtained from MeDIP-seq) for their epigenetic signature (hypomethylated or hypermethylated). The top 16 hypermethylated microRNA-DMRs were further separated (based on log2FC and p-value), and out of 16, the best three microRNA (based on log2FC and p-value) were selected for further study (**Table S2**).

4. Correlation of microRNA expression and hypermethylation of microRNA gene

To understand the association between hypermethylation of microRNA genes and its downstream dysregulated expression, we assessed the relative expression of selected microRNA in eight samples (EOC=6 and normal=2) used for MeDIP-sequencing. The relative expression level of microRNA-34a, let-7f, and microRNA-31 was significantly downregulated in six cancerous samples compared to normal with the drop-in expression level of 4.28 ($p<0.05$), 6.26 ($p<0.05$), and 4.07 ($p<0.05$), respectively (**Fig. S1**). Further, to validate this, we executed quantitative expression analysis of these microRNA in leftover samples (n=107).

5. Quantitative expression analysis of selected microRNA in tissue cohorts

The differential expression level of candidate microRNAs (microRNA-34a, microRNA-31, and let-7f) was assessed in tissue cohorts using qRT-PCR. In tissue cohort-I (n=74), significantly

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downregulated expression of miR-34a, let-7f and miR-31 was observed in EOC patients in contrast to controls with the drop-in expression level of 7.22 (p<0.0001), 7.11 (p<0.0001), and 7.40 (p<0.0001), respectively (**Fig. 3A**). Similarly, in cohort-II (n=71), miR-34a, let-7f, and

