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RESEARCH ARTICLE

Clinical significance of high expression of circulating serum lncRNA RP11-445H22.4 in breast cancer patients: a Chinese population-based study

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Abstract Long noncoding RNAs (lncRNAs) have been gradually confirmed to be tumor-associated biological molecules and became interesting new diagnostic targets of cancer. However, the clinical significances of most cancer-related lncRNAs are largely unknown. Here, we evaluated, for the first time, the feasibility and clinical significances of circulating serum lncRNA RP11-445H22.4 as biomarker for the detection of breast cancer (BC). In this study, the relative concentrations of breast cancer-associated lncRNA RP11-445H22.4 were investigated in a total of 136 serum samples by real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The correlations between the levels of serum lncRNA RP11-445H22.4 in breast cancer patients and the clinicopathological factors of these patients were further analyzed. Receiver operating characteristic (ROC) curve was constructed to evaluate the diagnostic values. In breast cancer patients, the expression level of lncRNA RP11-445H22.4 is significantly increased ($p<0.001$). The sensitivity and specificity of RP11-445H22.4 for BC were 92 and 74 %, respectively. Its expression levels were correlated with estrogen receptor (ER), progesterone receptor (PR), and menopausal status of the breast cancer patients ($p<0.05$). For the detection of breast cancer, the use of RP11-445H22.4 showed a remarkable improvement compared with the clinical serum carcinoembryonic antigen. In conclusions, lncRNA RP11-445H22.4 may be a new potential biomarker of breast cancer.

Keywords Breast cancer · lncRNA RP11-445H22.4 · Serum biomarker

Introduction

Breast cancer (BC) is the second leading cause of cancer-related death among women in the world [1]. According to immunohistochemical analysis for estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER-2) expressions, BC has been classified into four different molecular subtypes [2]. Most prediction and prognosis of BC therapy are associated with these subtypes. Therefore, determination of ER, PR, and HER-2 expression levels has become standard practice in the management of BC. Currently, although BC incidence and mortality rates in the western countries have been decreasing or stable during the past two to three decades, both rates have been increasing rapidly in many developing countries, including China [3]. Delayed diagnosis, recurrence, and metastasis are the main obstacles to the treatment of BC. Among the approaches currently used, blood-based testing is an ideal method for biomarkers in cancer care due to its ease and lower invasiveness [4]. However, conventional serum biomarkers, such as carcinoembryonic antigen (CEA) and carbohydrate antigen (CA125), are of limited clinical utility, and measuring them in BC patients remains controversial [5–8]. Thus, finding novel biomarkers for early detection, prediction of prognosis of BC have attracted attention.

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Long noncoding RNA (lncRNA) are novel class of molecules, with a length longer than 200 nucleotides (nt). Several studies have demonstrated that lncRNAs are involved in multiple biological functions and play a key regulatory role in cancer progression. They can act as oncogenes as well as tumor suppressor genes [9, 10]. For examples, lncRNA H19 is expressed at higher levels in gastric cancer cell lines and tissues [11]. However, lincRNA-21 was found directly activated by p53 in response to DNA damage and play a tumor suppressor role [12]. In decades, growing evidences showed that lncRNA expression levels were closely correlated with particular cancer states, and some specific lncRNA may be useful for cancer diagnosis, prognosis, and guided therapy [13]. In addition, noncoding RNAs are found stable in human body liquid and their expression levels may act as clinical indicators [14]. For example, the lncRNA prostate cancer gene 3 (PCA3) in urine samples may be selected as the diagnostic markers in prostate cancer [15]. To date, most studies describe the profiles of lncRNAs in BC cell lines and primary breast tumor tissues. There are only a few publications dealing with circulating lncRNAs in peripheral blood of BC patients. Considering blood-based testing is an ideal method for BC management, we analyze the serum lncRNA expression to explore new biomarkers for BC.

In our primary study, we identified the global lncRNA expression profile of BC by lncRNA microarray and then we verified that lncRNA RP11-445H22.4 was aberrantly expressed in BC tissues and may be related to BC occurrence. RP11-445H22.4 is a gene 862 nt in length and located in chromosome 20: 43,323,532-43,367,537. In the present study, we firstly measured circulating serum lncRNA RP11-445H22.4 expression levels in BC patients and healthy donors. Also, the potential correlations between circulating lncRNA and patient clinicopathological factors are further investigated. Our data shows that circulating serum lncRNA RP11-445H22.4 might be a potential novel biomarker for BC.

