# A three lncRNA set: AC009975.1, POTEH-AS1 and AL390243.1 as nodal efficacy biomarker of neoadjuvant therapy for HER-2 positive breast cancer

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## Abstract

# Background and purpose

LncRNAs have been found to play regulatory role in the chemoresistance after neoadjuvant therapy (NAT) of breast cancers. The breast pathological complete response (pCR) was different from the axillary nodal pCR (apCR) after NAT. And this difference was most significant in HER-2 positive (HER2+) subtype. The aim was to explore whether lncRNA expression in primary tumor had nodal predicting value for HER2 + breast cancer who received NAT.

# Methods

Total RNA was extracted from 103 HER2 + breast cancer tissues before NAT, as well as from 48 pairs of cancers and para-cancers tissues which did not receive NAT. LncRNAs were selected by microarray, validated by qPCR, and analyzed combined with related clinical factors to illuminate its potential as nodal efficacy biomarkers of NAT.

# Results

Our results demonstrated that three lncRNA set: lncRNA- AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were significantly up-regulated in non-apCR tissues, the AUC value was 0.789 (95%CI: 0.703–0.876). Combined with clinical factors and genomics, the multivariate analysis showed that the expression of lncRNA-AL390243.1 (OR 5.143; 95% CI: 1.570-16.847), tumor type (OR 0.144; 95% CI: 0.024–0.855) and nodal stage (OR 0.507; 95% CI: 0.289–0.888) were indicated as independent predictors for apCR after NAT in HER2 + patients (all $p < 0.05$). These three predictors were used to create a predictive nomogram. The AUC value was 0.859 (95%CI: 0.790–0.929). The calibration curve showed a satisfactory fit between predictive and actual observation based on internal validation with a bootstrap resampling frequency of 1000. Patients with higher level of lncRNA-AL390243.1 expression had a worse survival, especially in disease-free survival. The expression of lncRNA-AL390243.1 was significantly higher in nodal positive subgroup than in nodal negative subgroup ($p = 0.0271$).

# Conclusion

The expression of LncRNA-AC009975.1, POTEH-AS1, and lncRNA-AL390243.1 were upregulated in non-apCR tissues and acted as a new potential biomarker for the nodal efficacy prediction of NAT.

## 1. Introduction

Neoadjuvant therapy (NAT) has been the adapt therapy for patients with inoperable as well as some invasive and high-risk breast cancer, such as stage II–III HER-2 positive (HER2+) and triple-negative breast

cancer (TNBC) [1–2]. Downstaging of tumor is the major clinical benefit of NAT. As a result, clinical nodal positive ($cN_+$) patients might have more chance to achieve axillary nodal pathological complete response (apCR) after NAT and omit sub-sequent axillary lymph node dissection (ALND) [3]. The omission of ALND could prevent the short- and long-term side effects of surgery [4]. As the develop of sentinel lymph node biopsy (SLNB), the feasibility of SLNB after NAT has been confirmed in patients with initial $cN_+$ disease and after NAT clinical nodal negative ($ycN_0$) disease [5–7]. The 2021 St Gallen International Breast Cancer Consensus Conference also recommended that patients with initial $cN_1$ and $ycN_0$ disease after NAT are potential candidates for SLNB [8]. Patients who had more chance to reach apCR would have a significantly lower false negative rate (FNR) when performed SLNB after NAT [9]. So, optimizing candidate selection might be necessary to support the use of SLNB after NAT.

The apCR rates after NAT for hormone receptor positive/HER-2 negative (HR+/HER2-), HER2 + and TNBC subtype were 17.0-21.2%, 51.2–82.1% and 41.0–67.0%, respectively [1]. Compared with other subtypes of breast cancer, HER2 + patients might have more chance to achieve apCR. So, with the possibility of axillary de-escalating surgery for HER2 + patients achieved apCR, predicting nodal response of HER2 + patients after NAT had clinical implications.

Recently, with the development of high-throughput techniques, lncRNA has been gradually discovered and gained more and more attentions. LncRNA is category of RNA with over 200 nucleotides that don't have protein-coding ability, and it is characterized by low sequence conservation and complexity in regulatory mechanism [10]. Many studies have demonstrated the potential value of lncRNA as efficacy prediction biomarkers of breast cancer [11–13]. However, most studies focused on the efficacy prediction of breast primary tumor. To our knowledge, up to now, less researches explored whether lncRNA was associated with nodal efficacy after NAT. At the same time, the breast pathological complete response (bpCR) after NAT was different from the apCR. And this difference was most significant in HER2 + subgroup (Table 1). Therefore, it was not comprehensive to used efficacy prediction lncRNA of primary tumor to predict the nodal efficacy after NAT.

Table 1
The apCR and bpCR after neoadjuvant in different molecular subtypes

| | **overall** | | **HER2 + subgroup** | | **HR+/HER2- group** | | **TNBC group** | |
|---|---|---|---|---|---|---|---|---|
| | apCR | non-apCR | apCR | non-apCR | apCR | non-apCR | apCR | non-apCR |
| bpCR | 78 | 40 | 42 | 22 | 16 | 11 | 20 | 7 |
| non-bpCR | 77 | 221 | 39 | 69 | 28 | 130 | 10 | 22 |

apCR: axillary nodal pathological complete response; bpCR: breast pathological complete response

The purpose of our study was to explore whether lncRNA expression had a nodal predicting value for HER2 + subtype who received NAT, that might help to guide clinicians in treatment decisions and individualized treatment options. In current study, we firstly screened the differential lncRNAs between apCR group and non-apCR group in HER2 + subtype using microarray. Then we validated lncRNA in a larger cohort via qPCR based on which a three lncRNA set: lncRNA- AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 was selected. The diagnostics efficiency and predicting nodal metastasis assessment were analyzed, thus providing the evidence that these three lncRNA set as a nodal efficacy biomarker after NAT for HER2 + breast cancer.

## 2. Methods And Materials

## 2.1 Patients

Four hundred and sixteen patients who had histology confirmed initial clinical $T_{1-4}N_{1-3}M_0$ invasive breast cancer were analyzed for the consistency of apCR and bpCR. A total of 103 core biopsies fresh tissue samples (from April 2014 to 2019) who received NAT as well as 48 pairs of fresh tissue samples (from July 2020 to December 2020) who did not receive NAT from HER2 + breast cancer patients admitted at Shandong Cancer Hospital and Institute were enrolled in current study. Patients were ineligible if they had undergone therapy prior to NAT, concurrent cancer, bilateral breast cancer, or distant metastases.

The study was approved by the Shandong Cancer Hospital Ethics Committee (No. SDTHEC20110324). Informed consents were obtained from all patients, and all procedures were in accordance with the ethical standards of the responsible institutional committee on human experimentation and with the Helsinki Declaration.