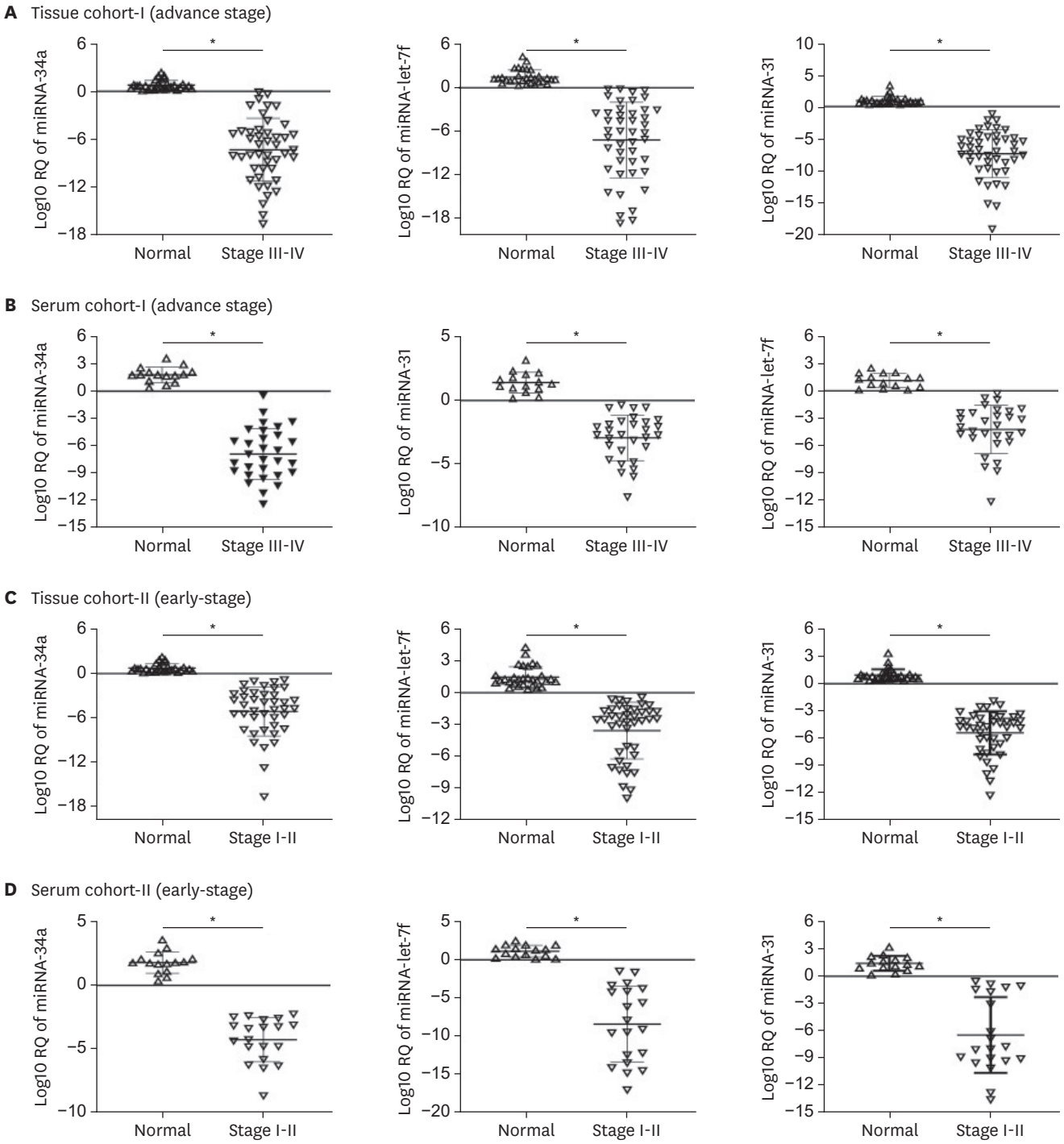

Fig. 3. Downregulation of miRNAs (miR-34a, let-7f and miR-31) in epithelial ovarian cancer. (A) In tissue cohort-I (mixed stage). (B) In serum cohort-I (mixed stage). (C) In tissue cohort-II (early-stage). (D) In serum cohort-II (early-stage). Statistical significance was determined by the Mann Whitney U-tests. Data represent mean ± standard deviation.
RQ, relative quantity.
*p<0.0001.

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miR-31 were notably under-expressed in EOC patients with a decreased fold change of 5.19 ($p<0.0001$), 3.56 ($p<0.0001$), and 5.35 ($p<0.0001$), respectively (**Fig. 3C**).

6. Quantitative expression analysis of selected microRNA in serum cohorts

The constancy of microRNAs downregulation was validated in 50 EOC and 15 normal serum samples. In serum cohort-1 (n=45), microRNA-34a, let-7f, and miR-31 exhibited significantly reduced expression in EOC with respect to controls with a reduced level of expression of 6.85 ($p<0.0001$), 4.15 ($p<0.0001$), and 2.96 ($p<0.0001$), respectively (**Fig. 3B**). Moreover, in serum cohort-II, we observed significant downregulation of microRNA-34a, let-7f, and miR-31 in EOC with a reduced fold change of 4.24 ($p<0.0001$), 8.37 ($p<0.0001$), 6.43 ($p<0.0001$), respectively (**Fig. 3D**).

7. Performance of tissue microRNAs as diagnostic biomarker in EOC

We performed a univariate binary logistic regression analysis of each microRNA in tissue cohort-I (n=74) and tissue cohort-II (n=71) to signify the involvement of microRNA with disease (OC). The downregulation of microRNA-34a, microRNA-31, and let-7f from both tissue cohorts were significantly associated with disease (**Table S3** - A).

Further, the diagnostic potential of each microRNA was assessed by performing ROC curve analysis in both tissue cohorts. In tissue cohort-I (advanced-stage III-IV), the area under curve (AUC) of microRNA-34a, let-7f, and microRNA-31 was 97.0, 92.1, and 92.1, respectively (**Fig. 4A**). Similarly, in tissue cohort-II (early-stage), the AUC of microRNA-34a, let-7f, and microRNA-31 was 96.9, 87.1, and 86.6, respectively (**Fig. 4C**). Besides, a multivariate binary logistic regression analysis was conducted for assessment of the diagnostic performance of multi-microRNA panel (microRNA-34a, let-7f, and microRNA-31) to envisage the possibility of ovarian cancer. The cumulative predicted probability of the multi-microRNA panel was obtained from the regression analysis model of cohort-I and cohort-II and was used to generate the ROC curve. The diagnostic value of the multi-microRNA panel in tissue cohort-I and cohort-II was 98.6 and 98.9, respectively (**Fig. 5A and B**). The diagnostic potential of independent microRNA and multi-microRNA panel in the discovery phase (both tissue cohorts) exhibited greater AUC, sensitivity, and specificity for the prediction of EOC. Detailed diagnostic performance results such as sensitivity, specificity, cut-off value, and 95% confidence interval (CI) values are depicted in **Table S4** - A.