Materials and methods

Patient and sample collection

Incident BC cases were recruited at the time of the diagnosis among patients treated at Breast Cancer Surgical Department of Nanjing Maternity and Child Health Care Hospital, Jiangsu Province, China, between 2012 and 2013. This study was approved by the ethical review committee of Nanjing Maternity and Child Health Care Hospital affiliated to Nanjing Medical University, and informed consent was obtained from all subjects. Only histologically confirmed new BC cases that had not been previously diagnosed for cancer were included into our study. Collected clinical data which contained information were available for all patients. We classified all participants into two groups. The first group involved 68 patients who were sampled for peripheral blood taken at the time of diagnosis (before surgery and any therapy). Second group is a control group including 68 healthy donors, recruited from healthy volunteers. Only subjects with no previous diagnosis of BC or any malignant disease and without manifestation of any acute disease or injury at the time of blood collection were enrolled. Controls had not been exposed to any potentially harmful chemicals except for those common to everyday environmental sources and were sampled without any particular requirement for fasting prior to blood sampling. Structured questionnaire was received from all blood donor BC patients and healthy controls. They reported their diet habits, alcohol consumption, body mass index (calculated from weight and height), and presence of diabetes. All participants are Chinese.

Stock of serum samples

To obtain plasma, the whole peripheral venous blood samples were collected in EDTA gel tubes (9.0 ml) and centrifuged (3000×*g* for 10 min) within 20 min after collection. The cellular fraction was separated from plasma. To prevent contamination of nucleic acid, each plasma sample was labeled and divided into three corning tube (2.0 ml) and stored at −80 °C until further RNA isolation.

RNA extraction and complementary DNA synthesis

Total RNA from blood was isolated from 400 μl of serum samples by using TRIzol LS reagent (Life Technologies) and was eluted into 30 μl of pre-heated (55 °C) elution solution following the manufacturer's procedure. All the RNA samples were stored at −80 °C until further processing. RNA concentration and purity was measured on a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). The OD260/280 ratios for all samples were between 1.8 and 2.0. Complementary DNA (cDNA) was synthesized from 2500 ng of total RNA by using a PrimesScript TM RT Master Mix Kit (Applied TaKaRa, Da Lian, China) with random hexamer primers in a final volume of 50 μl. The reverse transcription reaction were performed at 37 °C for 13 min, 85 °C for 5 s, and 4 °C for 10 min. The cDNA was stored at −20 °C.

Quantitative real-time reverse transcription PCR

The selected lncRNAs and the primers used for quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) were designed and synthesized by Generay Biotech (Shanghai, China). U6 was used as an internal control for tissue samples. The sequences of the PCR primers for U6, RP11-445H22.4 were as follows: 5′-CTCGCTTCGGCAGCACA-3′ (sense) and 5′-AACGCTTCACGAATTTGCGT-3′ (antisense) for U6 and 5′-GTAAAGCCATCACCAGGACA

ACC-3′ (sense) and 5′-CTCCCTAACAGAAGCCCACCA-3′ (antisense) for RP11-445H22.4; qRT-PCR analysis was performed using ABI Viia7 (Applied Biosystems) with SYBR expression assay system (TaKaRa, Dalian, China). The PCR reaction conditions were at an initial denaturation at 95 °C for 30 s, followed by 40 PCR cycles at 95 °C for 5 s and 60 °C for 34 s. Finally, annealing and extension at 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. Each sample was assayed in triplicates. We used the $2^{-\triangle\triangle Ct}$ method to determine the fold change in gene expression in the cancer samples relative to the control samples.

Statistical analysis

The data were analyzed using SPSS 20.0 software package (SPSS, Chicago, IL, USA). Differential expression levels of lncRNAs were compared by independent samples *T* test between two groups. All values are expressed as the mean± S.E.M. All experiments were repeated at least three times. Statistical significance was accepted for $p<0.05$.

Results

General characteristics of study population

Clinicopathological characteristics of BC group and control group are depicted in Table 1. All the participators are female. Average age of BC donors was 51 years (range 32–67), and controls were of similar age distribution as BC patients, with average of 49 years (range 34–64, $p=0.14$) The body mass index (BMI) and menarche age are considered as risk factors for BC; thus, we compared the BMI and menarche age between BC group and control group. No significant different was observed ($p=0.4$; $p=0.3$).

