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Plasma long non-coding RNAs (lncRNAs) serve as potential biomarkers for predicting breast cancer

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Abstract. – OBJECTIVE: Recent studies have suggested that lncRNAs play important regulatory roles in occurrence and progression of many cancers including breast cancer. However, only a small number of lncRNAs have proved to be related to breast cancer. Moreover, the effect of lncRNAs on breast cancer is yet unclear. We aimed at examining whether the expression level of these lncRNAs in our breast cancer patients could be different to normal people, and whether these lncRNAs could serve as potential biomarkers for the diagnosis of breast cancer.

PATIENTS AND METHODS: We selected twelve lncRNAs as the research targets, which were previously found to be abnormally expressed in plasma of other cancers. The expression levels of these lncRNAs were measured by Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) and compared between breast cancer patients and normal people.

RESULTS: The expression levels of plasma lncRNAs (H19, HOTAIR, and RP11-445H22.4) are found to increase significantly in breast cancer patients. The expression levels of other 9 lncRNAs were no significant changed compared with normal people.

CONCLUSIONS: lncRNAs may be related to the occurrence of breast cancer and serve as potential biomarkers for its diagnosis.

Key Words:
lncRNA, Plasma, Breast cancer, Biomarkers.

Introduction

Breast cancer is one of the five prevalent cancers in China (the other four are colorectal, lung, stomach, and esophageal cancers)[1]. At present, surgical resection is still the main method to treat it. The keys to the prognosis of breast cancer are early discovery, diagnosis and treatment. After surgery treatment, the recurrence rate of breast cancer is up to 0.156% and the 5-year survival rate is less than 25%[1,2]. It is well known that early diagnosis of breast cancer could increase the 5-year survival rate, but there were no easy, non-invasive methods to diagnose breast cancer early. Therefore, the major problem that clinicians are facing today is to find a new diagnostic approach that can detect breast cancer at an early and very likely curable stage. Plasma biomarkers, which can predict prognosis, evaluate therapy response and provide selection tools for hypoxia-modifying treatments, are thus being sought to predict breast cancer earlier than current methods. Recently, some reports have shown that lncRNAs, which are present in plasma, could serve as biomarkers for liver[3], lung[4,5], lymphoma[6], gastric[7], esophageal[8] and cancers, as well as heart failure[9], kidney injury[10] and depression[11]. LncRNAs transcript are longer than 200 nucleotides and regulate the gene expression. Some of them have been shown to play important regulatory roles in cancers biology, including breast cancer[12-14]. LncRNAs are closely related to biological behaviors such as the occurrence, development, invasion and metastasis of tumor. Abnormal expressions of some lncRNAs have been found in blood samples from cancer patients[12-14], but their study for an early diagnosis of breast cancer is at an early stage and the mechanism is yet unclear. LncRNAs are attractive biomarker candidates since they can be monitored non-invasively, and a large number of lncRNAs have been found to be expressed abnormally in blood samples of cancer patients. Among of them, only a few of lncRNAs could be used in breast cancer diagnosis. Here

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we determined whether plasma lncRNAs could serve as biomarkers for metastatic breast cancer. We aimed at examining whether the expression level of these lncRNAs in our breast cancer patients could be different to normal people, and to explore whether these lncRNAs could serve as potential biomarkers for the diagnosis of breast cancer.

Patients and Methods

Patients and Plasma Collection

Volunteers (experimental group) were recruited at the time of the diagnosis among patients treated at Breast Cancer Surgical Department of Linyi First Hospital. The healthy volunteers (control group) were recruited from healthy volunteers. Breast cancer diagnoses were confirmed by a histopathological examination (Figure 1). Patients with no previous diagnosis of breast cancer or any malignant disease at the time of blood collection were enrolled. All participants were Chinese. The study was approved by the Linyi First Hospital Ethical Committee, and individual permission was obtained using standard informed consent procedures. The investigation was conformed to the principles that are outlined in the Declaration of Helsinki regarding the use of human tissues.