8. Performance of serum microRNAs as diagnostic marker in EOC

We evaluated the persistent diagnostic potential of independent microRNAs and multi-microRNA panels in the validation phase, i.e., serum cohort (50 EOC and 15 normal). The univariate and multivariate analysis of individual microRNA and multi-microRNA panels in serum cohort-I (n=45) and cohort-II (n=35) revealed a significant association of miR-34a, let-7f, and miR-31 with disease occurrence (**Table S3** - B).

Further diagnostic potential of individual miR-34a, let-7f, and miR-31 was evaluated using ROC curve analysis. The AUC of miR-34a, let-7f, and miR-31 in serum cohort-I was 92.0, 87.9, and 85.6, respectively (**Fig. 4B**). Similarly, in cohort-II, miR-34a, let-7f, and miR-31 have AUC of 82.7, 82.0, and 81.0, respectively (**Fig. 4D**). The combined diagnostic potential of the microRNA panel in serum cohort-I and cohort-II was 95.5 and 96.9, respectively (**Fig. 5C and D**). In both serum cohorts, miR-34a, let-7f, and miR-31 turned out to be the strongest predictor of epithelial ovarian cancer. Moreover, the diagnostic potential of an independent and multi-microRNA panel from serum was consistent with the tissue cohorts. Detailed information of microRNAs

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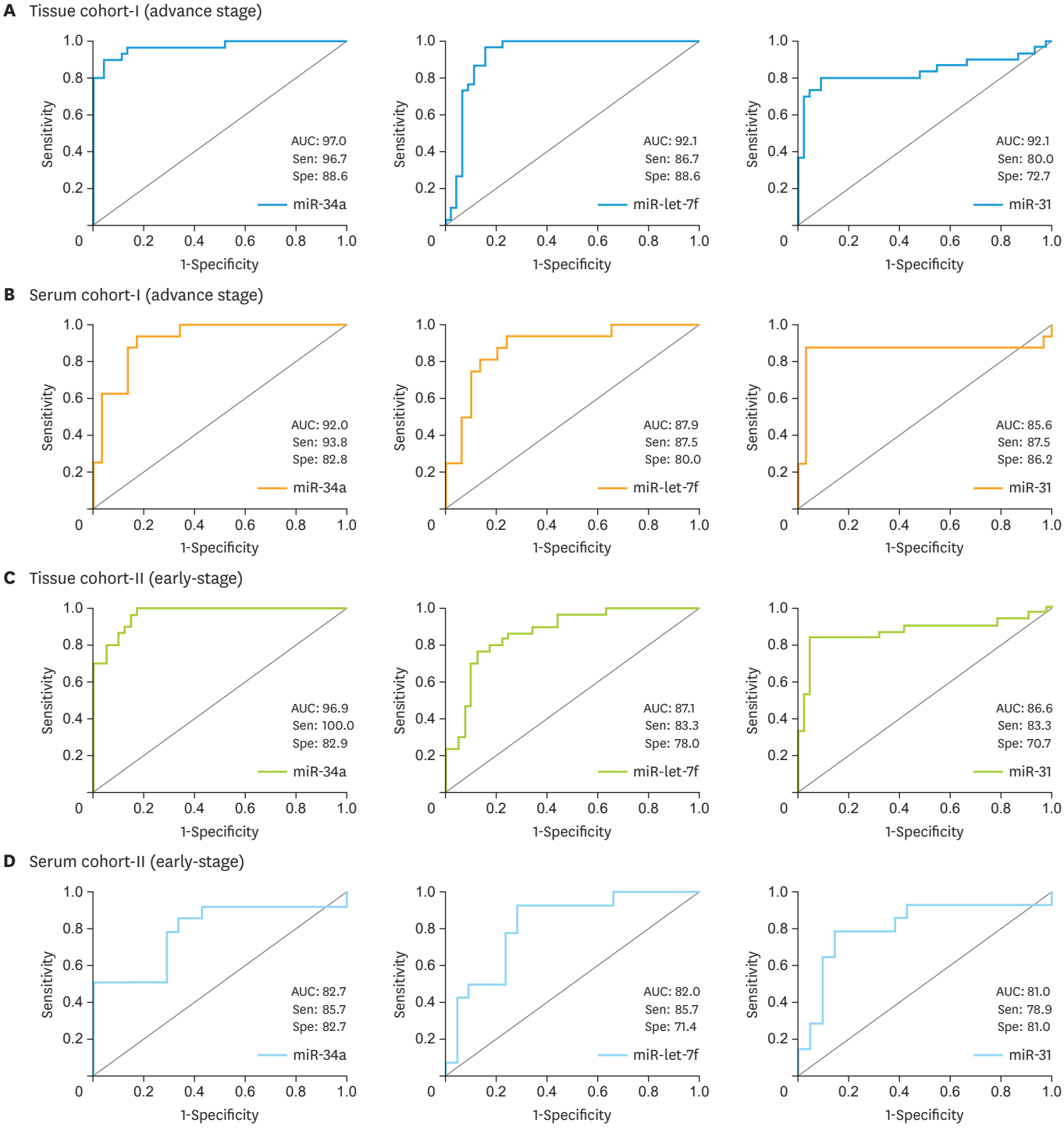