Table 1 Characteristics of the study groups comprising patients with BC and cancer-free controls

	BC	Control	*p* value
Total	68	68	
Gender	Female	Female	
Age (year)	51±1	49±1	0.14
High (cm)	160±1	157±2	0.15
Weight (kg)	63±1	60±2	0.18
BMI	32±8	25±0.4	0.4
Menarche age	14±0.2	14±0.2	0.3
Smoking	0	0	

No significant different was observed between BC group and control group

Circulating serum lncRNA relative expression

The relative level expression of circulating lncRNA RP11-445H22.4 was analyzed in each serum sample. As shown in Fig. 1, we found that in BC patients, the expression levels of serum RP11-445H22.4 were significantly raised than in healthy donors ($p<0.001$). The effect of possible confounding factors (age, body mass index, menarche age, and smoking) was assessed; none of the confounders influenced lncRNA expression, neither in cancer group nor in control group.

Diagnostic value of lncRNA and other biomarkers in breast cancer

To assess the feasibility of using serum RP11-445H22.4 as a diagnostic tool for the detection of breast cancer, receiver operating characteristic (ROC) curves were used. As shown in Fig. 2, the value of the area under the ROC curve was 0.904 for the RP11-445H22.4. In this model, optimal cutoff points of RP11-445H22.4 were indicated at 0.3 (sensitivity 92 % and specificity 74 %). To better understand the potential value of lncRNAs as clinical biomarker for BC screening, we compared sensitivity and specificity of other clinical serum biomarkers and lncRNA RP11-445H22.4. ROC curves and the area under the ROC curve (AUC) of AFP, CEA, CA125, and CA153 were shown in Fig. 3; sensitivity and specificity of AFP was indicated as 73 and 40 %. Sensitivity of CEA was 72 % and specificity of which is 47 %. The sensitivity of CA125 and CA153 was 72 and 42 % and specificity was 48 and 69 %. Among all the serum biomarkers, RP11-445H22.4 has the best sensitivity and specificity (Table 2), which means RP11-445H22.4 may probably work as a biomarker to

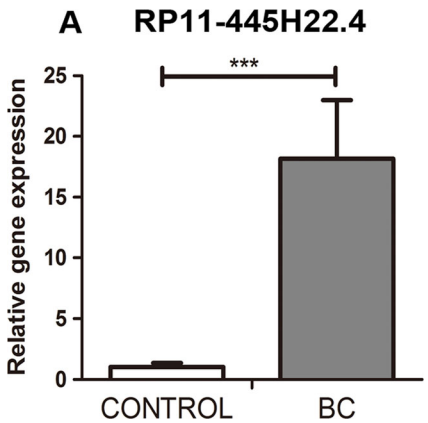

Fig. 1 Relative expression levels of circulating serum lncRNA RP11-445H22.4 between BC group and control group. Circulating serum lncRNA RP11-445H22.4 level in BC group was significantly higher than those in cancer-free controls ($n=68$). Real-time qRT-PCR was used to determine the expression level. The △Ct value was determined by subtracting the U6 Ct value from the lncRNA Ct value. $2^{-\triangle\triangle Ct}$ method was used to determine the fold change in gene expression in the cancer samples relative to the control samples

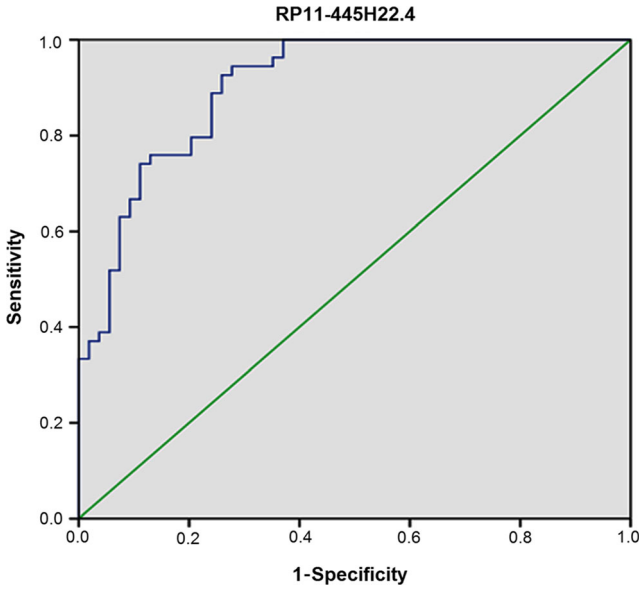

Fig. 2 Value of the area under the ROC curve for lncRNA RP11-445H22.4. In this model, optimal cutoff point of RP11-445H22.4 was indicated at 0.3 (sensitivity 92 % and specificity 74 %)

increase the detection rate of BC and may have better diagnostic value than current circulating markers.

