Differential Gene-Based Predictors of Neoadjuvant Chemotherapy Efficacy in Breast Cancer

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Abstract: Background and objective: *Chemotherapy is the most common treatment in breast cancer, and neoadjuvant chemotherapy (NAC) is wildly used because of it's efficiency and safety. To identify significantly differentially expressed genes and select the most suitable breast cancer patients for neoadjuvant chemotherapy (NAC) before treatment.* ***Methods:*** *We collected a total of 60 breast cancer patient samples before and after NAC. All the samples were subjected to high-throughput RNA sequencing (RNA-seq). Then, we identified AHNAK, CIDEA, ADIPOQ, and AKAP12 as candidate genes related to tumour chemotherapeutic resistance. Next, we analysed the expression levels of AHNAK, CIDEA, ADIPOQ, and AKAP12 by logistic regression and based on the result, we constructed a predictive model visualized by a nomogram.* ***Results:*** *The RNA-seq results show that AHNAK, CIDEA, ADIPOQ and AKAP12 are upregulated in residual disease after NAC ($P<0.05$), and compared with the pathological complete response (pCR) group, the non-pCR group presented high AHNAK, CIDEA, ADIPOQ and AKAP12 expression levels ($P<0.05$). Logistic analysis showed that high AHNAK, CIDEA, ADIPOQ and AKAP12 expression levels significantly reduced the pCR rate of NAC for breast cancer ($P<0.05$). In addition, our prediction model, which included AHNAK, CIDEA, ADIPOQ and AKAP12, showed a good fitting effect with the H1 test ($\chi2=6.3967$, $P=0.4945$) and the receiver operating characteristic (ROC) curve (area under the curve (AUC) 0.8249, 95% CI 0.722–0.9271).* ***Conclusion:*** *High expression*

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of AHNAK, CIDEA, ADIPOQ and AKAP12 indicates poor treatment response in breast cancer patients treated with NAC. The efficacy prediction model based on these results is expected to be a new method to select the optimal population of breast cancer patients for NAC.

Key words: Breast neoplasms; Neoadjuvant chemotherapy; Chemotherapeutic efficacy; Gene expression; Predictive model

Introduction

Breast cancer is the most common cancer in women, and its morbidity and mortality are ranked first globally [1]. Chemotherapy is one of the most effective treatments in breast cancer, and neoadjuvant chemotherapy (NAC) or preoperative chemotherapy, increases the chance of breast-conserving surgery for those who have a large tumour at initial diagnosis and has equal efficacy compared to adjuvant chemotherapy [2].

More importantly, the tumour in vivo can be used to directly measure the cancer response to NAC, and we can obtain information about the biological roles of breast cancer as well. Altogether, NAC provides a platform for biomarkers to explore and predict treatment prognosis and outcome[3]. The most reliable indicator of NAC is pathological complete response (pCR) [4], which is defined as ypT0 ypN0 or ypT0/is ypN0. Patients can obtain an 80% decreased recurrence rate if they are assessed as pCR after NAC, regardless of their molecular subtype [5]; furthermore, pCR helps these patients obtain a long-term outcome [6].

However, how to select patients who are most likely to achieve pCR after NAC in breast cancer is still an unsolved question. With the development of medical techniques

and precision medical demands, traditional molecular subtypes by immunohistochemistry in breast cancer no longer meet the needs of individualized clinical treatment. Research shows that gene signatures help to predict outcomes [7], so it is significant to detect differential genes before and after NAC and identify resistance genes in breast cancer patients. Here, we compared gene expression differences before and after NAC as well between the pCR and non-pCR groups of breast cancer. We found that AHNAK, CIDEA, ADIPOQ and AKAP12 most likely induce chemoresistance in NAC. Furthermore, we constructed a gene prediction model to select the optimal population of breast cancer patients for neoadjuvant therapy. Our work revealed that AHNAK, CIDEA, ADIPOQ and AKAP12 are efficacy*-related genes causing chemoresistance in breast cancer.*

1. Materials* and *Methods

1.1 General information

Ethics approval was granted by the hospital research ethics committee (Ethics Approval Number KYLX202134). A total of 60 breast cancer patients were prospectively enrolled from Yunnan Cancer Centre. All samples were collected from volunteers who were pathologically diagnosed with invasive breast carcinoma by the Pathology Department of Yunnan Cancer Centre, China. All of them provided informed consent and signed the consent form. Cancer tissues before and after NAC and clinical information were collected between September 2018 and June 2019 for all patients. If the tumour size was more than 2 cm, NAC treatment was administered based on anthracycline combined with taxane regimens, and the total number of chemotherapy

cycles was no less than 6 cycles.