Fig. 4. Represent receiver operating characteristics curve analysis of individual miRNA (miR-34a, let-7f, and miR-31) signature for prediction of epithelial ovarian cancer. (A) Tissue cohort-I (advance stage) (n=74). (B) Serum cohort-I (advance stage) (n=45). (C) Tissue cohort-II (early-stage) (n=71). (D) Serum cohort-II (early-stage) (n=35). Diagonal line: performance measure of the diagnostic test.
AUC, area under curve; Sen, sensitivity; Spe, specificity.

diagnostic performance, AUC, sensitivity, specificity, 95% CI, and optimal cut-off values are depicted in **Table S4** - B.

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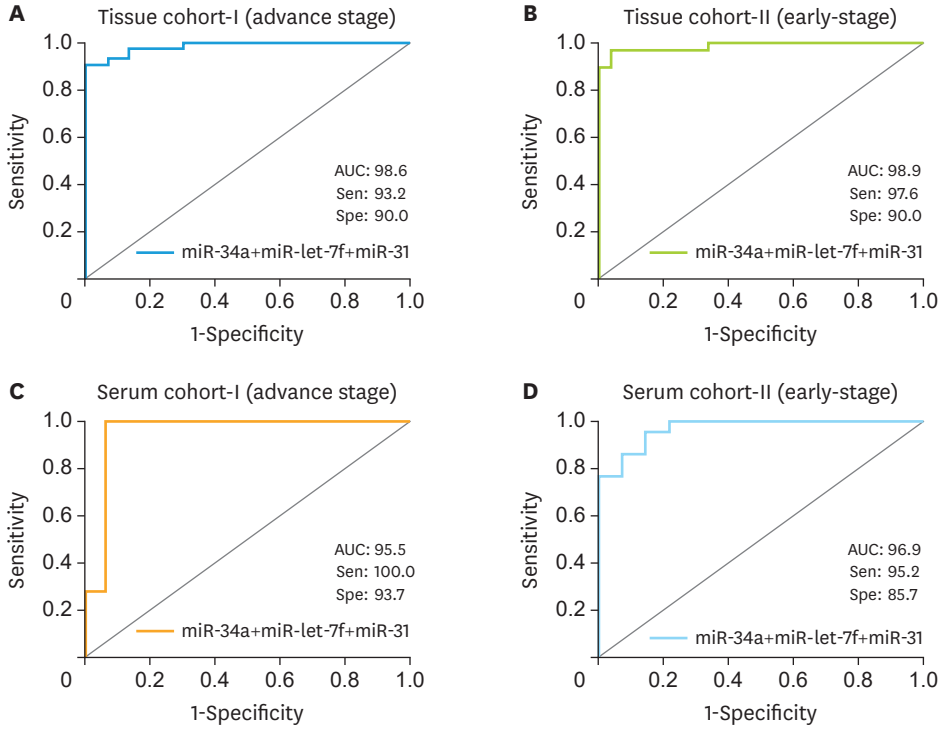

