Plasma miR-1273g-3p acts as a potential biomarker for early Breast Ductal Cancer diagnosis

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Abstract: Circulating miRNAs presenting in plasma in a stable manner have been demonstrated their potential role as a promising biomarkers in many human diseases, such as Alzheimer’s disease, melanoma and ovarian carcinoma. However, few circulating miRNAs could be used for breast ductal cancer diagnosis. Here, we identified miR-1273g-3p as a biomarker for detecting breast ductal cancer. We detected miR-1273g-3p levels in the plasma of 39 sporadic breast ductal cancer patients and 40 healthy donors by Stem-loop Quantitative Real-time PCR (qRT-PCR). The results showed the plasma miR-1273g-3p level were significantly up-regulated in breast ductal cancer patients compared with healthy donors (p=0.0139). Receiver operating characteristic (ROC) curve also revealed the significantly diagnostic ability of miR-1273g-3p in patients (p=0.0414). In addition, the plasma level of miR-1273g-3p was closely related to IIIB-IIIC TNM stage. We also confirmed the higher expression level of miR-1273g-3p in breast cancer cell lines MCF-7 (4.872±0.537) than normal breast cells (Hs 578Bst). Taken together, miR-1273g-3p could represent as a potential biomarker for early breast ductal cancer diagnosis.

Key words: breast ductal carcinoma, plasma, miR-1273g-3p, circulating miRNA, biomarker

INTRODUCTION

Breast cancer is the most frequently diagnosed cancer in any of the five continents (Bidoli et al. 2019). A higher frequency of early onset female breast cancer has been observed in low income countries than in high income countries (Bidoli et al. 2019), such as some Arab or Asian countries. Chinese Cancer Society estimated that the incidence and mortality of female breast cancer in china is increasing from 2000 to 2011, especially in women younger than 45 years (Chen et al. 2016). Furthermore, breast cancer is also the leading cause of death in women younger than 45 years (Chen et al. 2016). The high mortality rate of breast cancer is due to rapid cancer cells proliferation and a great degree of malignancy, which has not been early detected and treated. Early detection of breast cancer can reduce the high mortality rate and improve treatment outcomes (Chatterjee and Zetter 2005). Conventional diagnostic method (including gold standard, mammography) is not adequately sensitive for early tumor detection (Torre et al. 2015, Bleyer & Welch 2012, Weigelt et al. 2010, Kalinich & Haber 2018). Therefore, finding new diagnostic methods, such as identification of plasma biomarkers of breast cancer could serve as non-invasive biomarkers to prolong patient survival.
Currently, small non-coding RNAs (microRNAs) have opened a new avenue for early cancer diagnosis (Calin & Croce 2006). MicroRNAs (miRNAs) are short, 18-24 nucleotide noncoding RNAs, which could regulate gene expression by binding to 3’-untranslated region (3’-UTR) of messenger RNAs (mRNAs) (Eulalio et al. 2008) and protein-coding exons regions (Forman et al. 2008, Haussler et al. 2009, Hendrickson et al. 2009) leading to the inhibition of mRNAs translation or mRNAs degradation. MiRNAs are involved in a broad range of biological processes including cell proliferation, development, apoptosis, and cancer progress as well (Hamam et al. 2017). Recent studies have reported that miRNAs were released into the blood circulation by various cell physiological events under both normal and pathological conditions (Chen et al. 2012). Compared with normal donors, the expression profiles of miRNAs in malignant tumors are different, and different stage of tumors have different expression profiles. The miRNAs that released into plasma or serum can be protected from endogenous RNase activity (Schwarzenbach 2017, Schwarzenbach et al. 2014). Moreover, plasma miRNA profiles of cancer with high sensitivity and specificity could reflect disease development, tumor load, malignant progression towards metastasis (Calin et al. 2004). Several studies have demonstrated that circulating miRNAs in plasma or serum are stable and detectable, and the levels of circulating miRNAs specifically elevated in the patients with lung cancer (Cho 2012), colorectal cancer (Yuan et al. 2017), gastric cancer, (Konishi et al. 2012) breast cancer (Hannafon et al. 2016, Cuk et al. 2013). Therefore, plasma miRNAs are promising biomarkers for the early and minimally invasive diagnosis of breast ductal cancer (Mitchell et al. 2008). The assessment of plasma miRNAs disregulation may serve as an additional tool for breast cancer detection following by mammography screening and core needle biopsy.