1.2 Experimental *methods*

1.2.1 Methods of sample collection

Approximately 0.5 g of cancer tissue was obtained with the Mammotome system aspiration biopsy. Each sample was cut and then placed into tubes with RNA Later. Every tube was labelled, including the sample number and collection date, before or after NAC. All samples were preserved in -80 ℃.

1.2.2 High-throughput RNA sequencing (RNA-seq)

This part was performed by the Novogene company. The detailed methods are as follows.

1.2.2.1 RNA quantification and qualification

RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using a NanoPhotometer® spectrophotometer (IMPLEN, CA, USA). RNA concentration was measured using a Qubit® RNA Assay kit on a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

1.2.2.2 Library preparation for lncRNA sequencing

A total amount of 1.5 μg of RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations, and index codes were added to attribute sequences

to each sample. In brief, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primers and M-MuLV Reverse Transcriptase (RNaseH-). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. The remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of the 3' ends of DNA fragments, NEBNext adaptors with hairpin loop structures were ligated to prepare for hybridization. To select cDNA fragments of the right length, the library fragments were purified with the AMPure XP system (Beckman Coulter, Beverly, USA). Then, 3 μl of USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 ℃ for 15 min followed by 5 min at 95 ℃ before PCR. Next, PCR was performed with Phusion High-Fidelity DNA polymerase, universal PCR primers and Index (X) Primer. Finally, the products were purified (AMPure XP system), and library quality was assessed on the Agilent Bioanalyzer 2100 system.

1.2.2.3 Clustering and sequencing

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using the HiSeq 4000 PE Cluster Kit (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq 4000 platform, and 150 bp paired-end reads were generated.

1.2.3 The detailed grouping criteria

pCR [4] is defined as ypT0 ypN0 or ypT0/is ypN0. pCR was regarded as sensitive to

NAC, *and non-pCR was* regarded as resistance to NAC.

1.2.4 Statistical analysis

Correlations of clinical characteristics between the pCR group and the non-pCR group were tested by the chi-square test. The Mann-Whitney U-test or Wilcoxon test was used to test differences in AHNAK, CIDEA, ADIPOQ, and AKAP12 before and after NAC and in the two groups. Univariate and multivariate logistic regression tests were used to analyse the associations between AHNAK, CIDEA, ADIPOQ, and AKAP12 expression and non-pCR. Receiver operating characteristic (ROC) curve analysis was used to assess the discriminative ability of the nomogram. Statistical results were considered significant with a P value <0.05. All statistical analyses were carried out using SPSS statistics (version 25.0). The nomogram was drawn in R software (R version 3.6.0).

2. Results

2.1 Basic clinical features and treatment outcomes of patients

A total of 60 patients were enrolled, and after NAC treatment, 23 patients (38.3%) achieved pCR, suggesting sensitivity to NAC. Thirty-seven patients (61.7%) had non-pCR, meaning resistance to NAC. There were no statistically significant differences in age, TNM stage, Ki-67 expression level or menstrual status between these 2 groups (Table 1).

Table 1 Basic information of the pCR group and non-pCR group before NAC

Variables	pCR group (n=23)	Non-pCR group (n=37)	All patients (n=60)	*P* value
Age(years)				0.195
<50	9(15.0%)	20(33.3%)	29(48.3%)	
>=50	14(23.3%)	17(28.3%)	31(51.7%)	

T stage				0.056
2	12(20.0%)	28(46.7%)	40(66.7%)	
3	11(18.3%)	6(10.0%)	17(28.3%)	
4	0(0%)	3(5%)	3(5%)	
N stage				0.483
0	3(5%)	1(1.67%)	4(6.67%)	
1	11(18.3%)	23(40.0%)	34(56.67%)	
2	8(13.3%)	7(21.7%)	15(25%)	
3	1(1.67%)	6(10.0%)	7(11.67%)	
Ki-67(%)				0.456
<=15	5(8.3%)	14(23.3%)	19(31.7%)	
15-30	3(5.0%)	3(5.0%)	6(10.0%)	
>=30	15(25.0%)	20(33.3%)	35(58.3%)	
Menstrual status				0.419
Yes	11(18.3%)	20(33.3%)	31(51.7%)	
No	12(20.0%)	17(28.3%)	29(48.3%)	

2.2 Changes in RNA-seq in 60 patients before and after NAC

2.2.1 Expression changes in RNA-seq before and after NAC

For the non-pCR group, we collected cancer tissue before and after NAC. In the non-pCR group, the RNA-seq results showed that 953 genes were upregulated *and 2041 genes were* downregulated more than two times *than* before NAC treatment ($P<0.05$), *and noncoding RNAs were excluded*. Some differentially expressed genes are displayed in Figure 1.