Fig. 5. Receiver operating characteristic curve analysis in tissue and serum cohort to evaluate the ability of the combined miRNA panel (miR-34a+let-7f+miR-31) to diagnose epithelial ovarian cancer. (A) In tissue cohort-I. (B) In tissue cohort-II. (C) In serum cohort-I. (D) In serum cohort-II.
AUC, area under curve; predicted probability, miR-205+miR-200c+miR-141; Sen, sensitivity; Spe, specificity.

9. Correlation between microRNA expression and clinicopathologic factors in EOC patients

We evaluated the association between reduced expression of candidate microRNA with different clinical parameters in tissue (EOC=85) and serum (EOC=50) samples. We found a significantly lower expression of miR-34a ($p<0.005$), let-7f ($p<0.005$), and miR-31 ($p<0.005$) in patients (tissue samples) with combined FIGO stage III-IV compared to early-stage I-II (**Fig. S2A**). Similarly, miR-34a was significantly downregulated in stage III-IV in the serum cohort ($p<0.05$). However, significant downregulation of let-7f ($p<0.005$) and miR-31 ($p<0.05$) was observed in stage I-II patients (serum samples) (**Fig. S2B**). Besides this, significantly downregulated expression of let-7f ($p<0.05$) and miR-31($p<0.05$) was observed in a serous subtype of EOC when compared to other histotypes in tissue samples, except miR-34a (**Fig. S3A**). Moreover, the most significant downregulation in the level of microRNA-34a was observed in serous subtypes, while significant downregulation of let-7f ($p<0.05$) was observed in mucinous subtype compared to others in serum samples. Similarly, miR-31 was significantly downregulated in clear cell and serous subtype of EOC in the serum cohort ($p<0.05$) (**Fig. S3B**). In addition, downregulation of all three microRNAs was observed in patients with distant metastases in both tissue and serum cohorts except let-7f, which did not exhibit any significant difference between metastases and non-metastases patients in serum samples (**Fig. S4A and B**).

In addition, we performed the Spearman's rank-order correlations test to establish any relatedness between downregulated expression of microRNA in tissue and serum with clinical features. In tissue cohort-I and cohort-II, the patient's age and menopausal status

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didn't establish any correlation with candidate microRNA expression level, except miR-34a, which established a negative correlation from tissue cohort-II.

In tissue cohort-I and cohort-II, the mean expression level of microRNA-34a, miR-31, and let-7f established a negative correlation with CA125 level and distant metastases (**Table S5** - A).

Likewise, in serum cohort-I, the expression level of microRNA-34a, let-7f, and microRNA-31 exhibited a strong negative correlation with CA125, menopausal status, and distance metastasis except for age. Similarly, in serum cohort-II, miR-34a, let-7f, and miR-31 have a significant negative correlation with CA125 and distance metastasis. The age and menopausal status from cohort-II did not significantly correlate with candidate microRNA expression level (**Table S5** - B).

10. microRNA-target enrichment analysis

We further tried to investigate the functional role of hypermethylated selected microRNAs in EOC. We identified putative target genes of selected hypermethylated microRNA and only highly enriched overlapping target-genes (~243 genes) were sorted, and microRNA-target enrichment analysis (using miRnet-V2.0) was performed to create the microRNA-target regulatory network. The filtering capability of tools allowed us only to highlight target genes associated with candidate microRNA (miR-34a, let-7f, and miR-31) in OC. In this analysis, SMAD4, MYC was highly enriched central molecules, while key genes involved in cancer progression (such as TP53, BRCA1, and SOX9) were enriched in the regulatory network (**Fig. S5B**). Further, we also performed microRNA-disease enrichment analysis to observe relatedness between microRNA and ovarian cancer. To construct a microRNA-disease network, the miRnet-v2.0 tool was used with filter ovarian cancer only. Around 429 microRNAs were coupled with EOC with 531 edges, comprising our selected microRNA (**Fig. S5A**).

