# RESEARCH ARTICLE

# Differential microRNA Expression by Solexa Sequencing in the Sera of Ovarian Cancer Patients

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## Abstract

MicroRNAs are a class of small noncoding RNA which play important regulatory roles in a variety of cancers. MiRNA-specific expression profiles have been reported for several pathological conditions. In this study, we combined large scale parallel Solexa sequencing to identify 11 up-regulated miRNAs and 19 down-regulated miRNAs with computational techniques in the sera of ovarian cancer patients while using healthy serum as the control. Among the above, four miRNAs (miR-22, miR-93, miR-106b, miR-451) were validated by quantitative RT-PCR and found to be significantly aberrantly expressed in the serum of ovarian cancer patients ($P<0.05$). There were no significant differences between samples from cancer stage I/II and III/IV. However, the levels of miR-106b ($p=0.003$) and miR-451 ($p=0.007$) were significantly different in those patients under and over 51 yearsof age. MiR-451 and miR-93 were also specific when analyzed with reference to different levels of CA125. This study shows that Solexa sequencing provides a promising method for cancer-related miRNA profiling, and selectively expressed miRNAs could be used as potential serum-based biomarkers for ovarian cancer diagnosis.

Keywords: Ovarian cancer - serum - microRNAs - Solexa sequencing - diagnostic biomarkers

## Introduction

Ovarian cancer is the fifth commonest cancer in women worldwide and the leading cause of gynecologic cancer-related deaths in the United States (Siegel et al., 2013). Despite advances in detection and therapies, less than 30% of patients with late-stage diagnosis have a five-year survival rate (Smith et al., 2013). Cancer progression has been reported to be associated with high levels of TP53, HER-2/ER (Rossing et al., 2010) and AKT2 gene mutations and a low incidence of KRAS and BRAF gene mutations (Iorio et al., 2007; Nam et al., 2008) .

Ovarian cancer at its early stages (I/II) is difficult to diagnose, so that it often spreads and advances to later stages (III/IV) (Kurman et al., 2008). This is because most of the symptoms are non-specific and of little use in the direct diagnosis of the disease (Rossing et al., 2010). Prognosis is usually very poor due to the late diagnosis and frequent metastasis to remote organs. It is disproportionately deadly because it lacks any useful test for early detection or screening, meaning that most cases can not be diagnosed until they have reached advanced stages. More than 60% of women diagnosed with this cancer have reached stage III or stage IV when the cancer has already spread beyond the ovaries. The five-year survival rate for all stages of ovarian cancer is 47% (Visintin et al., 2010). However, for cases where a diagnosis of the disease is made early, when the cancer is still confined to the primary site, the five-year survival rate is 92.7% (Ries et al., 2003; Sankaranarayanan and Ferlay, 2006). Hence, there is a great need for identification of novel non-invasive biomarkers for early tumor detection. Due to the lack of reliable markers for detection and classification, more investigation needs to be done in this area (Cortez and Calin, 2009; Brase et al., 2010; Iorio and Croce, 2012).

MicroRNAs are the smallest functional, non-coding RNAs of plants and animals. Mature miRNAs are a class of small (~22 nucleotides) single-stranded non-coding RNA molecules (Lee et al., 2001), which were first discovered as critical regulators of developmental timing events in Caenorhabditis elegans (Visone et al., 2008). Studies have shown that miRNAs were processed from 70–100 nucleotides hairpin pre-miRNAs, and involved the post-transcription level to regulate gene expression. MiRNA binds through partial sequence homology to the 3'untranslated region (3'-UTR) of target mRNAs, causing blockade of translation and mRNA degradation (Calin and Croce et al., 2006). It has been suggested that sequence-specific, posttranscriptional regulatory mechanisms