2.2.2 Differential expression in RNA-seq in the pCR and non-pCR groups

Compared with the pCR group, 457 genes were expressed more than 2-fold and 1361 genes were expressed less than 2-fold in the non-pCR group ($P<0.05$*), excluding noncoding RNAs. The two groups showed differentially expressed mRNA pairs* (Figure 2*).*

2.2.3 Potential drug resistance-related genes in breast cancer treated with NAC

Furthermore, we selected genes that were more than 2 times upregulated in residual disease after chemotherapy in the non-pCR group and 2 times more highly expressed in the non-pCR group than in the pCR group. Finally, 28 genes met the requirements (Figure 3). *We next selected the 4 most relevant genes of cancer drug resistance from the 28 genes, considering the gene expression count, gene functions and relevant research of cancer treatment. Finally, we identified AHNAK, CIDEA, ADIPOQ, and AKAP12 as the 4 most relevant genes of NAC resistance in breast cancer, as these genes may influence the response to NAC in breast cancer*

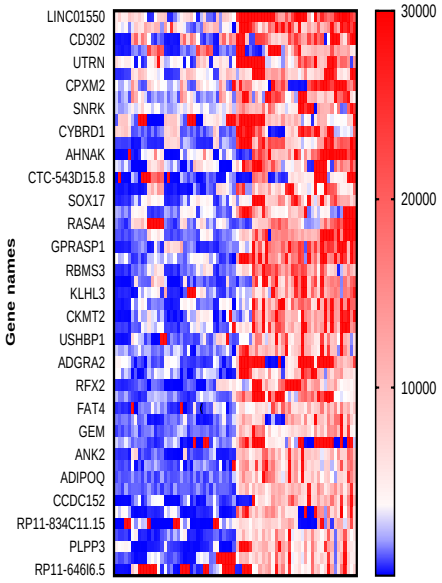

Figure 1 Genes expression level changes before (left half part) and after (right half part) NAC

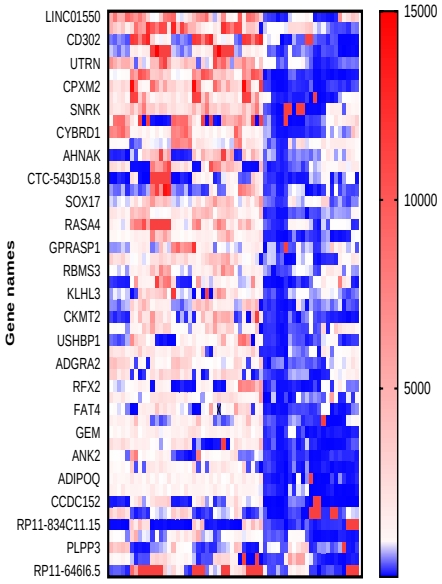

Figure 2 The non-pCR group (the left 37 columns) has a high expression compared with the pCR group (the right 23 columns) in some genes

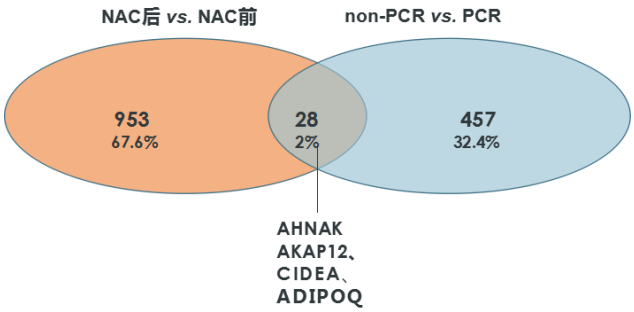

Figure 3 Screening of potential drug-resistant genes in breast cancer for NAC

2.2.4 Expression of AHNAK, CIDEA, ADIPOQ, and AKAP12 in the pCR and non-pCR groups before and after NAC

A box plot showing the expression of AHNAK, CIDEA, ADIPOQ, and AKAP12 in the non-pCR group (37 patients) and the pCR group (23 patients) before treatment is shown in Figure 4. Then, the Mann-Whitney U test showed statistically significant differences in AHNAK, CIDEA, ADIPOQ, and AKAP12 expression in the two groups (P<0.05).

