Circulating miR-221 and miR-222 as Potential Biomarkers for Screening of Breast Cancer

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Breast cancer is the second most common cancer in women with approximately 522,000 deaths annually worldwide. MicroRNAs have recently been studied as potential biomarkers that regulate gene expression and are involved in tumorigenesis. Here we evaluated circulating miR-221 and miR-222 as potential biomarkers for breast cancer by quantitative reverse transcription PCR using blood plasma of 30 healthy controls and 30 breast cancer patients. The TNM stage on circulating miR-221 and miR-222 was also investigated. Circulating miR-221 and miR-222 were significantly up-regulated in breast cancer patients compared to those in healthy controls (P < 0.0022 and P = 0.0058, respectively). Furthermore, the relative expression level of circulating miR-221 in patients with stage III breast cancer was higher than in those with stage I and II. Taken together, we have shown circulating miR-221 and miR-222 could be useful biomarkers for the screening of breast cancer patients.

Key Words: Breast cancer, Screening, miR-221, miR-222, Biomarker

Breast cancer is the most frequently occurring disease in females and the second leading cause of death worldwide (Siegel et al., 2018). According to the World Health Organization (WHO), approximately 522,000 women die each year from breast cancer (Ferlay et al., 2015). Current methods used to diagnose breast cancer such as mammography, computed tomography (CT), and magnetic resonance imaging (MRI) have been hindered by the cost and expertise needed. In addition, tumor serum markers such as carbohydrate antigen 15-3 (CA15-3) and carcinoembryonic antigen (CEA) are nonspecific and have limited sensitivity and specificity (Duffy, 1999; Ng et al., 2014). Therefore, it is necessary to discover new non-invasive biomarkers for the diagnosis of breast cancer.
microRNAs are small non-coding RNAs consisting of 18–25 nucleotides that play a role in regulating gene expression by binding the target messenger RNAs (mRNAs). Numerous recent studies have reported that microRNAs, which are involved in tumorigenesis, are a potential diagnosis, prognosis and therapeutic biomarker in various cancers (Achkar et al, 2016). miR-221 and miR-222 are located on the X chromosome with the same base sequence. Researchers have shown that miR-221 and miR-222 are over-expressed in breast cancer cells and function as oncomiRNAs (Garofalo et al., 2012). Previous studies also show that miR-221/222 promotes breast cancer cell proliferation, migration, and invasion by targeting PTEN, a tumor suppressor gene (Li et al., 2016). However, the clinical relevance of miR-221/222 in plasma is still poorly investigated. In this study, circulating miR-221 and miR-222 as potential biomarkers for breast cancer were investigated using the blood plasma of 30 healthy controls and 30 breast cancer patients.

A total of 30 blood samples were obtained from breast cancer patients who had been diagnosed with breast cancer at the Yonsei University Severance Hospital, Seoul, Republic of Korea from 2000 to 2015. For the healthy controls, a total of 30 blood samples were obtained from healthy donors who had never been diagnosed with breast cancer. All patients and healthy volunteers provided written consent and the study was approved by the institutional ethics committee at Yonsei University Severance Hospital (approval no. 4-2011-0011) and Yonsei University at Wonju (approval no. 1041849-201603-BR-010-04).

For separation of plasma, the whole blood samples were centrifuged for 15 min at 600 × g and stored at -80°C in the deep freezer until use. For extraction of small RNAs from the plasma of the study population, the NucleoSpin® miRNA Plasma kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's protocol.

miRNA expression was quantified by using the TaqMan® small RNA assays (Applied Biosystems by Life Technologies) with miRNA specific primers (miR-221 and miR-222) to measure the cycle threshold (Ct) - the number of PCR cycles in which fluorescence exceeds the background fluorescence value. The samples were repeated twice and the data were analyzed using the comparative ΔCt method (2^−ΔCt) with miR-16 as an endogenous control (Pfaffl et al., 2002).

