Overexpression of circulating miRNA-21 and miRNA-146a in plasma samples of breast cancer patients

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Breast cancer is the second most common malignant disease affecting Indian women and is the leading cause of cancer-related mortality. MicroRNAs (miRNAs) are remarkably stable in blood, which makes them novel and promising biomarkers for cancer detection and diagnosis. In this study, we investigated whether expression levels of circulating specific miRNAs, such as microRNA-21 (miR-21) and microRNA-146a (miR-146a) could be used as potential biomarkers in plasma of breast cancer patients and healthy individuals. We compared the expression levels of breast cancer associated specific miRNAs — miR-21 and miR-146a in plasma samples of histopathologically reported 14 patients (aged 35-61 yrs) with breast cancer and an additional 8 healthy volunteers (aged 35-54 yrs). The miRNA expression level was determined by TaqMan quantitative PCR (qPCR) assay. The miRNA expression level of each sample was normalized to that of miR-16 as reference and expressed as relative expression (2^{-∆Ct}). Our results showed that the circulating level of miR-21 and miR-146a were significantly higher in plasma samples of breast cancer patients, when compared to those of healthy controls (p<0.0004 and p<0.005, respectively). Thus, analyzing expression of miR-21 and miR-146a from plasma samples of breast cancer patients might be useful in the diagnosis of breast cancer. However, further studies with larger number of patients and control individuals are needed to confirm the results.

Keywords: Breast cancer, Biomarkers, MicroRNAs, miR-21, miR-146a, Plasma

Breast cancer is an increasingly serious public health problem in developing countries. It is becoming the most common form of cancer in Indian women. Incidence of breast cancer is constantly increasing than that of cervical cancer and it is becoming the most common cancer in women in India1-3. In 2008, 80 new cases of breast cancer per 100,000 population have been recorded3. Thus, the development of newer blood/serum based biomarkers is important as alternative to traditional cancer diagnosis methods4.

Recently, it is reported that miRNAs are remarkably stable in blood stream against endogenous RNase activity and hence can act as novel promising blood-based biomarkers for cancer detection and diagnosis5,6. miRNAs comprise a large family consisting of small (~21-23 nt in size) regulatory non-coding, single-stranded RNA molecules that regulate the multiple modes of gene expression by translational inhibition, increasing mRNA deadenylation and degradation and/or mRNA sequestration7. They play an important role in a broad range of cellular physiological and pathological processes8. In addition, they play a role in almost all aspects of cancer biology, such as cell proliferation, apoptosis, invasion/metastasis and cell cycle regulation9.

The expression profiles of miRNAs are found to be tissue-/cell-specific, which is directly reflective of various pathophysiological processes and thus indicating the different stages of cancer10,11. Based on the elevated level of miRNAs in body fluids, blood-based diagnostic tool could be developed for early detection of cancer12,13. Recently, it is has been found that tumor-associated miRNAs are present in plasma (miR-15b, miR-16, miR-24 and miR-141)5 and serum (miR-155, miR-210, miR-21 )14 of cancer patients. For example, it is reported that the ratio of miR-92a/miR-638 in plasma is a very sensitive marker for acute leukemia15. Increased level of miR-141 has been found in the serum of patients with prostate cancer in relation to healthy controls5. Therefore, circulating miRNAs are emerging as biomarkers for various diseases, including cancer16.

In the present study, we have selected two miRNAs (miR-21 and miR-146a) based on previous work that
are reported to be upregulated in breast cancer\textsuperscript{17,18}. We have attempted to analyse plasma miRNAs as possible biomarkers for the diagnosis of breast cancer. The expression levels of both miRNAs in patients with breast cancer and healthy volunteers have been determined using qPCR assay.