To date, several studies explored circulating miRNAs as breast cancer indicators (Li et al. 2018, Bertoli et al. 2015). However, the correlations between circulating miR-1273g-3p levels and breast cancer have not been identified. In this study, we detected that the plasma miR-1273g-3p level were significantly up-regulated in breast ductal cancer patients compared with healthy donors (p=0.0139). Receiver operating characteristic (ROC) curve also revealed the significantly diagnostic ability of miR-1273g-3p in patients (p=0.0414). In addition, the plasma level of miR-1273g-3p was closely related to IIB-IIIC TNM stage. We also confirmed the higher expression level of miR-1273g-3p in breast cancer cell lines MCF-7 (4.872±0.537) than normal breast cells (Hs 578Bst). Taken together, miR-1273g-3p could represent as a promising candidate biomarker for early breast ductal cancer diagnosis.

MATERIALS AND METHODS

Study population

Blood samples were collected between 2015 and 2016 from patient at first clinic diagnosis from Gansu Province Cancer Hospital. All patients (n=39) and healthy controls (n=40) were Han women from Gansu province, China. All the patients had never got any surgery, radiation or systemic therapeutic procedures. For patients, the surgical resection tumor specimens were confirmed through histopathologic analysis and the tumor stage was determined according to the tumor-node-metastasis (TNM) system. Healthy control blood samples were collected from healthy women donors with no history of malignant diseases, no blood donations received in the previous 3 years and no current inflammatory condition. Patients’ clinical
outcomes were listed in Table I. This study
was approved by the Ethical Committee (the
certificate number: A201503180009, Date: 03-
18-2015) of Gansu Province Cancer Hospital in
Lan Zhou, China. Written informed consent was
obtained from all participants involved in this
study.

Plasma samples
Blood samples were withdrawn in EDTA tubes
and processed within 2 hours. Plasma were
separated after twice centrifugation at 4 °C
(1000g for 10 minutes and 12000g for 10 minutes)
and stored at -80 °C.

RNA extraction
Total RNA containing small RNA was extracted
from 400µl plasma using miRVana™ RNA isolation
Kit AM1561 (Invitrogen, ThermoFisher scientific,
CA, USA) according to the manufacturer’s
instructions with a minor modification: 25 fmol
of a C. elegans miR-39 (Cel-miR-39) was spiked-
in of validation participants and eluted with 30
µl of nuclease-free water. The concentration of
all RNA samples were quantified by NanoDrop
ND-2000 (Thermo scientific, CA, USA).

MiRNA quantification
Stem-loop Quantitative Real-time PCR (qRT-
PCR) assay was used for miRNAs validation. In
brief, miRNAs were transcribed to cDNA using the
SuperScript™ III Reverse Transcriptase kit (Invitrogen, ThermoFisher scientific, CA, USA) and Bulge-loop™ miRNA RT primer (Bulge-
loop™ miRNA qRT-PCR Primer Sets: one specific
RT primer and a pair of qPCR primers for each set) specific for miRNA purchased from Ribobio
(Guangzhou Ribobio Co., Guangzhou, China).
Then, qRT-PCR was performed on Quantstudio™
12k Flex Real-Time PCR system (Applied Biosystems, ThermoFisher scientific, CA, USA).
The reaction was performed at 95 °C for 2 min,
followed by 40 cycles at 95 °C for 15 s, 60 °C for
30 s and 72 °C 15 s, and then ramped from 65 °C
to 95 °C to obtain the melting curve. Each sample
was run in duplicates for analysis. The relative
expression level of miRNA was normalized to
Cel-miR-39, and fold change of miRNA was
calculated by the 2^ΔΔCt method.