## Table 1. Clinical Serum Samples of Ovarian Pathological Information

| Variable | Clinicopathologic parameter | Number of RNA samples | | |
|---|---|---|---|---|
| | | Malignant | Benign | Normal |
| Case | | 31 | 23 | 8 |
| Age | <51 | 7 | 17 | 1 |
| | >= 51 | 24 | 5 | 7 |
| | Unknown | 0 | 1 | 0 |
| Clinical stage | I | 3 | 0 | 0 |
| | II | 4 | 0 | 0 |
| | III | 20 | 0 | 0 |
| | IV | 4 | 0 | 0 |
| Differentiated | Low-differentiated | 9 | 0 | 0 |
| | Primary-differentiated | 10 | 0 | 0 |
| | Middle-differentiated | 1 | 0 | 0 |
| | Well-differentiated | 1 | 0 | 0 |
| | Unknown | 10 | 0 | 0 |
| CA125 | <=35 (U/ml) | 2 | 11 | 0 |
| | >35 (U/ml) | 29 | 12 | 0 |
| Therapy | No | 2 | 0 | 0 |
| | Operation or chemotherapy | 29 | 23 | 0 |

mediated by miRNAs are more commonly previously appreciated. In 2008, Chen et al. (2008) introduced miRNAs as a new class of biomarkers for cancer. Several studies showed that some miRNAs can regulate cellular differentiation, proliferation and apoptosis processes that are important in cancer aggravation (Bartel., 2004; Corney and Nikitin, 2008; Dahiya et al., 2008; Iorio et al., 2012). Recently, differences have been found between miRNA pattern in serum or plasma of patients with a number of malignancies and healthy controls (Chen et al., 2013; Xu et al., 2013).

Traditional sequencing of cDNA libraries made from short RNA is very laborious and preferentially identifies abundant miRNAs. However, deep sequencing technology has now come into use, which enables the simultaneous sequencing of up to millions of DNA or RNA molecules, and provides a promising option for profiling miRNAs (Wyman et al., 2009). Deep sequencing overcomes many of the disadvantages of microarrays, which can be affected by background noises, nonspecific cross-hybridization problems and limited measurement of the relative abundances of previously discovered miRNAs (Wu et al., 2011). Deep sequencing measures absolute abundance (over a much wider dynamic range than possible with microarrays) and is not limited by array content, allowing for the discovery of novel miRNAs or other small RNA species (Creighton et al., 2009). A number of other next-generation sequencing technologies are currently widely used, including pyrosequencing (454 sequencing, Roche), which provides up to 400,000 sequences of up to 250 nt in length for a single read, and Illumina/Solexa and AB SOLiD generate shorter reads (35 bp) but generate >1 Gbp of sequence data per run (Moxon et al., 2008).

To better understand the role of miRNAs in ovarian cancer development and progression, comprehensive analysis of the expression and abundance of miRNAs in this cancer is required. The strategy of deep sequencing provides a powerful approach that allows rapid identification of miRNA expression profile even at very low level or in a small number of cells and sera. However, it should be corroborated using independent validation methods such as northern blotting or RT- qPCR (Resnick et al., 2009).

## Materials and Methods

*Sample Collection*

Serum samples were collected from 62 women: 31 patients diagnosed with ovarian carcinoma, 23 patients diagnosed with benign ovarian tumors, and 8 other control serum samples from anonymous women donated without cancer. All the serum samples were obtained following standard procedure at clinic-labs from Zhejiang Cancer Hospital, Hangzhou, Zhejiang, China. All the women provided written informed consent to participate in the study which was approved by the institutional review board. The clinical data of all patients and controls are shown in Table 1. Human blood was collected directly into serum collection tubes. The whole blood samples were allowed to stand for 1 h at room before being centrifuged at 1800 g for 10 min. The resultant serum was transferred into Eppendorf tubes. The sera were then separated and transferred into another Eppendorf tube (200 μl of serum each) on a cryostat under aseptic conditions. All the serum samples were snap frozen in liquid nitrogen and stored at -80 °C until RNA extraction. No patients had received chemotherapy or radiotherapy prior to blood collection.

*RNA Extraction and miRNA library preparation*

The frozen serum samples were homogenized in Qiagen Lysis solution using an Omni-Mixer Homogenizer. Qiagen kits (NO. 217400) are commonly used for total RNA purification, including small RNA fraction. The RNA concentration and purity of all samples were measured with NanoDrop (ND2000 spectrophotometer, USA).