## 2.2 Treatment

Clinical stage was determined in accordance with the 8th TNM staging system by the American Joint Committee on Cancer. All clinicopathological factors and laboratory indexes, including age, clinical tumor stage, clinical nodal stage, tumor type, molecular subtypes, neutrophil count, lymphocyte count, monocyte count, plasma blood sugar, platelet count, hemoglobin, plasma fibrinogen, and D-dimer, were estimated before the NAT and were collected from the patients' medical record.

Before NAT, core biopsies of the breast tumor were taken guided by ultrasound. Following core biopsies, excised tissue specimens were immediately placed in liquid nitrogen and subsequently frozen at − 80°C. Suspicious positive axillary lymph node (ALN) was accessed by fine needle aspiration also prior to initiation of NAT. HR was defined as positive with one percent expression rate. HER-2 was considered as positive with immune-histochemical staining of 3+, or fluorescence in situ hybridization that was amplified [14]. After this evaluation, patients received full course of anthracycline and paclitaxel-based chemotherapy regimens plus trastuzumab and (or) pertuzumab as the preferred regimens among the

available treatments. According to the efficacy of NAT, these specimens were divided into four groups: $ypT_0N_0$, $ypT_+N_+$, $ypT_0N_+$ and $ypT_+N_0$.

The primary endpoint of this study was apCR, which means that defined as no residual carcinoma in axilla. The bpCR was defined as no residual invasive carcinoma in the breast.

# 2.3 RNA isolation

Tissue samples were cut into small pieces with weight of 40–50 mg, then thoroughly ground in a dedicated mortar and transferred into a 1.5 mL centrifuge tube. Then 1 ml Trizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added to each centrifuge tube to extract RNA according to the procedure.

# 2.4 LncRNA Microarray

The Arraystar human lncRNA microarray V5.0 was designed for the global profiling of human lncRNA. About 19307 lncRNAs can be detected by our third-generation lncRNA microarray. Arraystar maintains high quality proprietary lncRNA transcriptome databases that extensively collect lncRNA through all major public databases and repositories, knowledge-based mining of scientific publications, including RefSeq, UCSC Knowngenes, Ensembl, and other related sources in the literature.

Briefly, a total of 2μg RNA from each sample isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) was labeled with the Agilent Gene Expression Hybridization Kit (Agilent), after which, the slides were scanned with the Agilent Microarray Scanner (Agilent). A mixture of equal amounts of total RNAs from each group was used as the reference pool. The Feature Extraction software (version 10.7.1.1, Agilent) was used to analyze array images to get raw data and Genespring software (version 14.8, Agilent) was employed to finish the basic analysis with the raw data. R language was utilized for analyzing the differentially expressed lncRNA.

# 2.5 Reverse transcription and Quantitative PCR (RT-qPCR)

The 5μg isolated RNA was reverse-transcribed into complementary DNA (cDNA) using Takara PrimeScript RT reagent Kit (Takara Bio, Kusatsu, Japan) in 10μl reaction system according to the manufacturer's instructions. Furthermore, LightCycler 480 qPCR system (Roche Diagnostics, BALE, Germany) was used for qPCR with a 20μl reaction system, including 10μl of SYB-Green Premix Ex Taq II Reagent (TakaRa Bio, Nojihigashi, Kusatsu, Japan), 7.2μl of RNase-free water, 0.4μl of upstream and 0.4μl of downstream primers, and 2μl of cDNA template. The reactions started at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s, 60°C for 1 min. The qPCR primers are listed in Table 2. All experiments were carried out in duplicate, and then the median Ct value was calculated, β-actin was used as an internal reference gene. The relative expression of lncRNA was evaluated by the comparative cycle threshold (ΔCt) method: ($\Delta Ct = Ct_{lncRNA} - Ct_{\beta-actin}$) as described previously [15].

Table 2
The qPCR primers

| Gene | Forward primer | Reverse primer |
|---|---|---|
| lncRNA-AC009975.1 | CAGTGCTGCCATCCCTAGTTGC | AACTCCAAGAGGTGGTTGCATAGC |
| POTEH-AS1 | TTATGGATATGTGCCGCCGAAGC | GGACTCAACAGAGCCAAGCCTTG |
| lncRNA-AL390243.1 | GCTGCCTGCTGATGCCATCG | TCCACTGCTGAGAGTCCTGTAAGG |
| GAPDH | CCTCG CCTTT GCCGA TCC | GGATC TTCAT GAGGT AGTCA GTC |

## 2.6 Statistical analysis

Statistical analyses were carried out using SPSS Statistics 22.0 software (IBM Corporation, Armonk, NY, USA) or GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA). The Kolmogorov-Smirnov test was carried out to check the normality of the distribution. If the data followed normal analysis, t test would be used; if not, Mann-Whitney test would be used. In paired data, the normally distributed numeric variables were evaluated by paired t-test, whereas non-normally distributed variables were analyzed by Wilcoxon rank-test. Univariable comparisons were performed using the Kruskal-Wallis test for continuous variables and Pearson $\chi^2$ test or Fisher exact test for categorical variables. Multivariable analyses of associations with apCR were conducted using logistic regression models.

A nomogram was developed based on variables in the final model with $p < 0.05$ using “rms” package for R. Calibration of the nomogram was carried out by internal validation using the bootstrap resampling approach and was displayed using a calibration curve. The receiver operator characteristic (ROC) analysis was used to determine the sensitivity, specificity and area under curve (AUC) was calculated to evaluate diagnostic efficiency. Data were shown as mean ± Standard Deviation, and $p$ value < 0.05 was considered statistically significant, and all tests were set as double-tailed.

# 3. Results

## 3.1 The consistency of apCR and bpCR

Firstly, we explored the consistency of apCR and bpCR in 416 patients who received full course of anthracycline and paclitaxel-based chemotherapy regimens plus trastuzumab and (or) pertuzumab followed by surgery from April 2014 to 2019. The apCR and bpCR rate was 37.3% (155/416) and 28.4% (118/416), respectively. The consistency coefficient of apCR and bpCR was 0.719 ($p$□0.001), and it was 0.789, 0.746 and 0.645 among HR+/HER2-, TN and HER2 + subtype, respectively (Table 1).

## 3.2 Identification of differentially expressed lncRNA using microarray in HER2 + subtype

To explore the differentially expressed lncRNA, six HER2 + fresh core biopsies tissues before NAT were collected (3 tissues from $ypT_0N_0$ group and 3 tissues from $ypT_+N_+$ group) and subjected to lncRNA

microarray. A total of 48 significant up-regulated genes and 42 significant down-regulated genes in $ypT_0N_0$ cases were screened and drawn in a volcano map as shown in Fig. 1A, meanwhile the expression patterns of these differentially expressed lncRNA were shown as the heatmap using hierarchical cluster analysis (Fig. 1B), and the Gene Ontology functional classification was performed to gain overall insight into the functions of annotation genes (Fig. 1C). Finally, six up-regulated and six down-regulated with the significance was selected from these differentially expressed lncRNA as the candidates for next validation (as shown in Fig. 1D).