**Materials and Methods**

**Clinical samples**

Blood samples of 14 women patients (between 35-61 yrs age group) diagnosed with breast carcinoma and 8 healthy volunteers (between 35-54 yrs age group) without any known abnormality of the breast were collected from Gynecology and Radiation Oncology Departments of Government Mohan Kumaramangalam Medical College Hospital (GMKMCH) in Salem, India, from December 2010 to March 2011. The study was approved by the Clinical Research Ethics Committee (Ref no: 7713/MEI/2010). Prior written consent was obtained from all participants/donors for the use of their blood samples in this study.

Breast cancer of the 14 patients was diagnosed and confirmed by analysing the core biopsy samples of the patients. After surgery, the grades were recorded and analysed according to the modified Bloom-Richardson grading system. Blood samples (up to 3 ml per patient) were collected via a direct venous puncture into K2 EDTA-treated tubes (sprayed, 5.4 mg, BD Hemogard TM BD vacutainer) during the routine samplings. The anonymised samples were stored at -80°C until total RNA isolation. Samples of breast cancer patients were collected before any treatment was performed.

**Plasma separation**

From the collected blood samples, plasma was separated by centrifuging the whole blood sample at 3000 rpm for 10 min at 20°C and the supernatant was transferred into diethylpyrocarbonate (DEPC)-treated 1.5 ml microcentrifuge tubes. The supernatant was then aliquoted into eppendorf tubes and immediately frozen at -80°C until isolation of total RNA.

**Isolation of total RNA**

miRNA was isolated from 1 ml thawed plasma using mirVana\textsuperscript{TM} miRNA Isolation Kit (Ambion, USA, AM1560), following the manufacturer’s instructions. The concentration and purity of all RNA samples were analyzed using synergy H4-Multimode microplate (BioTek, US).

<table>
<thead>
<tr>
<th>miRNAs</th>
<th>Primer sequence</th>
<th>AB</th>
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</table>

**Quantitative PCR (qPCR)**

qPCR was performed using an ABI 7900 Thermal Cycler system (Applied Biosystems), miR-21 and miR-146a TaqMan MicroRNA assays. The 20 µl reaction mix contained 1.33 µl of cDNA, 7.67 µl with nuclease-free water, gene-specific primer 1 µl and 10 µl of 2x TaqMan Universal Master Mix, No AmpErase HUNG (Applied Biosystems). The components were mixed gently and PCR reaction plate was dispensed with 20 µl of complete PCR master mix and placed in Applied Biosystems 7900HT fast real-time PCR system. The reaction mixture was first incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All the reactions were performed in duplicate. The miRNA expression level in each sample was normalized by that of miR-16 as reference and expressed as relative expression (2\(-∆C_t\)). The tumor and normal relative expression data were further evaluated using the non-parametrical Mann-Whitney U test. The statistical significance was set at p<0.05.

**Results**

Expression levels of plasma miRNAs are consistent among normal individuals

Eight healthy volunteers (35-54 yrs) were randomly selected and expression levels of miR-21 and miR-146a in their plasma were analyzed by qPCR. Expression levels of plasma miRNAs among
healthy subjects were quite consistent (Figs 1 & 2). These results showed that the miRNA level was constant in different healthy volunteers. Next, we examined the expression level of miR-21 and miR-146a from the plasma of patients diagnosed with breast cancer.

miRNA expression in the plasma of breast cancer patients

Using qPCR, we found circulating miRNAs viz; miR-21 and miR-146a detectable and amplified in all samples from 14 breast cancer patients. The circulating levels of miR-21 and miR-146a were normalized to that of miR-16 as a reference and were found to be significantly elevated compared to normal controls (15-times higher, p<0.0004 and 5-times higher, p<0.0005 respectively). The significant difference in miRNA profiles between breast cancer and normal samples are summarized in Figs 1, 2 & 3.

Discussion

Optimization of plasma-based biomarkers towards early detection of breast cancer is crucial for treatment strategy. The research in search of blood based biomarkers towards breast cancer diagnosis during the past decades has resulted in limited progresses. Therefore, more organized and intensive research is needed for the early development and standardization of plasma-biomarker technique.