Total RNA of each sample was size-fractioned on a 15% PAGE gel, and a 16-30 nt fraction was collected and extracted. The 5' RNA adapter was ligated to the RNA pool with T4 RNA ligase. Ligated RNA was size-fractionated using 15% agarose gel, and a 40-60 nt fraction excised. The 3'RNA adapter was subsequently ligated to precipitated RNA using T4 RNA ligase. Ligated RNA was size-fractionated on 10% agarose gel, and the 70-90 nt fraction (small RNA + adaptors) excised. The eight ovarian cancer samples were pooled together and the eight health control samples were also pooled together, respectively. The mixed RNAs ligated with adaptors were subjected to RT-PCR (Superscript II reverse transcriptase, 15 cycles of amplification) to produce sequencing libraries. PCR products were purified and small RNA libraries were sequenced using Solexa, a large scale parallel sequencing technology. The purified cDNA library was used for cluster generation on Illumina's Cluster Station and then sequenced on Illumina GAIIx at LC Bio-Hangzhou the following vendor's instructions for operating the instrument. Raw sequencing reads were obtained using Illumina's Sequencing Control Studio software version 2.8 (SCS v2.8) following real-time sequencing image analysis and base-calling by Illumina's

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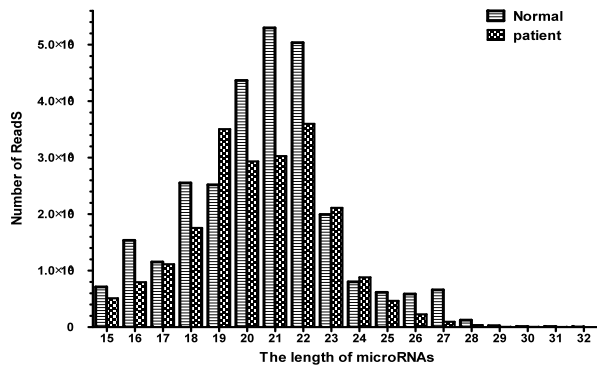


**Figure 1. Length Distribution of Reads of Normal and Patient Sample after 3ADT Cut**

**Table 2. miR-seq Data in the Normal Controls and Patients**

| samples | Raw read | Mapped reads (ratio) | Reads after filting | Mapped miRBase reads |
|---------|----------|---------------------|---------------------|----------------------|
| Normal | 31203799 | 27984023(89.7%) | 23578171 | 451 |
| Patients | 24607070 | 20965062(85.2%) | 17942497 | 767 |

Real-Time Analysis version 1.8.70 (RTA v1.8.70).

*MicroRNA Quantification by Real-Time PCR.*

The four microRNAs (miR-22, miR-93, miR-106b, miR-451) were selected by the expression for reverse transcription PCR analysis in serum samples. Mature miRNA-specific primers, containing stem-loop primers, were designed by Primer 5.0. Each reaction was performed in a final volume of 5 μl containing 2.5 μl of total RNA, 10 mM of each dNTPs (Takara), 5 U/μl of M-MLV reverse transcriptase (Takara), 1 U/μl of RNase Inhibitor (Takara), and 0.25 μl of each stem-looped primer. The mixture was incubated at 42 °C for 60 minutes, and 70 °C for 15 minutes. All RT reactions included no-template controls were run in a PCR S1000 Thermocycler (Bio-Rad).

MicroRNA quantification was performed by SYBR green qRT-PCR array. In brief, serum RNA containing miRNA was polyadenylated by poly (A) and reverse transcribed to cDNA using miScript reverse transcript kit (Qiagen) according to the manufacture's instructions. Real-time qPCR was performed using miScript SYBR® GreenI (Takara, Code No.DRR-041A) with the manufacturer-provided miScript universal primer and the miRNA-special forward primers in ABI PRISM 7500 real time System (Applied Biosystems). MiR-16 was used as an internal standard control for normalization, and is also commonly used as reference miRNA in sera in Real-time PCR analysis. The reactions were amplified for 30 seconds at 95 °C, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 31 seconds. All reactions were run in triplicate, including no-template and no reverse transcription controls for each gene. The cycle threshold (Ct) is defined as the number of cycles required for the fluorescent signal to cross the threshold in qPCR. The fold changes of miRNA expression in each ovarian cancer serum sample relative to the average expression in normal control were calculated based on the threshold cycle (CT ) value using the following formula (Livak and Schmittgen, 2001): RQ = $2^{-\Delta\Delta CT}$