# 3.3 The lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were associated with apCR significantly in HER2 + subtype

Next, we analyzed the differential expression of above selected lncRNA expression by qPCR in HER2 + cohort with 39 cases of $ypT_0N_0$ group and 31 cases of $ypT_+N_+$ group. After Mann-Whitney tests analysis, the expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were elevated significantly in $ypT_0N_0$ group compared with in $ypT_+N_+$ group ($p = 0.0008$, $p = 0.0339$ and $p = 0.0230$, respectively, Fig. 2A). Then, we analysis the expression of these three lncRNA in apCR group (n = 62) and non-apCR group (n = 41). As shown in Fig. 2B, the RT-qPCR result showed that the expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 was elevated significantly in non-apCR group compared with in apCR group ($p = 0.0003$, $p = 0.0022$ and $p = 0.0090$, respectively). Therefore, we selected lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 as potential biomarker for nodal efficacy prediction.

Then we assess the diagnostic efficiency of these three lncRNA in predicting nodal efficacy. To evaluate diagnostic performance of the nodal efficacy prediction, a ROC curve was calculated via comparing these samples. The AUC of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 was 0.685 (95%CI: 0.581–0.790) with 75.8% sensitivity and 46.1% specificity (Fig. 3A), 0.674 (95%CI: 0.564–0.783) with 69.4% sensitivity and 58.5% specificity (Fig. 3B), 0.683 (95%CI: 0.579–0.787) with 72.6% sensitivity and 56.1% specificity (Fig. 3C), respectively (all $p < 0.05$). When combined, the AUC reached 0.789 (95%CI: 0.703–0.876) with 80.6% sensitivity and 61.0% specificity (Fig. 3D).

The relationship between these three lncRNA expression level and clinical characteristics was also analyzed in Table 3. The expression of lncRNA AL390243.1 in tissues of breast cancer was irrelevant with age, HR status, tumor stage, nodal stage and tumor type. POTEH-AS1 was correlated with progesterone receptor change, but irrelevant with other characteristics. The lncRNA-AC009975.1 was associated with tumor stage and nodal stage, but irrelevant with other characteristics.

Table 3
The relationship between lncRNA expression level and clinical characteristics

| Characteristics | Cases | lncRNA-AL390243.1 | POTEH-AS1 | lncRNA-AC00975.1 |
|---|---|---|---|---|
| | | *p* value | *p* value | *p* value |
| T stage | | 0.401 | 0.252 | 0.049 |
| $T_1$ | 8 | | | |
| $T_2$ | 56 | | | |
| $T_3$ | 19 | | | |
| $T_4$ | 20 | | | |
| ER status | | 0.073 | 0.129 | 0.466 |
| positive | 59 | | | |
| negative | 44 | | | |
| ER change | | 0.899 | 0.632 | 0.585 |
| yes | 13 | | | |
| no | 52 | | | |
| PR status | | 0.755 | 0.716 | 0.882 |
| positive | 37 | | | |
| negative | 66 | | | |
| PR change | | 0.162 | 0.037 | 0.240 |
| yes | 6 | | | |
| no | 59 | | | |
| HER-2 change | | 0.785 | 0.403 | 0.907 |
| yes | 3 | | | |
| no | 63 | | | |
| N stage | | 0.204 | 0.110 | 0.012 |
| $N_1$ | 48 | | | |
| $N_2$ | 23 | | | |
| $N_3$ | 32 | | | |
| Tumor type | | 0.087 | 0.734 | 0.405 |

| Characteristics | Cases | lncRNA-AL390243.1 *p* value | POTEH-AS1 *p* value | lncRNA-AC00975.1 *p* value |
|---|---|---|---|---|
| IDC 1 | 3 | | | |
| IDC 2 | 94 | | | |
| IDC 3 | 3 | | | |
| others | 3 | | | |

Taken together, these data suggested that the three lncRNA can be promising biomarkers for nodal efficacy prediction after NAT of HER2 + breast cancer. Next, we want to combine with clinical factors and genomics to analysis the influence factors of apCR.

# 3.4 The analysis of apCR influence factors

The optimal cut-off values of apCR influence factors were calculated according to the ROC analysis. The apCR rate after NAT was 60.2% (62/103). Combined with clinical factors and genomics, the apCR was associated with the expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1, tumor type and nodal stage (all $p$ 0.05). Furthermore, according to the multivariate analysis result, the expression of lncRNA-AL390243.1 (OR 5.143; 95% CI: 1.570-16.847, $p = 0.007$), tumor type (OR 0.144; 95% CI: 0.024–0.855, $p = 0.033$) and nodal stage (OR 0.507; 95% CI: 0.289–0.888, $p = 0.018$) were indicated as independent predictors for apCR after NAT in HER2 + patients (Table 4).

Table 4
The logistic regression analyses of apCR after NAT

| Factors | apCR | non-apCR | univariable *p* value | multivariable *p* value |
|---|---|---|---|---|
| Tumor stage | | | 0.485 | |
| $T_1$ | 6 | 2 | | |
| $T_2$ | 36 | 20 | | |
| $T_3$ | 10 | 9 | | |
| $T_4$ | 10 | 10 | | |
| Nodal stage | | | 0.005 | 0.018 |
| $N_1$ | 37 | 11 | | |
| $N_2$ | 13 | 10 | | |
| $N_3$ | 12 | 20 | | |
| Tumor type | | | 0.022 | 0.033 |
| IDC 1 | 2 | 1 | | |
| IDC 2 | 60 | 34 | | |
| IDC 3 | 0 | 3 | | |
| others | 0 | 3 | | |
| ER status | | | 0.066 | |
| positive | 31 | 13 | | |
| negative | 31 | 28 | | |
| ER change | | | | |
| yes | 25 | 27 | 0.103 | |
| no | 3 | 10 | | |
| PR status | | | | |
| positive | 38 | 28 | 0.468 | |
| negative | 24 | 13 | | |
| PR change | | | | |
| yes | 25 | 34 | 0.719 | |

| Factors | apCR | non-apCR | univariable *p* value | multivariable *p* value |
|---|---|---|---|---|
| no | 3 | 3 | | |
| HER-2 change | | | | |
| yes | 27 | 36 | 0.417 | |
| no | 2 | 1 | | |
| POTEH-AS1 level | | | 0.003 | 0.074 |
| < 11.225 | 42 | 38 | | |
| ≥ 11.225 | 20 | 3 | | |
| AC00975.1 level | | | 0.011 | 0.362 |
| < 12.57 | 33 | 32 | | |
| ≥ 12.57 | 29 | 9 | | |
| AL390243.1 level | | | < 0.001 | 0.007 |
| < 13.02 | 24 | 31 | | |
| ≥ 13.02 | 38 | 10 | | |
| TRBV level | | | 0.092 | |
| < 12.15 | 20 | 20 | | |
| ≥ 12.15 | 42 | 21 | | |
| RUVBL1-AS1 | | | 0.066 | |
| < 13.34 | 31 | 28 | | |
| ≥ 13.34 | 31 | 13 | | |
| D-dimer | | | 0.180 | |
| < 0.4 mg/L | 31 | 15 | | |
| ≥ 0.4 mg/L | 31 | 26 | | |
| Fibrinogen level | | | 0.547 | |
| < 2.96 g/L | 34 | 20 | | |
| ≥ 2.96 g/L | 28 | 21 | | |
| NLR | | | 0.494 | |
| < 4.413 | 29 | 22 | | |

| Factors | apCR | non-apCR | univariable *p* value | multivariable *p* value |
|---|---|---|---|---|
| ≥ 4.413 | 33 | 19 | | |