By using sensitive techniques, the expression levels of many miRNAs can be determined, which can be used for diagnostic purposes. The circulating miRNAs are relatively stable, very accessible, easily testable, promising and stage-specific biomarkers for non-invasive diagnosis in various tumors. Compared to the stable, reproducible and species-specific and consistent tissue or cellular miRNAs, the plasma and serum miRNAs are found to be more resistant against endogenous RNase activity. The secreted circulating miRNAs are encapsulated by membrane-bound lipid vesicles (exosomes: 50-100 nm in diameter), which protect them from the degradation against RNase digestion. Recent findings have shown that majority of the circulating miRNAs are associated with extracellular Argonaute2 (Ago2) protein complex and the complexes serve as a significant carrier of miRNAs stability in plasma. Although previous works have also reported use of miRNA as biomarker in breast cancer patients, in this study, we focused on standardizing the miRNA expression ratios in plasma of breast cancer patients and healthy volunteers as a diagnostic tool.

It is reported that miR-21 is significantly upregulated in breast cancer. Another tissue-based study has shown that miR-21, miR-365, miR-181b, let-7f, miR-155, miR-29b, miR-181d, miR-98 and miR-29c are up-regulated more than two-fold in breast cancer, when compared to normal adjacent tumor tissues. Among the nine miRNAs, miR-21 is the most significantly up-regulated, when analyzed by qPCR.
miR-21 has emerged as a key oncomir, being overexpressed in a wide range of malignant tumors. It is involved in various cancer-related processes, such as invasion, migration and metastasis. Potential target genes of miR-21 such as tropomyosin1 (TPM1), phosphatase and tensin homolog (PTEN), programmed cell death 4 (PDCD4), Maspin (mammary serine protease inhibitor), BCL-2, p53 and Ankyrin repeat domain 46 (ANKRD46) have been identified. The regulatory role of miR-146a has also been found in breast cancer tissue samples. It is reported that miR-146a is an important potential regulator targeting the genes of BRCA1 and BRCA2 in breast cancer and plays a role in metastasis. In addition, a recent study has demonstrated that miRNA-146a/b negatively regulates the NF-κB activity, when expressed in MDA-MB-231, a highly metastatic human breast cancer cell line. To the best of our knowledge, there is no other report to date about miRNA-146a expression levels from the plasma samples of breast cancer patients.

In this study, we determined the expression levels of plasma miRNAs (miR-21 and miR-146a) of patients with breast cancer and healthy volunteers by qPCR assay. We found miR-21 and miR-146a were significantly overexpressed in the plasma of breast cancer patients, when compared to control cases. Circulating miRNAs are considered to be released from cancer tissues as well as from normal tissues. Recent reports suggest that plasma miRNAs might be protected in a complex with other molecules, such as exosomes, proteins and lipids. The protection of miRNAs might be higher in breast cancer patients than that of controls. miR-21 and miR-146a exhibited increased level in the plasma samples possibly, because breast cancer cells might selectively secrete or release cellular miRNAs, such as miR-21 and miR-146a into the plasma. The importance of miR-146a in plasma of patients diagnosed with breast carcinoma was not demonstrated earlier.

Apart from their use as general breast cancer screening tool, plasma miRNA assays have two more potential clinical uses: screening high risk patients for breast cancer and monitoring breast cancer patients during the follow-up period after breast cancer treatment. These miRNA biomarkers might also be powerful in evaluating the efficacy of adjuvant therapies. The qPCR results fully supported that plasma miRNA expression profiles were changed in patients with breast cancer. Thus, elevated expression levels of miR-21 and miR-146a in plasma might be considered as blood-based markers of breast cancer, which could be easily detected by qPCR.

In conclusion, our results showed that tumour-derived miRNAs in the blood plasma might serve as an important tool for the blood-based detection of human breast cancer. However, studies with larger number of patients and healthy controls are needed to validate our findings.

Acknowledgments

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References