Where $\Delta\Delta CT = (C_{T\,(miRNA)} - C_{T\,(miR-16)})_{tumor} - (C_{T\,(miRNA)} - C_{T\,(miR-16)})_{mean\,normal}$

## Results

*Solexa sequencing of miRNAs from the serum of the ovarian cancer patients*

In the key miRNA screening step, Solexa sequencing-based miRNA expression profiling was performed to identify differential expression patterns of miRNAs from the sera of the patients and sera of the healthy control group (Garmire and Subramaniam, 2012).

We obtained raw data by sequencing eight small RNA pools of ovarian cancer patients' serum and healthy serum as control. The low quality reads were filtered according to base quality value. We trimmed the adaptor sequence at the 3' primer terminus and cleaned up 5'adaptor contaminants formed by ligation, and finally collected the small RNAs and analyzed size distribution. From the patient samples, 24,607,070 effective reads were obtained and 31,203,799 effective reads were obtained from the normal controls. After filtration by rRNA, tRNA, snRNA, and snoRNA, the remaining effective reads were mapped to miRNA Precursor Library-Human. From the patients and normal controls, 17,942,497 and 23,587,171 reads were obtained, respectively (Table 2). In these reads, the most abundant length was the 22 nt size class in both cancerous and normal serum samples, and the percentages were 17.1% and 18%, respectively (Figure 1). Compared to the miRBase (17.0), 451 precursor miRNA out of 1733 known miRNAs were identified in patients' samples, and 767 miRNAs in normal control samples. To study the differential expression profiles of the serum miRNAs in patient samples and control samples, fold-change of the reads of sequenced miRNAs was employed. Using 2-fold expression difference as the cut off level, 11 up-regulated miRNAs and 19 down-regulated miRNA were identified (Garmire and Subramaniam, 2012).

*Validation of Key miRNA expression in Serum of Ovarian Cancer Patients*

Known miRNA expression files in patients and normal control samples were compared to determine the differentially expressed miRNAs. The expression of miRNAs in paired samples was shown by calculating the log2 Ratio (control/patient). The procedures are as follows: (1) Normalize the expression of miRNAs in two samples (control versus patient) to get the expression of transcript per million (TPM). Normalized expression = (Actual miRNA count/ Total count of clean reads)*1000000. (2) Calculate fold-change and p value from the normalized expression. Then calculate fold-change = log2 Ratio (control/patient).

*MiRNA expression and clinical pathologic features of ovarian cancer*

The differences of miRNAs expression in sera from normal control and from ovarian cancer patients were analyzed. MiR-22 and miR-93 were consistently up-regulated more than 2-fold in cancer sera compared with the normal control sera. MiR-106b (*P*=0.000) and miR-
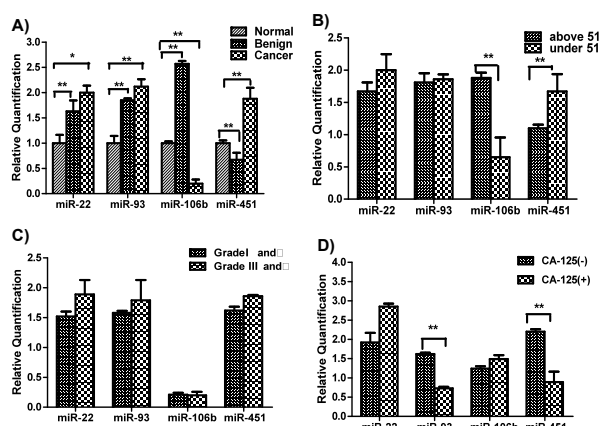
**Figure 2. Correlation of Biopathologic Features of Invasive Ovarian Cancer with the Relative Expression Level of Four Selected miRNA in Sera**

451 (*P*=0.000) were significantly differently expressed between cancer sera and normal sera. In benign tumor patient sera, miR-22, miR-93, miR-106b, and miR-451 increased in relative expression to 1.63 fold, 1.85 fold, 2.57 fold and 0.67 fold of the normal controls, respectively (Figure 2A).