Based on data from the multivariate analysis result, we created a nomogram to predict patients with apCR after NAT (Fig. 4A). The scores for these three factors were added up to calculate the probability of apCR. And the total scores and bottom risk scale were referenced.

The overall performance of the nomogram was internal validated with a bootstrap resampling frequency of 1000. The calibration curve showed a satisfactory fit between the predictive and actual observation (Fig. 4B). The discriminatory capability of the nomogram was revealed with ROC curve and AUC value. The ROC curve of nomogram was depicted in Fig. 4C. The AUC value was 0.859 (95%CI: 0.790–0.929, $p < 0.001$). These results indicated that the expression of lncRNA-AL390243.1, tumor type and nodal stage might be an efficacy prediction biomarker. Integrating clinical factors and genomics might help to predict apCR and guide individualized treatment options.

As the panel of 2021 St. Gallen International Consensus Conference recommended that patients with initial $cN_1$ and $ycN_0$ disease are potential candidates for SLNB after NAT [8]. So, we performed a subgroup analysis of $cN_1$ disease. Among $cN_1$ disease, the qPCR showed that lncRNA-AL390243.1 was elevated significantly in non-apCR group compared with in apCR group ($p = 0.0041$, Fig. 4D). The AUC value was 0.806 (OR 0.668, 95%CI = 0.668–0.944, $p = 0.002$, Fig. 4E). And the multivariate analysis showed that lncRNA-AL390243.1 was indicated as independent predictors of apCR after NAT for $cN_1$ subgroup ($p = 0.012$).

## 3.5 LncRNA-AL390243.1 facilitate monitoring survival after NAT

The median follow-up was 55 months (33–81 months), with the last follow-up in August 2021. Six cases were lost to follow-up, and the effective follow-up rate was 94.2% (97/103). The median overall survival (OS) in patients with low level and high level of lncRNA-AL390243.1 expression was 50.0 months and 30.5 months, respectively ($p = 0.686$). The median disease-free survival (DFS) was 43.0 months and 18.5 months, respectively ($p = 0.028$) in patients with low level and high level of lncRNA-AL390243.1 expression (Fig. 5A). Patients with higher level of lncRNA-AL390243.1 expression had worse survival compared to patients with a lower lncRNA-AL390243.1 expression. However, the expression of POTEH-AS1 and lncRNA-AC009975.1 were not associated with OS or DFS (all $p > 0.05$, Fig. 5B-C).

## 3.6 The lncRNA-AL390243.1 might predict nodal metastasis of breast cancer

As the three lncRNA might be nodal efficacy biomarkers after NAT of HER2 + breast cancer, so we proposed a hypothesis that the three lncRNA might predict nodal metastasis of breast cancer.

Next, the expression of lncRNA-AC009975.1, POTEH-AS1, and lncRNA-AL390243.1 were analyzed in a HER2 + disease cohort which did not received NAT among 48 pairs of breast cancer tissues and para-cancer tissues. The lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were significantly elevated in breast cancer tissue compared to the para-cancer tissues (Fig. 6A). And the *p* values were 0.0003, 0.0009, and 0.0241, respectively.

Then, we analyzed the nodal status in cancer tissues. In these 48 cancer tissues, we found that the expression of lncRNA-AL390243.1 was significantly higher in nodal positive subgroup than in nodal negative subgroup ($p = 0.0271$), whereas POTEH-AS1 and lncRNA-AC009975.1 showed no significant difference between nodal positive and negative group (all $p > 0.05$, Fig. 6B). The AUC value of lncRNA-AL390243.1 in predicting nodal metastasis was 0.727 (95%CI: 0.554–0.859, $p = 0.0153$, Fig. 6C). These data revealed that the lncRNA-AL390243.1 might play an important role in predicting nodal metastasis.

# 4. Discussion

In current study, lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 expression level were statistically increased in core biopsies tissues from non-apCR group compared to those from apCR group, possessing rather high diagnostic efficiency, the AUC of three-lncRNA set was 0.789 with a sensitivity of 80.6%, and a specificity of 61.0%. More importantly, according to the multivariate analysis, lncRNA-AL390243.1 expression level, nodal stage and tumor type from HER2 + patients were independent predictors of apCR after NAT, which also possessed a higher diagnostic efficiency with 0.859 (95%CI: 0.790–0.929). The nomogram might be especially helpful in predicting which patients might gain a benefit from NAT with regard to nodal response, allowing a more individualized assessment of nodal conversion probability. The lncRNA-AL390243.1 was significantly expressed higher in nodal positive breast cancer than in nodal negative cancer, suggesting lncRNA-AL390243.1 expression level might be a novel biomarker for nodal metastasis diagnostics. In addition, the high level of lncRNA-AL390243.1 was associated with poor DFS, indicating that lncRNA-AL390243.1 might take part in the tumor progression after NAT.

The benefits of NAT for early breast cancers have been well described by others [16–17]. Furthermore, the achievement of pathological complete response (pCR) after NAT is a surrogate marker of improved oncologic outcomes, especially in HER2 + breast cancer [18–20]. The use of effective chemotherapy as well as targeted therapies such as trastuzumab and (or) pertuzumab for HER2 + disease in the neoadjuvant setting have led to an increase in the rate of pCR after NAT ranging from 30 to 63%. However, the bpCR was not completely consistent with apCR. There was difference of therapeutic effect between breast primary tumor and ALN. And this difference was most significant in HER2 + subgroup. So, we want to explore whether there were differentially lncRNAs in primary tumors that could predict apCR of HER2 + breast cancer.