Target prediction
The potential target genes of miR-1273g-3p were
predicted by PicTar (http://pictar.mdc-berlin.
de/cgi-bin/PicTar_vertebrate.cgi), TargetScan7.1
(http://www.targetscan.org/) and microT-
CDS (http://www.microrna.gr/microT-CDS).
Considering that the predictn programs often
suffer from high false positive rates, only the
target genes predicted by both tools were taken
into account.

Gene ontology (GO) term analysis of target
genes
In order to infer the potential functions of the
differentially expressed miRNAs, the functional
analysis of their target genes was analyzed
by Shanghai OE Biotech Technology Co, Ltd.
(Shanghai, China). Functional categories were
clustered using the Functional Annotation
Clustering tool, and representative GO categories and KEGG pathways from each clustered set with a p-value<0.05 were selected and taken into
consideration for further analysis.

Cell culture
Cell lines were purchased from ATCC. Hs
578Bst is breast normal cell. MDA-MB-231, MCF-7, T47-D, ZR-75-1 are breast cancer cell. Hs
578Bst and MDA-MB-231 were cultured in RPMI
complete medium (RPMI/10% FCS/1% Sodium
pyruvate/2mM L-glutamine/PenStrep). MCF-7,
T47-D, ZR-75-1 were grown in DMEM complete
medium (DMEM/10% FCS/2mM L-glutamine/
PenStrep). Cells were maintained in an incubator with 5% CO$_2$, 21% O$_2$ at 37 °C.

**Statistical Analysis**

All statistical calculations were performed by GraphPad PRISM 7 software (GraphPad Software, CA, USA). The significance of plasma miRNA levels was determined by Mann-Whitney test, $\chi^2$-test or Kruskal–Wallis test where appropriate. ROC curve were established for determine the diagnostic value of miRNAs. All $p$-values are two-tailed and less than 0.05 was considered to be statistically significant.

**RESULTS**

**Clinical characteristics of patients**

The clinical characteristics and the pathology of the breast ductal cancer patients were summarized in Table I. Plasma samples from 39 patients and 40 healthy controls were used for this study. There were no significant differences in age between patients and healthy controls (49.91±9.795 vs 49.23±8.529, $p=0.7369$). All cases were histologically confirmed as ductal carcinoma of the breast with a tumor size ranging from 0.15 to 4.0 cm. Of the 39 patients...
with tumor ≤2 cm were 23.08% (n=9) and tumor >2 cm were 76.92% (n=30). For TNM stage in 39 patients, 33.33% were TNM stage I-IIa (n=13), 51.28% TNM stage IIB-IIIA (n=20) and 15.39% TNM stage IIIB-IIIC (n=6). 13 patients were ER-negative (33.33%) and 26 patients were ER-positive (66.67%). 21 patients were PR-negative (53.85%) and 18 patients were PR-positive (46.15%). 31 patients were Her2-negative (79.49%) and 8 patients were Her2-positive (20.51%). 8 patients were Ki67-negative (20.51%) and 31 patients were Ki67-positive (79.49%). 21 patients were P53-negative (53.85%) and 18 patients were P53-positive (46.15%). 20 patients were lymph node metastasis-negative (51.28%) and 19 patients were lymph node metastasis-negative-positive (48.72%).

Validation of miRNAs in Breast Ductal Cancer plasma
To identify the miRNAs candidates as biomarker in breast ductal cancer plasma, plasma levels of miR-21-5p and miR-1273g-3p were measured by Stem-loop qRT-PCR assays. The results showed that miR-21-5p (p=0.0043) and miR-1273g-3p (p=0.0139) in the plasma of patients had higher expression than healthy control (Fig. 1a).

Evaluate the diagnostic value for miRNAs
To further verify the discriminating power of two miRNAs for breast ductal cancer diagnosis, receiver operating characteristic (ROC) curve analysis was performed. The area under curve (AUC) closer to 1 reflected more substantial differences between breast cancer and healthy controls.