11. Functional enrichment of microRNA target genes

Gene ontology and KEGG analysis were executed to assess the potential function of predicted target genes of candidate microRNA using the freely available tool DAVID. In GO analysis, the enriched biological process was regulation of cell projection organization, cell development, regulation of gene silencing by microRNA. Similarly, in molecular function, zinc ion binding and microRNA binding terms were highly enriched, while in a cellular component, Synapse, cell projection, regulation of cell development were highly enriched terms (**Fig. S5C**). Further, we assessed KEGG pathways enrichment analysis of microRNA-target genes to unveil the preparatory course of ovarian cancer progression. KEGG analysis revealed several biologically important pathways relating to cancer progression. The Cell cycle, Hippo signaling pathways, and Wnt signaling pathways were highly enriched pathway terms in our analysis revealing the importance of these microRNA-targets in cancer progression ($p<0.05$) (**Table S6**).

DISCUSSION

Ovarian cancer is the fifth cancer morbidity causing gynecological malignancy among women and accounts for five percent of overall cancer-associated death in women [7]. Recently, microRNA has gained attention due to its association with tumorigenesis, cancer progression, and its stable presence in liquid biopsy during adverse cancer microenvironments [8]. Cancer disease progression is caused by multistage genome alteration. In the last two decades, cancer genome studies have been only focused on

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protein-coding genes, while only a few studies have been carried out in correlation to the altered function of non-coding sequences. microRNA dysregulation follows similar epigenetic regulation as of protein-coding genes. It has been well established that microRNA downregulation is caused by several factors, including homozygous deletion, heterozygous deletion, mutation, polymorphism, and an epigenetic mechanism [9]. A recent study by Adams et al. revealed epigenetic modification on CpG island and TATA boxes close to miRNA genes promoter are greatly affected and leading to dysregulated expression [10]. Recent studies revealed that around 63 miRNA genes were epigenetically deregulated in 21 different cancers, including leukemias, gastric cancer, colorectal cancer, bladder cancer, breast cancer, ovarian, cervical, prostate, and lung carcinoma. They reported that 6.9% of studied microRNAs are dysregulated due to epigenetic mechanism; moreover, methylation frequency of microRNA genes in at least two cancer types was 45.5%, while 54.5% of microRNA showed cancer specific methylation [11,12]. This study focused on epigenetic modification of genes in ovarian cancer and correlated their downstream expression toward developing a miRNA expression-based ovarian cancer diagnostic biomarker. Moreover, differential expression profiling study conducted on microRNA derived from patients' body fluid may establish microRNA as a promising non-invasive biomarker for predicting early-stage OC for enhanced management of the disease.

microRNA-34a from liquid biopsy has been documented as a biomarker in breast, lung, and ovarian cancer. Moreover, microRNA-34a involvement in cell proliferation and invasion in EOC is markedly reported; restoration of microRNA-34a could inhibit proliferation, motility, and invasion of OC cells by targeting MET (a hepatocyte growth factor) [13-15]. In contrast, elevated expression of microRNA-31 was found in endometrial cancer. The functional role of microRNA-31 is slightly obscured to understand due to its dualistic nature (act as a tumor suppressor or oncogenic) depending upon the cellular microenvironment [16-19].

let-7 family plays a critical role in carcinogenesis via acting as a tumor suppressor and frequent downregulated in gastric, papillary thyroid, renal, breast and ovarian carcinoma [20-23]. Conferring these studies, the downregulation of microRNA-34a, microRNA-31, and microRNA-let-7f seems to have a potential role in tumor advancement by targeting cancer-associated genes, and evaluating their dysregulated expression might be helpful in discriminating epithelial ovarian cancer patients from control at the early stage of the disease.

This study recruited three hypermethylated microRNAs (microRNA-34a, microRNA-31, and miR-let-7f) through MeDIP-Sequencing of six EOC and two control. We then investigated the differential expression of candidate microRNAs in tissue and serum cohorts of EOC patients compared to their normal counterparts. The mean expression of individual microRNA-34a, microRNA-31, and miR-let-7f was significantly downregulated in EOC samples with reference to normal controls in both tissue and serum cohorts. Consistent downregulated expression of candidate microRNAs was observed in early-stage tissue and serum cohort of EOC patients. Hypermethylation-induced downregulation of microRNA34a, let-7f, and miR-31 in ovarian cancer has been reported previously [13,24-28].