Previous studies demonstrated the potential value of lncRNAs as efficacy prediction biomarkers for breast cancer [11–13]. LncRNA H19 could promote drug resistance in HR + breast cancer cells through inhibiting BIK and NOXA expression [21]. Liang et al. [22] found that lncRNA PRLB could act as an oncogene through affecting the miR-4766-5p/SIRT pathway, and significantly increase breast cancer proliferation and chemoresistance. Zhang et al. [23] found that lncRNA ITGA9-AS1 had a high predictive value for pCR after NAT with an AUC value was 0.800, and the expression level was higher in non-pCR. Yuan et al. [24] observed that lncRNA ATB could promote trastuzumab resistance and invasion-metastasis cascade in breast cancer by competitively biding miR-200c, upregulating E-box-binding protein 1 and zinc finger protein 217, and then induced epithelial-mesenchymal transition and invasion. In addition, Shi et al. [25] also found that lncRNA ATB was much higher in trastuzumab resistance patients. Li et al. [26] screened a microarray of lncRNA involved in trastuzumab-resistant SKBR-3/Tr cells. The result showed that the expression of lncRNA GAS5 was decreased in SKBR-3/Tr cells. Further research showed that GAS5 suppresses cancer proliferation by acting as a molecular sponge for miRNA-21, leading to the de-repression of phosphatase and tension homologs, the endogenous target of miRNA-21. Moreover, mTOR activation associated with reduced GAS5 expression.

However, most studies focused on the lncRNA efficacy prediction value of breast primary tumor. The strength of our study was that our study might be first research to explore whether lncRNA had nodal efficacy prediction value. In the current study, we demonstrated lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were increased in non-apCR group significantly and stably. We believe these three lncRNAs play a crucial role in the tumorigenesis and nodal efficacy prediction of breast cancer, but the underlying molecular mechanisms needs further studies to illuminate.

For patients with initial $cN_+$ and $ycN_0$ disease after NAT, the feasibility of SLNB after NAT has been confirmed in NCCN guideline and St. Gallen international expert consensus [5–6, 8]. However, the overall FNR of SLNB after NAT was still high. Optimizing candidate's selection could improve the sensitivity of selection. If patients have better therapy response and higher apCR rate, they might have more chance to avoid ALND after NAT [27]. In other word, individualized surgical treatment could be chosen according to the therapy response after NAT. So, our nomogram could help to make clinical decision.

There were also several limitations. The most important was the retrospective design. And we involved a limited number of patients at a single institution, which increased the probability of selection bias. Second, we could not take some factors associated with apCR into account. The clinical application of genomics still needs to be validated using a large, independent, prospective cohort.

In conclusion, our study identified that lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 were upregulated in non-apCR breast cancer tissues and acted as a new potential biomarker for the nodal efficacy prediction of breast cancer. At the same time, lncRNA-AL390243.1 might act as a potential biomarker for nodal metastasis prediction of breast cancer.

# Declarations

**Ethics approval and consent to participate:** Written informed consent was obtained from all patients before participation in the study. The study protocol was approved by independent ethics committees at every participating center, and the study was undertaken in full accordance with the Declaration of Helsinki.

**Consent for publication:** All authors agreed to publish this article.

**Availability of data and material:**The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Competing interests:** The authors have stated that they have no conflicts of interest in this work.

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**Authors' contributions:** All authors made a substantial contribution to the conception or design of the work and/or the acquisition, analysis or interpretation of the data. Bi Z, Zhang Y and Song XG participated in drafting the work. Chen P, Qiu PF, and Xie L revised it critically for important intellectual content. Wang YS and Song XR have approved the final completed version of this paper and assume accountability for all aspects of the work.