LncRNA RP11-445H22.4 and ultrasound

Mammography and ultrasound are considered as two imaging methods to screening BC. In early research, our team already found that ultrasound rather than mammography has better sensitivity and specificity among Chinese women [16]. In consideration of better diagnostic meaning of ultrasound for Chinese women, we compared the sensitivity and specificity in scanning BC by ultrasound and lncRNA RP11-445H22.4 in this study. We found specificity in lncRNA RP11-445H22.4 and ultrasound was nearly, but lncRNA RP11-445H22.4 had better sensitivity (Table 2).

LncRNA expression levels with immunohistochemical analysis and menopausal conditions

According to immunohistochemical expression for ER, PR, and HER-2, BC was divided into different molecular subtypes which directly influenced treatment strategy. ER and PR were crucially involved in the regulation of mammary gland growth and development. Early study has suggested that a deregulation of the ER and PR may play a major role on BC development and progression [17]. Therefore, we divided BC patients into ER/PR(+) and ER(−)PR(−) groups to observed whether there was any difference of serum lncRNA RP11-445H22.4 levels. The result showed that RP11-445H22.4 level was significantly higher in ER/PR(+) group (Fig. 4a). This result suggests that lncRNA RP11-445H22.4 may be associated with female ER and PR levels involved in the development of BC. HER-2 is one of the most frequent amplified oncogenes in breast cancer. Gene amplification and protein overexpression of the HER-2 have been reported to be linked to the prognosis for BC [18]. Thus, we also investigated the difference of serum RP11-445H22.4 levels between HER-2(−) and HER-2(+) BC patients. Although no significant was observed, we found that there was an increasing tendency of RP11-445H22.4 expression level in HER-2(−) patients (Fig. 4b). This may due to de limited number of blood samples; further investigation on the relation between HER-2 levels and serum RP11-445H22.4 levels should be considered. Previous studies demonstrated that a higher BC incidence was found in postmenopausal women [19]. We further compared serum lncRNAs expression levels in pre- and postmenopausal BC patients. It is interesting to find a higher level of serum RP11-445H22.4 in postmenopausal BC patients (Fig. 4c). Our results suggest that lncRNA RP11-445H22.4 may have clinical significance and may have a great value in BC diagnose.

Discussion

Currently, various researches suggest that lncRNAs play a role in cell differentiation and development, and its expression can contribute to the cancer progression and prognosis [20, 21]. The discovery that lncRNAs are key regulators in cancer transformation and progression leads to its application possibilities for cancer diagnostics and therapeutics. Several significant associations have been found between aberrant lncRNA expression and human tumors. For example, lncRNA MALA T1 has a high expression in non-small cell lung cancer and its high expression has a close relation with metastasis and prognosis. LncRNA SChLAP1 is overexpressed in prostate cancer and its expression levels independently predict poor outcomes. LncRNA H19 and HULC have been found as vital players in gastric cancer and markedly increased in gastric cancer tissues and cells [22–25]. For the noncoding RNAs that have been found stable in human serum and mature product of lncRNA that is the final functional production, measuring the expression of lncRNA may directly represent the levels of the active molecule. Thus, lncRNAs have a potential value as a candidate for non-invasive clinical indicators [26]. All these results strongly demonstrated that lncRNAs can contribute to cancer management, understanding of which will be a key opportunity in cancer diagnosis and treatment.

Breast cancer is one of the most common cancers and cause of cancer death among women. Four different BC subtypes have been defined by the expression of hormone receptor. Previous study has proved that hormone receptor expressions are associated with the risk of BC [27]. HER-2 is one of the most frequent amplified oncogenes in breast cancer. HER-2 gene statuses and protein overexpression have been reported