Figure 1. Validation of miRNAs in breast ductal cancer and healthy controls (n = 79). (a) Box plot of plasma levels of miR-21-5p and miR-1273g-3p in breast cancer ductal patients. The expression of miRNAs was normalised to cel-miR-39. The lines inside the boxes represent the medians. The boxes mark the interval between the 25th and 75th percentiles. The whiskers denote the interval between the 10th and 90th percentiles. Statistically significant differences were determined using Mann–Whitney tests. (b) Receiver-operator characteristics (ROC) curve analysis of miRNAs to discriminate BC patients from healthy controls. *p<0.05.
control. ROC analysis of miR-21-5p (AUC=0.6583, 95% CI: 0.5345-0.7822, \(p=0.0154\)) and miR-1273g-3p (AUC=0.6333, 95% CI: 0.5114-0.7552, \(p=0.0414\)) were shown in Fig. 1b. Although miR-21-5p and miR-1273g-3p alone generated satisfactory ROC values, the AUC value was not elevated when the two miRNAs were combined (Fig. 1b).

The relationship between plasma miRNAs levels and clinical features

To determine if two plasma miRNAs level were associated with clinical features, patients were stratified (Table II). We found there were no differences of two miRNA levels among tumor size, ER status, PR status, Her2 status, Ki67, P53 and Lymph node metastasis. The plasma levels of miR-21-5p, miR-1273g-3p in breast ductal cancer patients at different TNM stages were also evaluated to determine if the plasma miRNAs could be detected in breast ductal cancer (Fig. 2a). Patients with high miR-21-5p expression were associated with high risk of breast malignancy compared to those with low miR-21-5p expression profiles (Stage IIb-Illa vs healthy control, \(p=0.0012\); Stage IIIb-IIIC vs healthy control, \(p=0.0168\), stage IIb-Illa vs

Table II. Correlation of the expression of plasma miRNA with clinicopathologic features.

| Clinicopathologic features | Relative expression of miR-21-5p | \(p\)-value | Relative expression of miR-1273g-3p | \(p\)-value |
|---------------------------|---------------------------------|----------|-----------------------------------|----------|
| Tumor size (cm)\(c\)      |                                 |          |                                   |          |
| \(\leq 2\) cm              | 1.564(0.9175-5.742)              | 0.5660   | 5.236(0.1916-241)                 | 0.8829   |
| >2 cm                      | 1.465(0.7072-8.103)              | 0.9883   | 3.159(0.3694-64.21)               | 0.8716   |
| TNM stage                  |                                 |          |                                   |          |
| I-IIa                      | 0.8423(0.3855-2.035)             | 0.0188   | 0.9511(0.241-4.112)               | 0.1212   |
| IIb-IIIa                   | 1.823(1.237-10.110)              | 0.4944   | 4.488(0.4059-80.34)               | 0.6061   |
| IIIb-IIIc                  | 7.519(0.9565-23.13)              | 0.9883   | 113.3(2.538-837.0)                | 0.0172   |
| ER status                  |                                 |          |                                   |          |
| Negative                   | 1.518(0.3855-12.5200)            | 0.9883   | 3.368(0.1242-466)                 | 0.8716   |
| Positive                   | 1.502(0.8433-5.318)              | 0.9883   | 3.246(0.6003-17.52)               | 0.8716   |
| PR status                  |                                 |          |                                   |          |
| Negative                   | 0.4944(0.6144-6.4990)            | 0.4944   | 2.949(0.241-34.03)                | 0.0172   |
| Positive                   | 1.823(0.8433-7.72)               | 0.4944   | 5.5(0.3694-99.77)                 | 0.8716   |
| Her-2                      |                                 |          |                                   |          |
| Negative                   | 1.564(0.8445-6.59)               | 0.2087   | 3.74(0.7432-56.15)                | 0.0172   |
| Positive                   | 0.737(0.3098-10.34)              | 0.2087   | 0.4186(0.07582-255)               | 0.0172   |
| Ki67                       |                                 |          |                                   |          |
| <14%                       | 3.267(0.9761-6.972)              | 0.5730   | 9.415(1.689-108.9)                | 0.3283   |
| >14%                       | 1.412(0.8423-5.899)              | 0.7487   | 1.718(0.3163-56.15)               | 0.1925   |
| P53                        |                                 |          |                                   |          |
| Negative                   | 1.412(0.8327-5.397)              | 0.7487   | 1.269(0.2169-34.1)                | 0.1925   |
| Positive                   | 1.541(0.7439-12.2)               | 0.7487   | 7.199(0.9798-120)                 | 0.1925   |
| Lymph node metastasis      |                                 |          |                                   |          |
| Negative                   | 1.406(0.8426-4.297)              | 0.3653   | 3.456(0.4579-45.13)               | >0.9999  |
| Positive                   | 2.295(0.7801-11.11)              | 0.3653   | 2.949(0.1657-88.4)                | >0.9999  |