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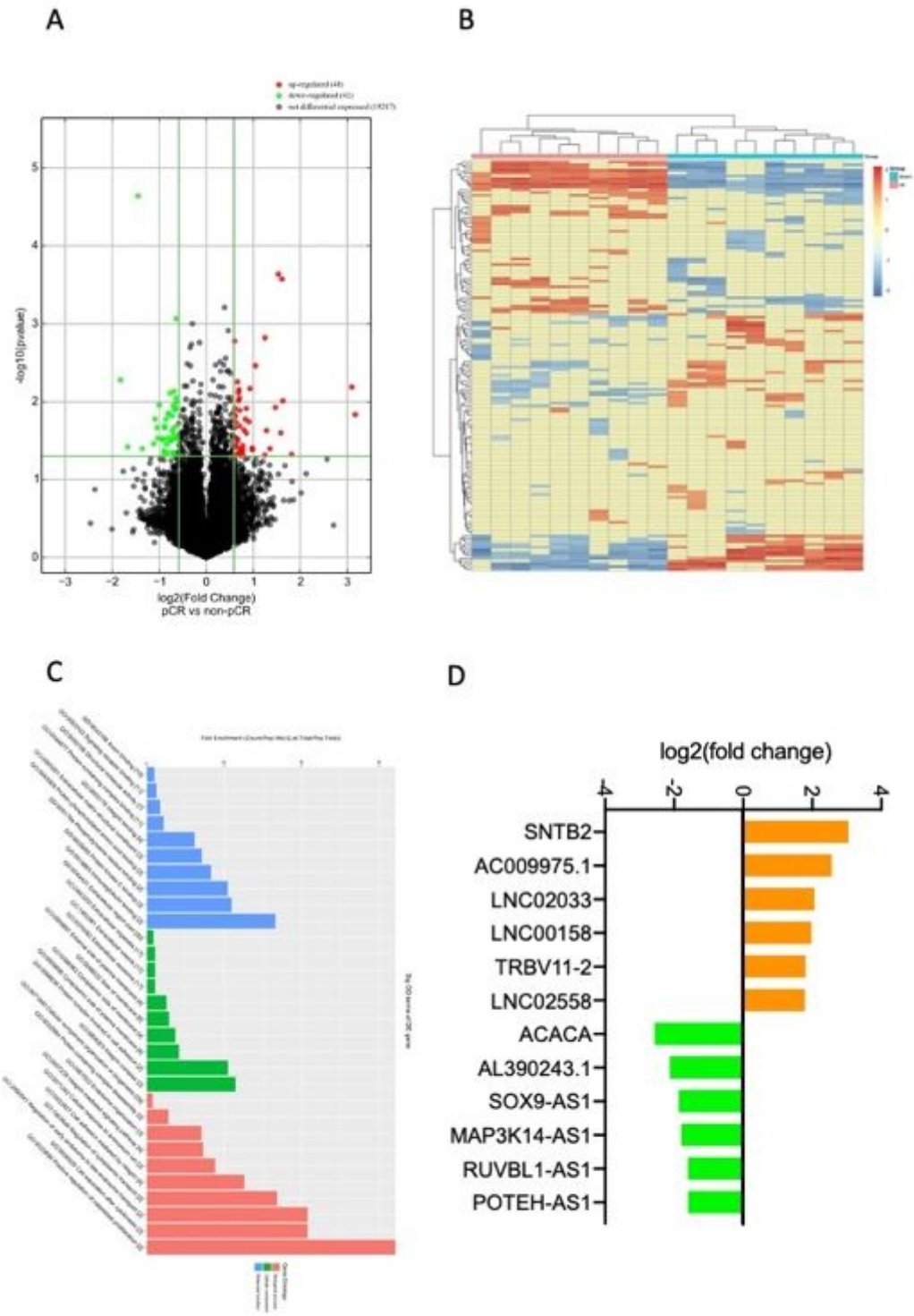
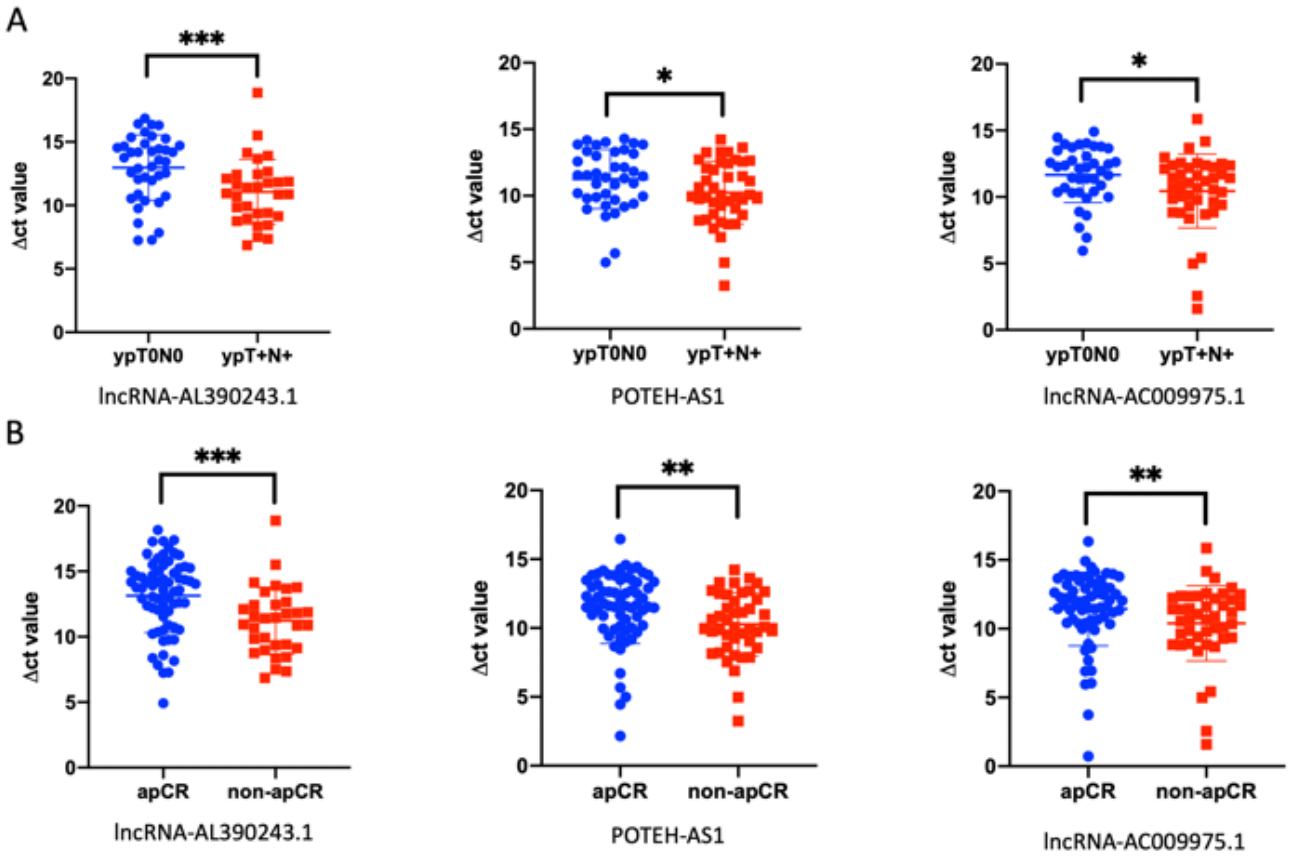
# Figures



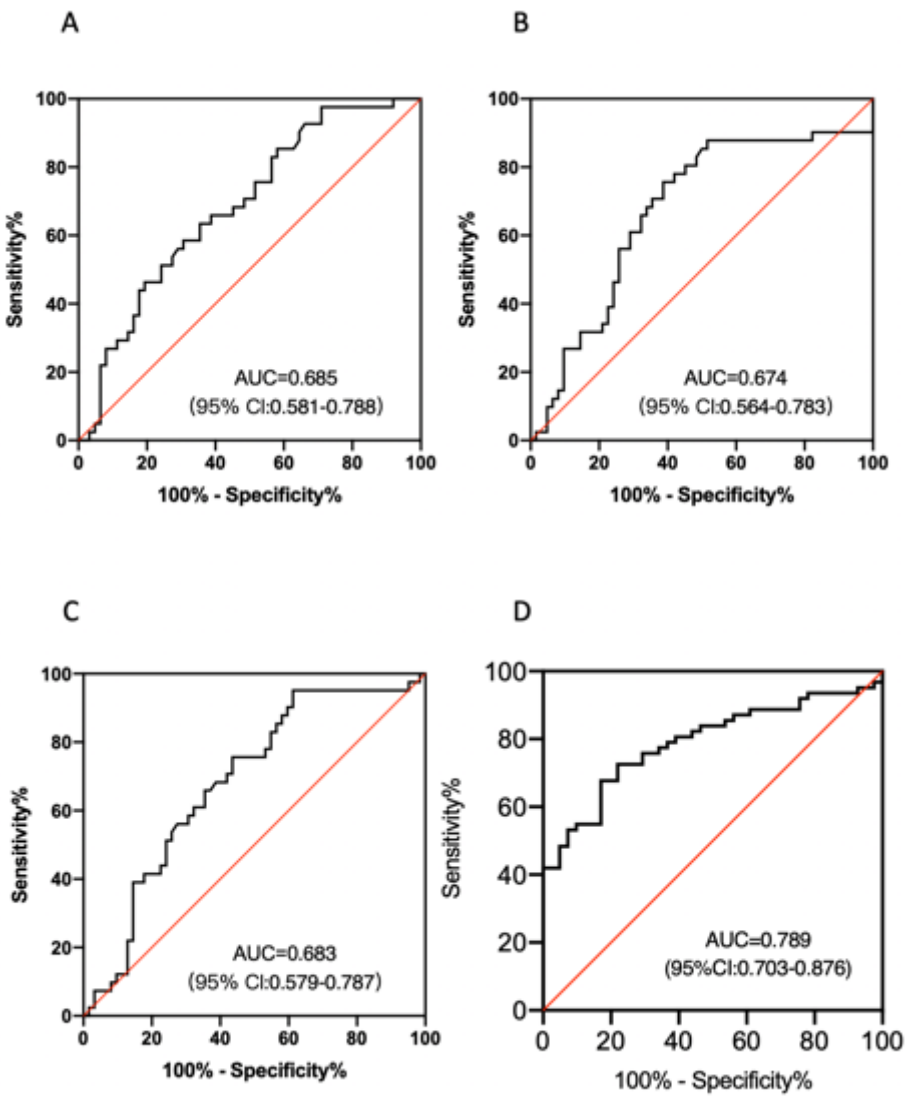

## Figure 1

Identification of differentially expressed lncRNA. (A) Volcano plot compared the expression fold change of lncRNA for ypT0N0 and ypT+N+ tissues; (B) a heat map was generated after supervised hierarchical cluster analysis. LncRNA is shown in red (upregulation) versus blue (downregulation); (C) GO analysis of lncRNA in ypT0N0 and ypT+N+ tissues; (D) 12 selected lncRNAs are shown in yellow (upregulation) versus green (downregulation).