We assessed the relative expression level of circulating miR-221 and 222 in 30 healthy control and 30 breast cancer patients. The characteristics of the patients are shown in Table 1. The age of the breast cancer patients ranged from 31 to 76 years old (mean ± SD, 55.03 ± 11.31 years; 95 CI, 50.73–59.34). Of 30 breast cancer patients, 10 (33.3%) were in stage I, followed by 10 (33.3%) in stage II, and 10 (33.3%) in stage III. The expression levels of circulating miR-221 and miR-222 in breast cancer patients were significantly higher than those in the healthy controls (P < 0.0022 and P = 0.0058, respectively) (Fig. 1). The mean expression levels of circulating miR-221 and miR-222 were 0.2569 (range, 0.0024 to 2.6240) and 0.0562 (range 0.0003 to 0.3530) in breast cancer patients and 0.0895 (range, 0.0011 to 0.3184) and 0.0230 (range 0.0027 to 0.0641) in healthy controls. The area under the ROC curve (AUC) of circulating miR-221 and miR-222 was 0.7267 (95% CI, 0.5983–0.8551, P = 0.0025) and 0.7056 (95% CI, 0.5683–0.8428, P = 0.0062), respectively.

Subsequently, the expression levels of circulating miR-221 and miR-222 in different stages were analyzed. There was a statistically significant difference between the miR-221 expression level of stage II and stage III and that of the healthy controls (P = 0.0279 for stage II and P = 0.0163 for stage III) (Fig. 2A). For miR-222, there also was a significant difference between stage II and the healthy controls (P = 0.0084) (Fig. 2B).

Many recent studies have reported blood-based biomarkers for diagnosing breast cancer, such as circulating tumor cells.

<table>
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<td>III</td>
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Table 1. Clinical characteristics of breast cancer patients
Fig. 1. Circulating miR-221, and miR-222 expression level in plasma of breast cancer patients and healthy controls. The relative expression of miR-221 (A), and miR-222 (B) in plasma of breast cancer patients and healthy controls was analyzed by RT-qPCR.

Fig. 2. Circulating miR-221, and miR-222 expression level according to TNM stage. The miR-221 (A) and miR-222 (B) expression levels were analyzed at different breast cancer stages.
(CTCs), circulating tumor DNA (ctDNA), exosomes, and microRNAs. Circulating miRNAs have many advantages as biomarkers, such as stability during pH changes, freeze-thaw cycles, and extended storage (Mitchell et al., 2008; Yu et al., 2011). In this study, circulating miR-221 and miR-222 were potential biomarkers showing 2.87-fold and 2.44-fold higher expression for breast cancer patients compared with healthy controls, respectively.

In this study, some healthy controls were shown high expression of miR-221 and miR-222 or some patients with stage III were shown low expression of miR-221 and miR-222. The reason for discrepancies of miR-221 and miR-222 expression in healthy controls and breast cancer patients was that microRNAs regulate multi-genes rather than a single gene. For example, miR-221/222 was associated with oncogenic process regulating tumor suppressor gene PTEN (Li et al., 2016). Also, miR-221/222 was associated with inflammatory immune response regulating peroxisome proliferator-activated receptor-γ coactivator-1α (Song et al., 2017).

There are limitations to our study. First, a small number of samples were included. Therefore, it is necessary to conduct further research using a larger number of samples from breast cancer patients at various stages to demonstrate the utility of circulating miR-221 and miR-222 for breast cancer screening and diagnosis. Second, we only included breast cancer patients with cancer stage I-III. Further study with stage IV patients is needed to investigate the expression of circulating miR-221 and miR-222 with breast cancer stage IV patients.

In conclusion, expression levels of miR-221 and miR-222 in plasma were significantly higher for breast cancer patients than for healthy controls. Notably, miR-221 in patients with stage III breast cancer was higher than in those with stage I and II. Taken together, circulating miR-221 and miR-222 can serve as potential non-invasive biomarkers for breast cancer screening and diagnosis.

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CONFLICT OF INTEREST
The authors have no conflicts of interest with regards to this study.

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