Using a stringent validation process and multivariate binary logistic regression model, we evaluated the diagnostic potential of three candidate microRNAs (microRNA-34a, microRNA-31, and let-7f) to predict the risk of EOC. Individually microRNA-34a, microRNA-31, and let-7f could detect EOC with greater AUC value, sensitivity, and specificity. Moreover, integrated panels of microRNA in early-stage tissues and serum samples were

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shown to have exceptional diagnostic value for early-stage prediction of EOC. Since our objective was to develop a minimally-invasive biomarker for EOC detection, we compared downregulated fold expression of candidate miRNA of tissue with serum samples. Our expression profiling of candidate miRNAs from both early and advanced-stage serum cohorts showed less variation in their expression profiles than tissue cohorts. Moreover, significant reduced expression of miR-31 and let-7f was observed in the case of early-stage serum samples than early-stage tissue samples. However, the combined diagnostic value of miRNA from early-stage serum samples almost had similar AUC, sensitivity, and specificity compared to early-stage tissue samples, making them the potential candidate biomarkers. These outcomes imply that an integrated microRNA panel from serum may be used as a non-invasive early detection marker for EOC. Similarly, the diagnostic potential of microRNA-34a and microRNA-31 is analogous to combined microRNA panels for the early diagnosis of EOC. Recent study showed combined diagnostic value of miR-34a and CA-125 could discriminate EOC from normal with an AUC of 0.818, while lower expression of let-7f has the diagnostic value of AUC 0.78 [24,29-32]. Moreover, the comparative expression analysis of let-7f in different cancers (colorectal, lung, prostate, papillary thyroid cancer) showed a higher diagnostic value for discriminating respective cancer from the normal [29,31-35]. Very few reports are available on exploring the diagnostic value of these selected microRNA in EOC; therefore, the discriminative potential of each microRNA and integrated microRNA panel from the present study could aid in predicting early-stage ovarian cancer.

Accordingly, we attempted to correlate microRNA expression level with clinicopathological features of patients. Our results suggest that downregulated expression of microRNA-34a in the advanced stage of tissue and serum samples could be used to staging EOC while miR-31 and miR-let-7f (in serum) lower in stage I-II in EOC [14,34]. Moreover, metastatic patients are observed to have a lower level of miR-34a and miR-31 during carcinogenesis. A strong negative correlation between downregulated expression of candidate microRNA with serous ovarian cancer subtype, metastatic nature of the disease, menopausal status, and CA125 level was observed, suggesting that these microRNA could be valuable as a prognostic biomarker [35].

Moreover, microRNA-target network enrichment revealed significant enrichment of cancer-associated genes (MYC, SMAD4, TP53, BRCA1, and SOX9), while GO revealed several important terms at biological, molecular, and cellular levels involved in carcinoma. In addition, Wnt signaling, Hippo signaling, cell cycle pathways in carcinoma were significantly enriched terms in KEGG analysis. In this context, Wnt and Hippo signaling pathways were reported to play crucial role in cellular events such as cell migration, epithelial-mesenchymal transition, cell proliferation, cells polarity, self-renewal, and apoptosis [36-40].

Overall, the present study provides a comprehensive report of microRNAs gene hypermethylation associated with downregulated expression of selected microRNA in EOC tissue and serum samples. Our results revealed that the downregulation of candidate microRNAs could detect EOC at an early stage with a higher diagnostic value. Moreover, this is probably the first report implicating the integrated diagnostic power of microRNA-34a, microRNA-31, and let-7f in the diagnosis of EOC at early-stage with higher sensitivity and specificity. However, this study has certain limitations; the samples size used for MeDIP-NGS sequencing is a bit low and could have induced sample biases during the screening of candidate miRNA. Therefore, screening of miRNA using MeDIP-NGS sequencing should be done with a large cohort of EOC samples. Moreover, sample biases can be reduced by incorporating samples with different histological subtypes and FIGO stage. In addition,

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samples included for expression analysis in this study were the only representative of the North Indian population, so more demographic samples are needed to be included for further experimental validation at a larger cohort size. Nevertheless, a liquid biopsy-based microRNA biomarker discover has several hurdles to overcome before accomplishing it as a clinical biomarker.