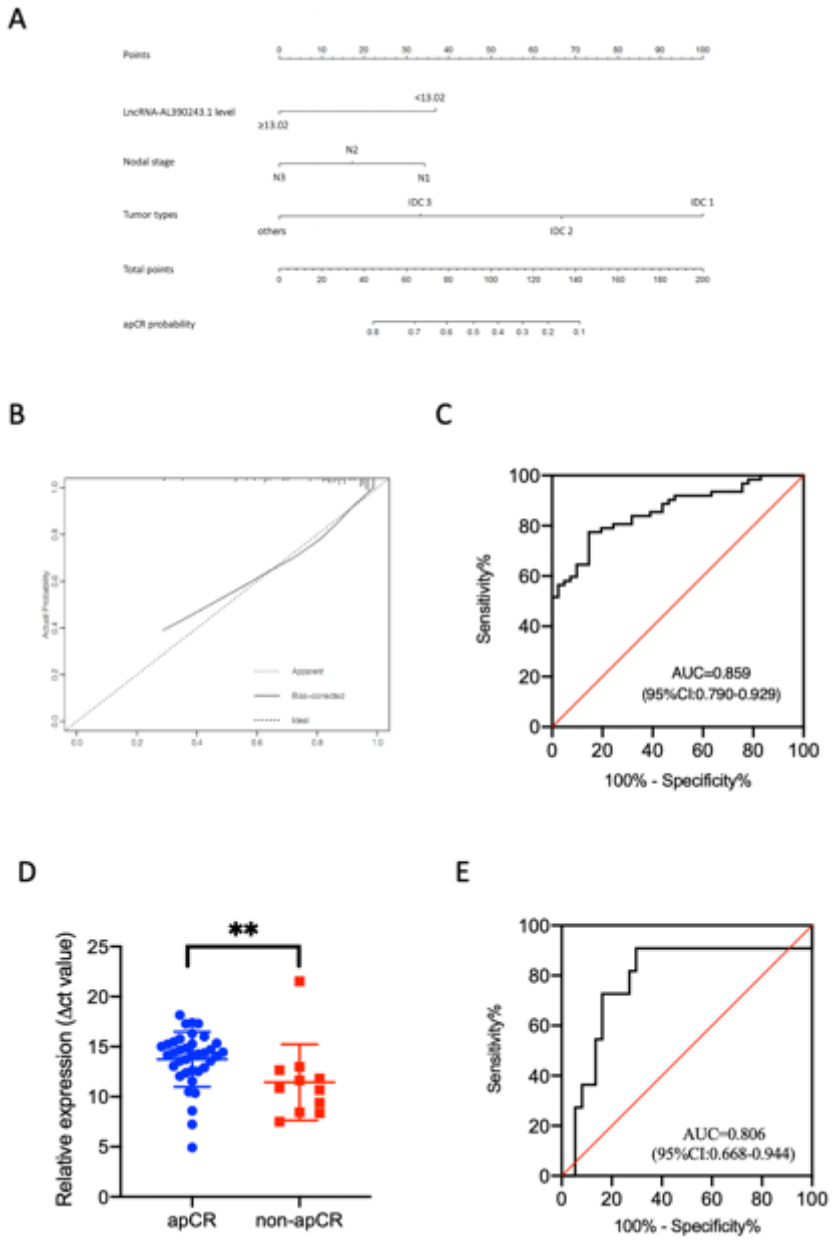

## Figure 2

The lncRNA was closely related with nodal efficacy. (A) The different expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 in tissues in 39 cases of ypT0N0 group compared with 31 cases of ypT+N+ group. (B) The different expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 in tissues in 62 cases of apCR group compared with 41 cases of non-apCR group.