The miRNAs expression level was examined for different ages of the patients. The median age of the patients was 51 years (range 23-76), and two groups (<51 and >51) were analyzed. As the age of the patients increased, miR-106b expression was significantly lower (*P*=0.003), but miR-451 expression was significantly higher (*P*=0.007) (Figure 2B). Cancer patient sera were also analyzed based on CA125 level (<35 U/ml and >35 U/ml). MiR-106b and miR-93 expression were both very significantly down-regulated in the patient sera with higher CA125 level (*P* < 0.01) (Figure 2C). The relative expression of miR-22, miR-93 and miR-106b also increased in sera of higher malignancy patients. The higher tumor grades (grade III and IV) showed higher expression of these miRNAs. However, no significant difference existed through statistical analysis (Figure 2D).

## Discussion

The development of high throughput deep sequencing technology provides the possibility of a near complete view of miRNA profiles (Rathjen, et al 2009). These data highlight the potential of a new large scale parallel sequencing strategy to profile miRNA expression in malignant tumors. The present results provide experimental evidence supporting the authenticity of 30 miRNAs with abnormal expression in ovarian cancer serum, including 11 up-regulations and 19 down-regulations. The expression of four miRNAs (miR-22, miR-93, miR-106b, miR-451) were identified which were significantly different in the sera of ovarian cancer patients compared with the healthy serum. These specific miRNAs may serve as noninvasive markers for ovarian cancer detection. Mitchell et al. have demonstrated that miRNAs are very stable in plasma and serum, which are protected from RNase and remain stable even in harsh conditions (Mitchell et al., 2009). Therefore, their stability makes miRNA expression well

suited for testing in samples. Furthermore, because of the simplicity and reproducibility of obtaining blood samples, these noninvasive and easily detectable biomarkers may have a great potential in cancer therapy and prognosis. Nam et al. have reported that the expression levels of some microRNAs were correlated with the survival of patients with serious ovarian carcinoma, and higher expression of miR-93 was significantly correlated with a poor prognosis (*P*<0.05) (Kurman et al., 2008). In additional, Fu et al. (2012) utilized microRNA array and real-time PCR to show that miR-93 was significantly up-regulated, and acted as a regulator of PTEN/Akt signaling pathway, in regulation of chemotherapeutic drug cisplatin chemosensitivity in ovarian cancer cells. MiR-451 was reported to be dysregulated in several kinds of diseases, such as polycythemia vera, stomach, leukemia, non-small-cell lung carcinoma. It was reported by Van Jaarsveld et al. that miR-451 may regulate the expression of MDR1 (*P*-glycoprotein), a drug transport protein implicated in paclitaxel resistance in ovarian cancer (Van Jaarsveld et al., 2010). In addition, down-regulation of miR-451 was associated with a worsening prognosis of the disease. Recently it has been shown that miR-22 is aberrantly expressed in several types of cancers, and it correlated with a tumor- suppressing pathway. It was reported that miR-22 may be involved in inhibiting ovarian cancer metastasis [30]. Increased expression of miR-22 may repress the protein level of ezrin, indicating that miR-22 may bear a potential role in inhibiting ovarian cancer metastasis in an ezrin-mediated way (Li et al., 2010).

MicroRNAs display aberrant expression patterns and functional abnormalities in several types of human cancer (Liu et al., 2012). The challenge for the future is to define the function and the potential targets of these miRNAs by using bioinformatics as well as genetics (Wan et al., 2012). A profound understanding of the expression, processing, and action of miRNAs may enable the development of more general methods to direct the regulation of specific gene targets and may also lead to new ways of detecting ovarian cancer.

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