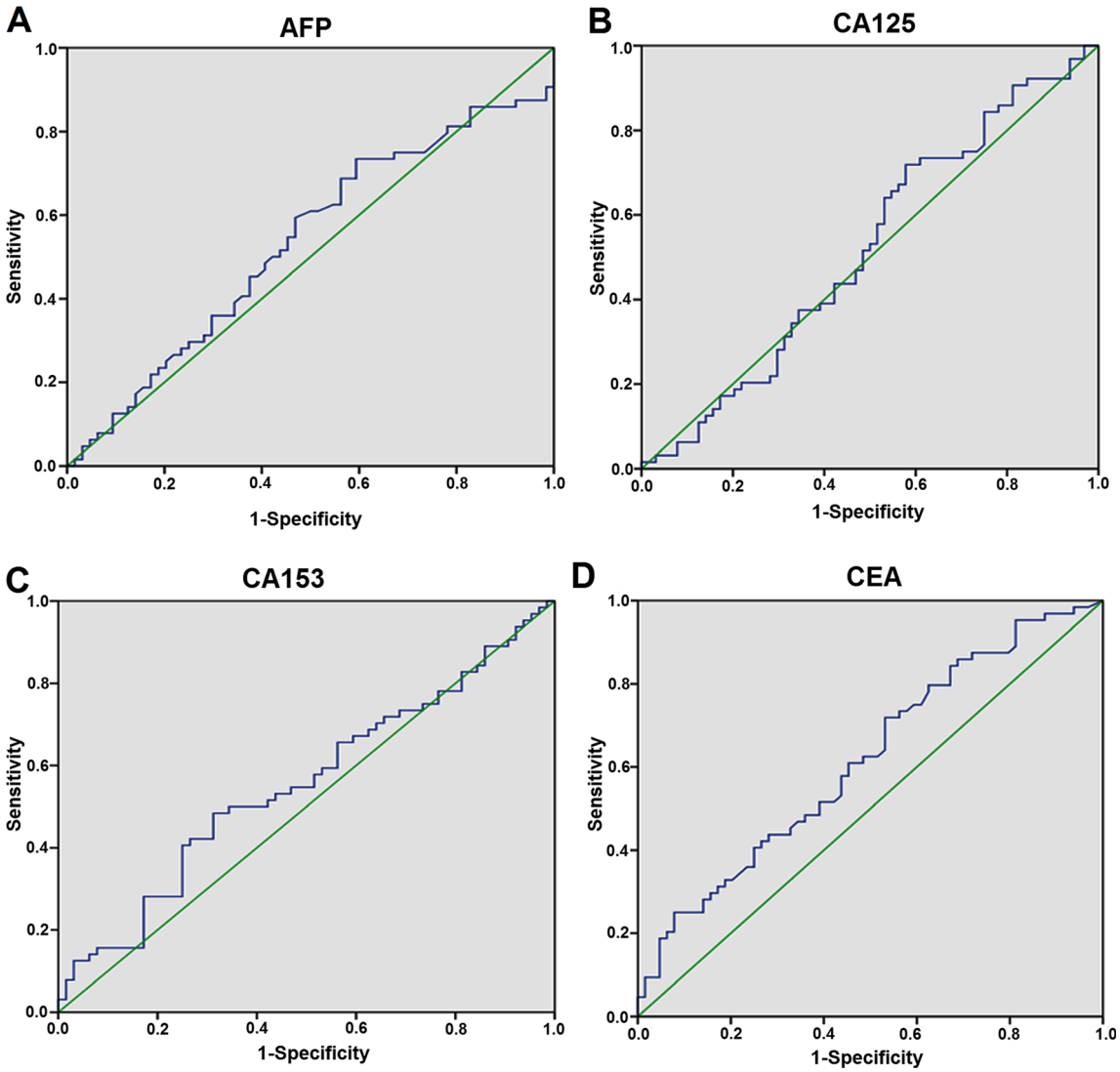

Fig. 3 Value of the area under the ROC curve for clinical used of BC screening biomarkers including AFP, CEA, CA125, and CA153. The ROC curve of AFP (**a**), CEA (**b**), CA125 (**c**), and CA153 (**d**). Optimal cutoff point of AFP was indicated at 1.48 (sensitivity 73 % and specificity 40 %). CEA cutoff point was 1.22, sensitivity was 72 %, and specificity 47 %. The cutoff point of CA125 and CA153 was 10.18 and 10.43 separately, sensitivity was 72 and 42 %, and specificity was 48 and 69 %

related to a poor prognosis, high frequency of recurrence, and reduced overall survival of BC [28]. In this study, we firstly found that circulating serum lncRNA RP11-445H22.4 expression level was overregulated in the serum of BC patients compared with the health controls. Then, we used ROC curve to identify whether RP11-445H22.4 could act as a candidate non-invasive clinical indicators. An ROC curve shows the sensitivity and specificity of RP11-445H22.4 for BC as 92 and 74 %, respectively, which were higher than conventional serum biomarkers. Further, we investigate the relationship between RP11-445H22.4 and ER, PR, and HER-2 states. The result showed that the expression level of RP11-445H22.4 is associated with ER/PR(+) BC patients. In addition, postmenopausal BC patients have a higher serum RP11-445H22.4 level than premenopausal BC patients. All these imply that RP11-445H22.4 is a dysregulative molecule that may be associated with female ER and PR levels play crucial roles in