\(\text{aMedian of relative expression, with 25th-75th percentile in parentheses. b}p<0.05\) was considered significant (Mann-Whitney U test between 2 groups and Kruskall-Wallis test for 3 groups). \(\text{cMaximal tumor diameter.}\)
stage I-IIa, $p=0.0127$, stage IIIb-IIIc vs stage I-IIa, $p=0.0365$). While compared stage I-IIa patient with healthy control, miR-21-5p expression showed no significant difference ($p=0.5741$). The plasma miR-1273g-3p showed marked difference when stage IIIb-IIIc compare with healthy control ($p=0.0392$) (Fig. 2b).

**Gene ontology (GO) term analysis of miR-1273g-3p**

To address how functions and pathways could be affected by the complex interactions between the miRNAs and their target genes, we inferred the functions of miRNAs from their target genes. The functional analysis showed that miR-1273g-3p target genes were involved in a variety of positive regulation processes, such as protein complex assembly, phosphatidylinositol-mediated signaling, regulation of gene expression, Wnt signaling pathway, regulation of transcription and DNA-templated (Fig. 3a). Further analysis found that the target genes of miR-1273g-3p were enriched in the pathways related to cancer, Hippo signaling pathway (Fig. 3b). These results indicate that miR-1273g-3p may play a key role in carcinogenesis by regulating various biological processes. For detailed functional analysis of the miRNA in breast ductal cancer will be studied in the future.

**Cell expression of miR-1273g-3p**

To test the level of miR-1273g-3p expression, we examined the miRNA expression in MCF-7, T47-D, ZR-75-1 and MDA-MB-231 cells. The results revealed relative high expression of miR-1273g-3p in MCF-7 (4.872±0.537) and in ZR-75-1 (1.551±0.410) cell, but low expression in T47-D (0.678±0.166) and in MDA-MB-231 (0.835±0.417) when compare with Hs 578Bst breast normal cell (Fig. 4).

**DISCUSSION**

According to the data from American Cancer Society, an estimated 252,710 cases of invasive breast cancer and an additional 63,420 new cases of in situ lesions of the breast were diagnosed in 2017, furthermore among them 40,610 patients would die from breast cancer (Siegel et al. 2017). In most cases, breast cancer is diagnosed after the symptoms, but in many cases the disease is already in advanced clinical stage when symptoms occur. Thus, early diagnosis plays a
very important role to improve the survival rate and life quality for breast cancer patients. When early stage of cancer, there are many biological changes appearing in the body, some genes are over-expressed, while others are under-expressed. Therefore, identifying differentially expressed genes as an effective biological marker, which, the expression of these genes is stable and reliable, for early diagnosis of cancer is a promising direction for current cancer research.