Quantitative Real-Time PCR (qRT-PCR) Analysis

12 lncRNAs (H19, POU3F3, HNF1A-AS1, SPRY4-IT1, hGAPDH, HOTAIR, RP11-445H22.4, ENSMUST00000119855, AK139989, AK153778, HULC, MALAT1), which were previously found to be abnormally expressed in the plasma of some cancer, were selected as candidate targets for subsequent plasma lncRNA assays. Total RNAs were extracted using TriPure Isolation Reagent (Roche, Basel, Switzerland). The first-strand cDNAs were synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA). RNA abundance was measured using ABsolute QPCR SYBR Mix (Life Technologies, Carlsbad, CA, USA) and Roche Light Cycler 480 Real-time PCR system (Roche, Basel, Switzerland). The expressions of individual genes were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene. Primers for Real-time qPCR were listed in Table I.

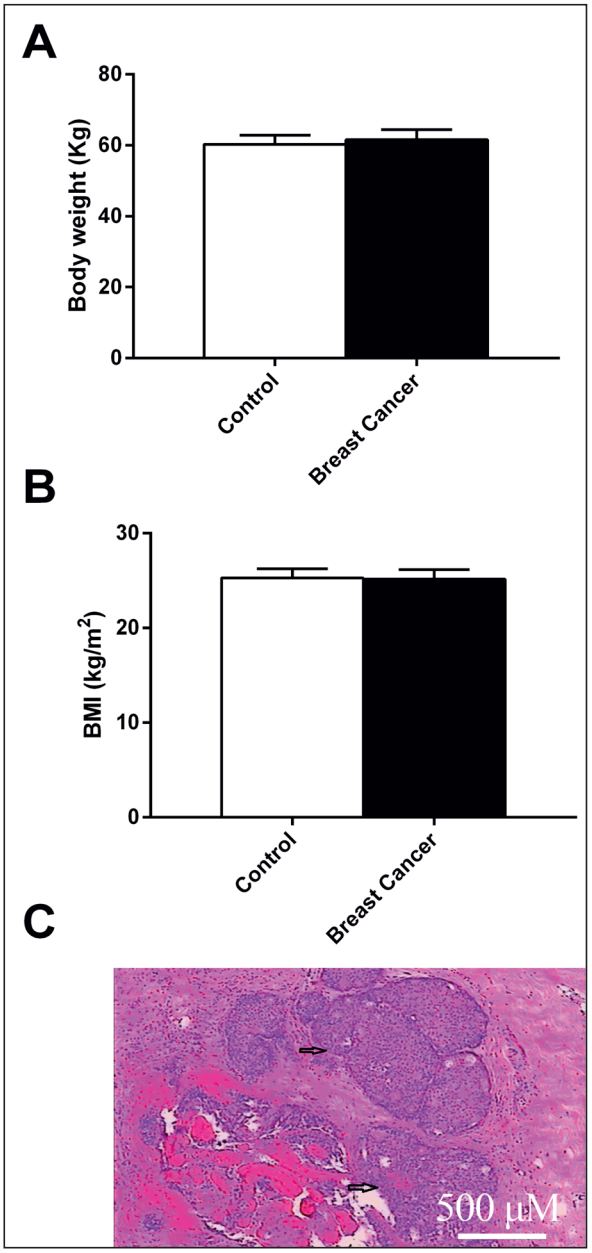

Figure 1. Characteristics of metastatic breast cancer patients. Healthy female volunteers and BC patients were chosen for this study. The body weight ***(A)*** and BMI ***(B)*** of these two groups are measured. All BC patients were confirmed by hematoxylin-eosin (HE) staining in a histopathological examination. ***(C)*** N=11-16.

Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Differences between groups were analyzed by two-tailed Student's *t*-tests. $p < 0.05$ was considered statistically significant.