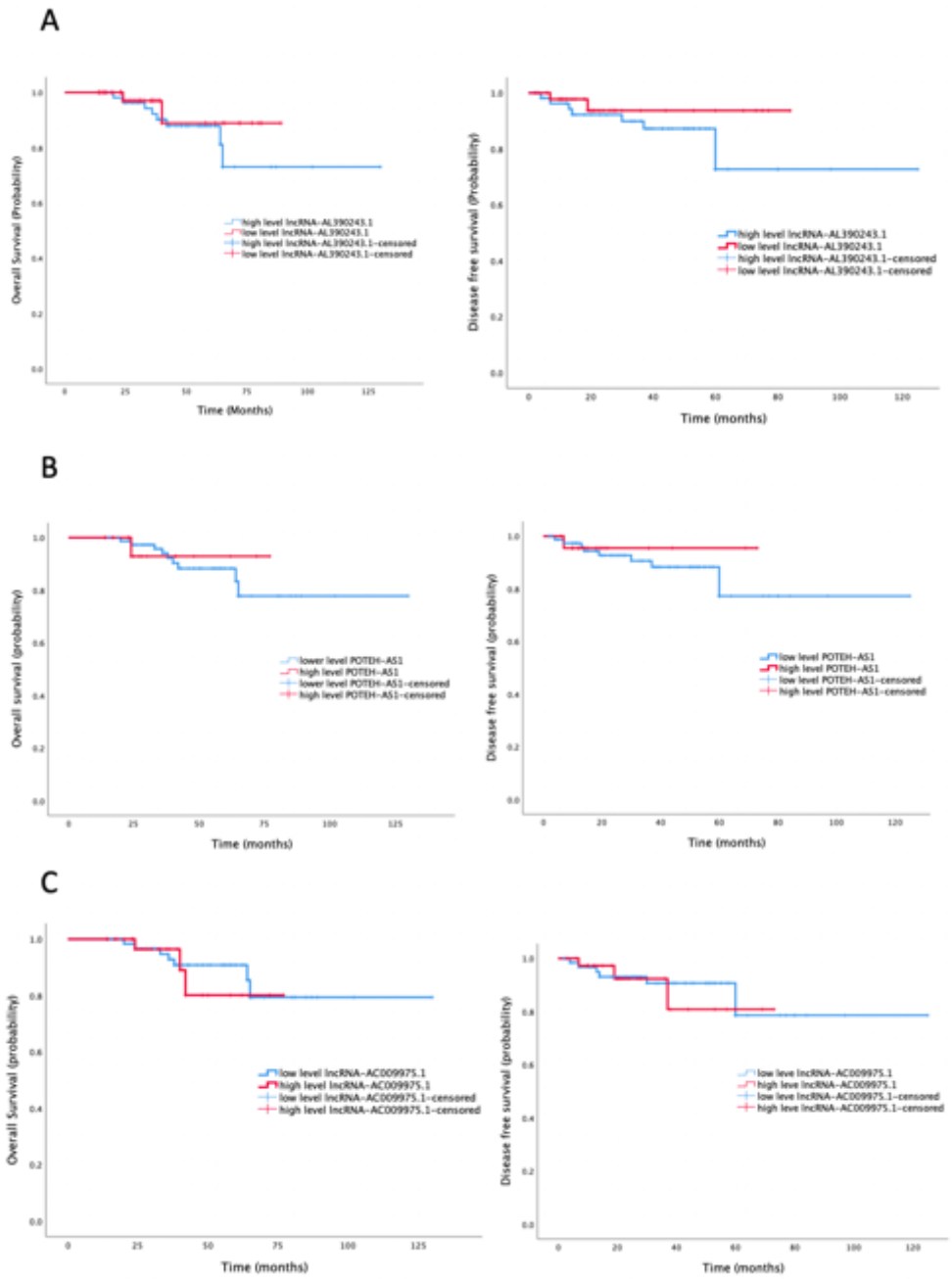

## Figure 3

LncRNA as the nodal efficacy biomarkers after NAT for HER2+ breast patients. (A) The AUC of lncRNA-AL390243.1 was 0.685. (B) The AUC of POTEH-AS1 was 0.674. (C) The AUC of lncRNA-AC009975.1 was 0.683. (D) The diagnostic performance for their combination demonstrated the AUC of 0.789.



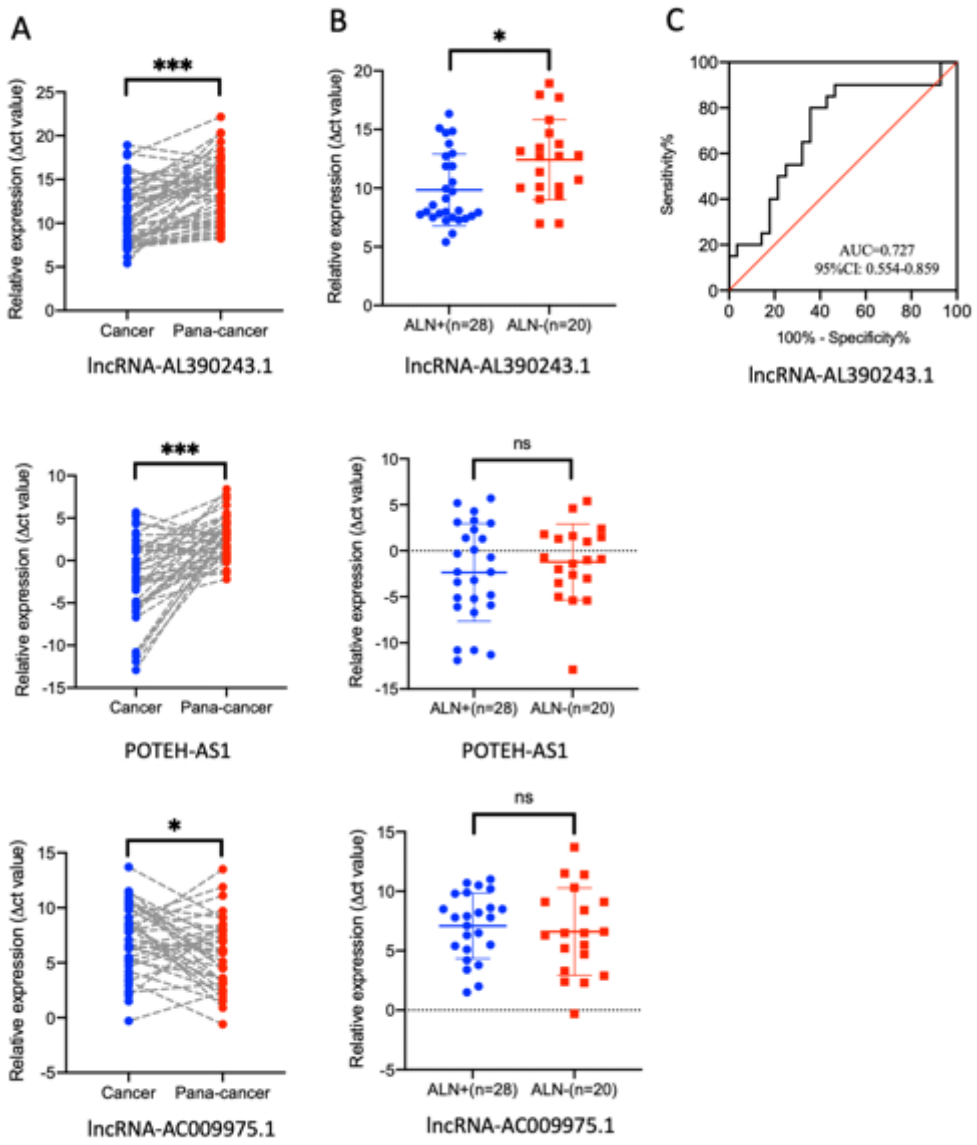

## Figure 4

(A) The nomogram to predict patients with apCR. To calculate the probability of apCR, the scores for the three factors were summed up. And the total scores and bottom risk scale were referenced. (B) The calibration curve showed a satisfactory fit between the predictive and actual observation. (C) The ROC curve of the nomogram. (D) The different expression of lncRNA-AL390243.1 in tissues in apCR group and non-apCR group among patients with cN1 disease. (E) The AUC of lncRNA-AL390243.1 was 0.806 among patients with cN1 disease.




## Figure 5

The survival analysis of three lncRNA. (A) The overall survival and disease-free survival of lncRNA-AL390243.1. (B) The overall survival and disease-free survival of POTEH-AS1. (C) The overall survival and disease-free survival of lncRNA-AC009975.1.




## Figure 6

The expression of lncRNA in tissues which did not received NAT. (A) The expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 in breast cancer tissue and para-cancer tissues. (B) The expression of lncRNA-AL390243.1, POTEH-AS1, and lncRNA-AC009975.1 in nodal positive tissues and nodal negative tissues. (C) The AUC value of lncRNA-AL390243.1 in predicting nodal metastasis was 0.727.