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SUPPLEMENTARY MATERIALS

Data S1
Supplementary materials and methods

Click here to view

Table S1
Represents MeDIP NGS mapping statistics of 6epithelial ovarian cancer and 2 normal samples

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Table S2
Represents list of top 16 hypermethylated miRNA

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Table S3
Binary logistic regression analysis of tissue Cohort-I (advance stage tissue sample; n=74), Cohort-II (early stage tissue samples; n=71) and serum samples (advance stage serum samples (n=50), early stage serum samples (n=35)

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Table S4
Represents data of receiver operating characteristic curve analysis for the prediction of epithelial ovarian cancer using expression level of miRNA in tissue and serum cohort respectively

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Table S5

Represents correlation of miRNAs with clinical characteristics in tissue and serum cohort of EOC

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Table S6

Represents top 13 KEGG enrichment analysis results of miRNA-target genes

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Fig. S1

Expression analysis of hypermethylated miRNA in samples used for MeDIP NGS analysis (6 EOC and 2 normal samples). miR-34a, let-7f and miR-31 was significantly downregulated in epithelial ovarian cancer compared to normal samples with drop=in fold change of 4.28 ($p<0.05$), 6.26 ($p<0.05$), and 4.07 ($p<0.05$), respectively.

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Fig. S2

Differential expression analysis of miRNA in different FIGO stages. (A) miRNA-34a, let-7f and miR-31 showed significant downregulation in FIGO stage-III-IV ($p<0.005$, $p<0.0005$, $p<0.005$, respectively) compared to stage I-II in tissue cohort. (B) In serum cohort, miRNA-34a was significantly reduced in combined stage III-IV ($p<0.005$) compared to early-stage I-II. Similarly, let-7f and miR-31 was significantly reduced in combined early-stage I-II ($p<0.005$, and $p\leq0.0071$) compared to stage III-IV. Statistical significance was determined by the Mann Whitney U-tests. Data presented as log10RQ;Data represent mean ± standard deviation.

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Fig. S3

Represents differential expression of miRNA in histological subtypes of EOC. (A) Relative expression oflet-7f was significantly reduced in serous compared to mucinous and clear cell histotype of EOC in tissue samples. Similarly, miR-31 was reduced in clear cell subtype as compared to serous and endometrioid histotype of EOC in tissue samples. However, miR-34a does not exhibit any significant change in tested groups(B) Relative expression of miR-34a and let-7f was significantly reduced in serous subtypes compared to endometrioid subtype of EOC in serum samples. Similarly, miR-31 was reduced in clear cell subtype compared to other histoypes. Statistical significance was determined by the One-way ANOVA test. Data presented as log10RQ; Data represent mean ± standard deviation.

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Fig. S4

Represents relative expression of candidate miRNA in tissue and serum samples of metastatic and non-metastatic patients. (A) In tissue samples, miRNA-34a, let-7f and miRNA-31 was significantly reduced in metastatic patients compared to non-metastatic patients ($p<0.005$, $p<0.005$, and $p<0.005$, respectively) (B) Similarly, In serum samples miRNA-34a and miR-31 showed significant downregulation in metastatic patient compared to non-metastatic

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patients (p<0.005, and p<0.05); however, let-7f does not exhibit any significant change in metastatic and non-metastatic patients. Statistical significance was determined by the Mann Whitney U-tests. Data presented as log10RQ; Data represent mean ± standard deviation.

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Fig. S5

Represents miRNA-disease enrichment, miRNA-target regulatory network and functional analysis of miRNA targets. (A) Represent miRNA-disease enrichment analysis where candidate miRNA (highlighted in red circle) were significantly enriched with other miRNA. (B) In regulatory network analysis SMAD4 and MYC were central target molecule along with SOX9, TP53, SMAD3, BRCA1. (C) represent GO analysis of candidate miRNA-target.

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