Table I. Primers for qRT-PCR.

lncRNAs	Forward	Reverse
H19	ATCGGTGCCTCAGCGTTCGG	CTGTCCTCGCCGTCACACCG
POU3F3	AATCACTGCAATTGAAGGAAAAA	CCTTGTTTTCCAACCCTTAGACT
HNF1A-AS1	TCAAGAAATGGTGGCTAT	GCTCTGAGACTGGCTGAA
SPRY4-IT1	ATCCGAAGCGCAGACACAATTCA	CCTCGATGTAGTCTATGTCATAGGA
hGAPDH	CACCAGGGCTGCTTTTAACTC	GACAAGCTTCCCGTTCTCAG
HOTAIR	GGCAAATGTCAGAGGGTT	GTGTAACAGGCAGGTGGA
RP11-445H22.4	GTAAAGCCATCACCAGGACAACC	CTCCCTAACAGAAGCCCACCA
ENSMUST00000119855	GCGGAATAGAAGTTAGTGTGGAAC	TGTAGCGGAGAAGTAGCATCATC
AK139989	CCCTGTGAGAACCCGATAGAAAC	TCTGGAAGTGTATAGTGCCTGTTG
AK153778	GGCTGTTATGGTCTGGCTCTG	CCTCATTCTCCTGCCTTCATCTC
HULC	ATCTGCAAGCCAGGAAGAGTC	CTTGCTTGATGCTTTGGTCTGT
MALAT1	AAAGCAAGGTCTCCCCACAAG	GGTCTGTGCTAGATCAAAAGGCA

Results

Characteristics of Metastatic Breast Cancer Patients

Breast cancer patients and healthy female controls were chosen for this study. Body mass index (BMI) and age have been considered as risk factors for breast cancer, so we chose healthy female volunteers with similar BMIs and ages as the patients (Figure 1A-B). The presence of breast cancer in the patients was confirmed by hematoxylin and eosin (HE) staining in a histopathological examination (Figure 1C). The data showed that eight of the twelve lncRNAs were examined (AK153778, H19, RP11-445H22.4, ENSMUST0000119855, HULC, SPRY-IT1, POU3F3, HOTAIR) and their bands were clear, while the other four lncRNAs (MALAT1, AK139989, PCAT18, and HNF1A-AS1) didn't show clear bands after 40 cycles' of amplification in both healthy controls and breast cancer patients.

Selection of Metastatic Breast Cancer-Related lncRNAs

About twelve plasma lncRNAs, which have been reported to have elevated expression in human cancers, were selected (Table I). Next, we tested whether these lncRNAs were expressed in normal human plasma. Regular RT-PCR analysis showed that eight (AK153778, H19, RP11-445H22.4, ENSMUST0000119855, HULC, SPRY-IT1, POU3F3, HOTAIR) of these twelve lncRNAs had clear bands while the other four (MALAT1, AK139989, PCAT18, and HNF1A-AS1) did not show clear bands after 40 cycles' of amplification. These four lncRNAs were thus not expressed at detectable levels in plasma.

Identification of Breast Cancer Related Plasma lncRNAs

qRT-PCR analysis was performed to further study whether these eight lncRNAs could serve as biomarkers for breast cancer. As shown in Figure 2, the expression levels of H19, HOTAIR, and RP11-445H22.4 were significantly increased in the plasma of breast cancer patients compared to healthy female controls. The expression of HULC, POU3F3, ENSMUST0000119855, and AK153778 didn't change. SPRY4-IT1 expression was slightly decreased in the plasma of breast cancer patients. Therefore, from what has been discussed above, H19, RP11-445H22.4, and HOTAIR could serve as biomarkers for breast cancer and may potentially be used to diagnose breast cancer.