MiRNAs is a class of small non-coding RNAs containing 22 nucleotides, which generally negatively regulate gene expression at the post-transcription stage. Recent studies revealed that miRNAs could have effect on cancer related processes, such as cell proliferation, differentiation and apoptosis(Jansson and Lund 2012). Consequently, many researchers using circulating miRNAs as cancer biomarkers are being carried out widely. In recent years, there is a great deal of studies demonstrated the genetic association between miRNAs and cancer, including lung cancer (Hu et al. 2016) colorectal cancer (Schetter and Harris 2009) and breast cancer(Takahashi et al. 2015, Kurozumi et al. 2017). Several previous studies have demonstrated the value of plasma/serum miRNA in breast cancer diagnosis (Lagendijk et al. 2018, Khodadadi-Jamayran et al. 2018, Li et al. 2017, Souza et al. 2019). MiR-21 is involved in regulating the expression of multiple tumor suppressor genes, and plays a crucial role in the occurrence and development of a variety of diseases (Folini et al. 2010, Peralta-Zaragoza et al. 2016). Matamala et al. (2015) reported that miR-21 had significantly higher expression in circulating of breast carcinoma patients compared to healthy donors. A plurality of studies have shown that up-regulation of miR-21 was detected in blood (Alunni-Fabbro et al. 2018), and that plasma miR-21 could be used as a non-invasive biomarker for breast cancer diagnosis, as well as an indicator for tumor invasiveness (Hannafon et al. 2016). Our results were consist with previous study. Therefore, miR-21 is a promising biomarker for breast cancer diagnosis in clinics. However, breast cancer is a very complicated disease according to their clinical characteristics. Identifying new miRNAs as biomarkers are urgent need for breast cancer diagnosis. In our study, we found miR-1273g-3p is an important regulator in breast cancer cell proliferation (data not shown), and we also focus on the capacity of miR-1273g-3p as biomarker.

Currently, little is known about the functional role of miR-1273g-3p in breast cancer. MiR-1273g-3p located on chromosome 1 at 1p32.3, is a member of the miR-1273 family. MiR-1273 consists of miR-1273a, miR-1273c, miR-1273d, miR-1273e, miR-1273f, miR-1273g-3p, miR-1273g-5p, miR-1273h-3p and miR-1273h-5p (Ivashchenko et al. 2014). Guo and his colleagues found that miR-1273g-3p participates in acute glucose fluctuation-induced autophagy, dysfunction, and proliferation attenuation in human umbilical vein endothelial cells (Guo et al. 2016). Sahu and his colleagues found that miR-1273g-3p had a relationship with megakaryocyte differentiation (Sahu et al. 2018). Niu et al. (2016) found that miR-1273g-3p was the most significantly up-regulated miRNA and correlated with the stage of HCV-related liver fibrosis. Fang et al. using next generation sequencing technology detected that miRNA1273g/miRNA382 was up regulated in breast cancer plasma (Fang et al. 2018).

In this study, we identify miR-1273g-3p and miR-21-5p as a potential biomarker for breast cancer diagnosis by Stem-loop Quantitative Real-time PCR (qRT-PCR). Our results also confirmed the plasma miR-1273g-3p level were significantly up-regulated in breast ductal cancer patients compared with healthy donors ($p=0.0139$). However, miR-21-5p has been
confirmed as a robust biomarker by many groups. It suggested that miR-1273g-3p has the same ability as miR-21-5p to distinct breast cancer from healthy person. Therefore, miR-1273g-3p could be a very promising candidate for breast ductal cancer diagnosis. However, the detection efficiency of breast cancer was not enhanced when we combined the two miRNAs into diagnostic panles.

Some researchers found that hsa-miR-1273g-3p regulates several significant genes (Niu et al. 2016, Gao et al. 2019). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) was known as a tumor suppressor gene and are low expressed in hepatocellular cancer (Qian et al. 2017), gastric cancer (Zhang et al. 2017), and breast cancer (Gao et al. 2019). Over-expression of miR-1273g-3p could inhibit translation of PTEN, increase the expression of

![Figure 3. Biological Process GO terms (a) and KEGG pathways (b) enriched in the target genes of miR-1273g-3p.](image)

![Figure 4. Relative Expression of miR-1273g-3p in breast cancer cell lines.*** p<0.001.](image)
α-SMA, Col1A1, and reduce apoptosis in HSCs. As PTEN can modulated tumor progression via PI3K/AKT pathway is tightly correlated with breast cancer aggressiveness, stage and prognosis (Gao et al. 2019). Here, we make a preliminary infer that hsa-miR-1273g-3p maybe have effect on tumorigenesis and progress of breast cancer by regulating the expression of PTEN. (Figure S1 - Supplementary Material).

In conclusion, we demonstrated that miR-1273g-3p is over-expressed and may contribute to promoting progression of breast cancer, the miR-1273g-3p may have potential utility as both prognostic and diagnostic biomarker.

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SUPPLEMENTARY MATERIAL

Figure S1. Relative expression of miR-1273g-3p and target gene PTEN.

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