Table 2 Sensitivity and specificity comparison between clinical screening method and lncRNA biomarkers

Method	Name	Sensitivity (%)	Specificity (%)
LncRNA biomarker	RP11-445H22.4	92	74
Clinical screening method	AFP	73	40
	CEA	72	47
	CA125	72	42
	CA153	48	69
	Ultrasound	68	81

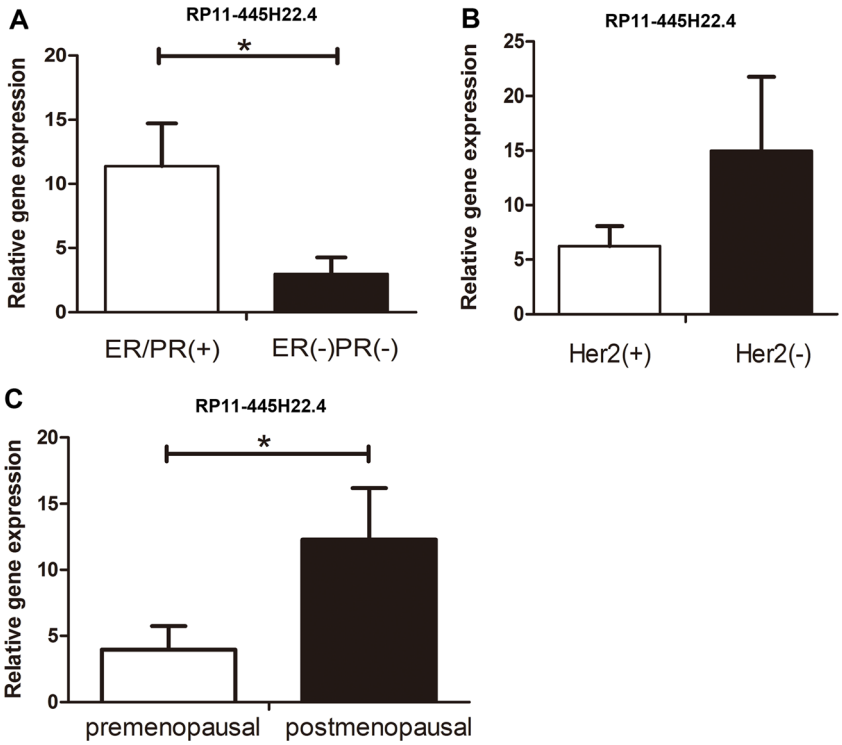

Fig. 4 LncRNA expression levels with immunohistochemical analysis and menopausal conditions. LncRNA expression levels were analyzed with immunohistochemical analysis (**a**, **b**) and menopausal conditions (**c**). RP11-445H22.4 level was significantly higher in ER/PR(+) group and has a decreased trend in HER-2(+) group. Postmenopausal BC patients have a higher serum RP11-445H22.4 level than premenopausal BC patients

BC, and circulating serum RP11-445H22.4 may be a potential biomarker for clinical evaluation. In the present study, we only measured serum lncRNA RP11-445H22.4 levels in Chinese. Unfortunately, we did not have evidence of serum RP11-445H22.4 levels in different ethnic groups. This may be crucial in terms of the application in other places of the world.

In conclusion, our research showed that one of lncRNAs, RP11-445H22.4, was significantly higher in the serum of BC patients and has better sensitivity and specificity than other conventional biomarkers. Its expression level was associated with ER, PR, and menopausal states of BC patients. This new finding suggested that detection of circulating serum lncRNA RP11-445H22.4 may serve as a novel complementary marker for BC diagnosis and prognosis. One limitation of this study is that all the patients are BC patients only; the expression level of serum lncRNA RP11-445H22.4 in other cancer patients, such as lung cancer and cervical cancer patients, would be discussed in the following study. In addition, further research is required to understand the mechanism of lncRNA RP11-445H22.4 in BC progression and evaluate significance of our findings in the clinical setting.

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Conflicts of interest None

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