Discussion

lncRNAs are detectable in human plasma[7,9,10] and are remarkably stable even under ribonuclease A (RNase A) digestion[7,15]. A possible explanation is that they are packaged in exosomes, which could protect from RNase A digestion[16,17]. Recently, plasma lncRNAs have been investigated as potential diagnostic markers in several diseases including hepatic ischemia/reperfusion injury, lung cancer, B-cell neoplasm, acute kidney injury, gastric cancer, and hepatocellular carcinoma (HCC)[3-10]. For example, the expression levels of AK153778, AK139989, and ENSMUST0000119855 are significantly increased in the plasma of mice with heart failure[9]. The level of HULC is dramatically increased in HCC patients[18]. In addition, PCAT18 has been shown

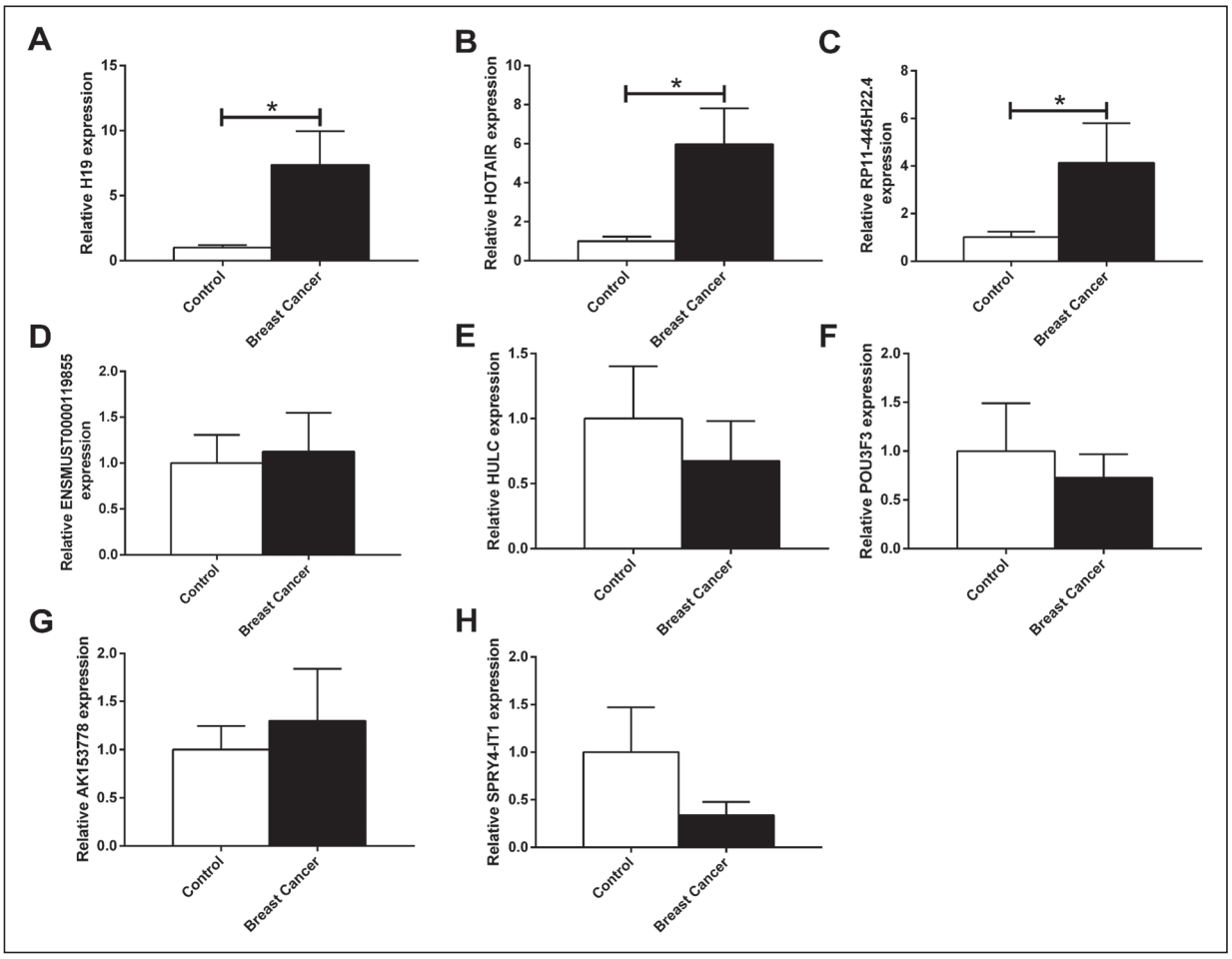

Figure 2. Identification of breast cancer related plasma lncRNAs. qRT-PCR analysis was performed to identify plasma lncRNAs which are associated with breast cancer. The relative expression levels of plasma H19 ***(A)***, HOTAIR ***(B)***, RP11-445H22.4 ***(C)***, ENSMUST0000119855 ***(D)***, HULC (E), POU3F3 ***(F)***, AK153778 ***(G)***, SPRY-IT1 ***(H)*** were measured in breast cancer patients and healthy female control by qRT-PCR. N=11-16. *$p < 0.05$.

to be upregulated in the plasma of metastatic prostate cancer patients[19]. Plasma expression levels of POU3F3, HNF1A-AS1 and SPRY4-IT1 are significantly higher in esophageal squamous cell carcinoma (ESCC) patients compared to normal controls[8]. RP11-445H22.4 is dramatically upregulated in the serum of breast cancer patients[20]. The expression of H19, HOTAIR and MALAT1 was shown to be significantly increased in plasma from gastric cancer patients compared to normal controls[7]. We also confirmed that the expression of H19, HOTAIR and RP11-445H22.4 increased in the serum of breast cancer patients. This indicated that the abnormal expressions of lncRNAs in cancer patients were lacking of specificity. LncRNAs can't be directly translated into proteins, so how are they closely related to health and disease? Recent studies[21-25] have suggested that lncRNAs play a role in the occurrence and progression of many tumors . LncRNAs are key regulators in cancer transformation and progression, leading to their application possibilities for cancer diagnostics and therapeutics. Previous researches[21-25] have shown that lncRNAs could suppress apoptosis of tumor cells and prolong their live time and regulate the growth cycle of tumor cell results in their excess proliferation[21]. Furthermore, an abnormal expression of lncRNAs may be closely related to the metastasis of tumor[25-27]. For all those reasons, lncRNAs are considered to be the new breakthrough to tumor diagnosis and treatment, but the work of lncRNAs as a therapeutic strategy in the diagnosis and treatment of breast cancer is in the primary stage, and little is known about its mechanisms. Therefore, it re-

mains to be further investigate if lncRNAs help us to understand the mechanisms of tumorigenesis and instruct clinical therapeutics.

We evaluated whether these previously reported plasma lncRNAs could serve as biomarkers for breast cancer. We first determined whether these twelve lncRNAs were expressed in the plasma of healthy controls as well as breast cancer patients. Regular RT-PCR data showed that eight of the twelve lncRNAs were found (AK153778, H19, RP11-445H22.4, ENSMUST0000119855, HULC, SPRY-IT1, POU3F3, HOTAIR) and they had clear bands while the other four lncRNAs (MALAT1, AK139989, PCAT18, and HNF1A-AS1) were given no clear band after 40 cycles' of amplification in both healthy controls and breast cancer patients. Their expression levels were further measured in plasma from breast cancer patients and healthy subjects. The results demonstrated that the levels of H19, RP11-445H22.4, and HOTAIR were significantly upregulated in plasma from breast cancer patients compared to normal controls, indicating that these upregulated plasma lncRNAs could be used as a potential non-invasive screening strategy for breast cancer patients.

The occurrence and progress of tumors are regulated by many genes and other factors. With the development of the research, more and more lncRNAs were found to be associated with the development of breast cancer, but is still lacking the specificity and the mechanism is unclear.

Conclusions

We provided new kinds of lncRNAs related to breast cancer with sensitivity and specificity, as biomarkers for breast cancer. However, it was difficult to collect enough tissues with multiple primary neoplasms. Furthermore, the study was not clarified the molecular mechanisms about how lncRNAs modulated the incidence and development of breast cancer. Further investigations are needed to explore the lncRNA function and the molecular mechanism that directly affect the tumor growth and development.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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