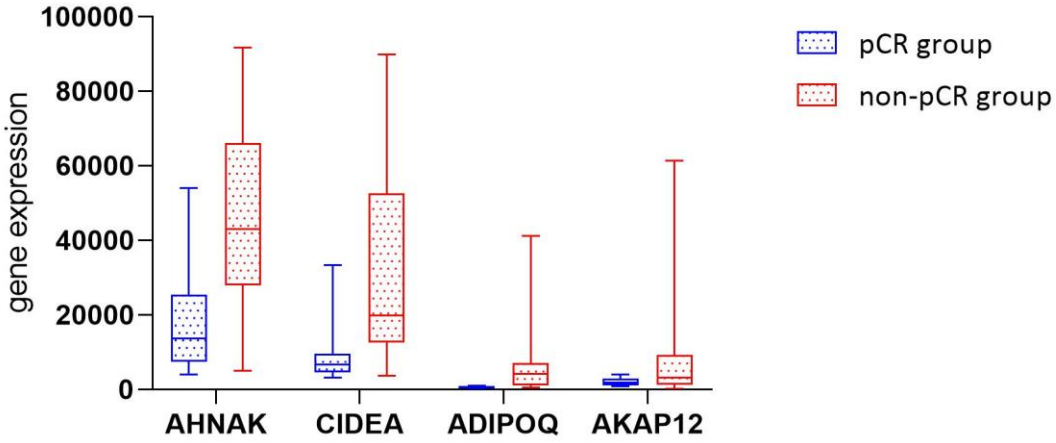

Figure 4 Box plot of AHNAK, CIDEA, ADIPOQ, and AKAP12 expression before NAC

Similarly, we made a *box plot* showing *AHNAK, CIDEA, ADIPOQ, and AKAP12 expression before and after NAC in the non-pCR group (Figure 4).* The *Wilcoxon test*

was used to compare the means (P<0.05).

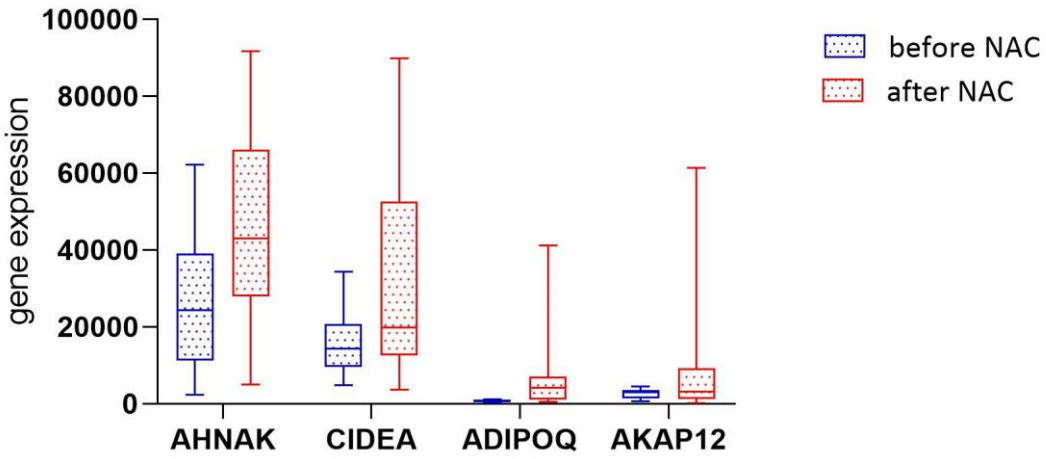

Figure 5 Box plot of AHNAK, CIDEA, ADIPOQ, and AKAP12 expression before and after NAC in the non-pCR group

2.3 *Logistic regression analysis for NAC efficacy*

All clinical indicators and potential resistance genes were used as candidate predictors. The univariate logistic regression results showed that there was no correlation between age, T stage, N stage, Ki-67 level, and menstrual status and non-pCR (P>0.05). However, univariate and multivariate logistic regression analyses indicated that the expression of AHNAK, CIDEA, ADIPOQ, and AKAP12 increased the risk of non-pCR (odds ratio (OR)>1, P<0.05), which means the high expression of AHNAK, CIDEA, ADIPOQ, and AKAP12 results in poor efficacy of NAC treatment in breast cancer (Table 2).

Table 2 Univariate and multivariate logistic regression analyses for NAC efficacy

Variables	Univariate logistic regression			Multivariate logistic regression		
	OR	95% CI	*P* value	OR	95% CI	*P* value

Age (years)	0.99	0.954–1.041	0.806	-	-	-
T stage	0.502	0.208 – 1.213	0.126	-	-	-
N Stage	0.953	0.490 – 1.853	0.887	-	-	-
Ki-67 (%)	0.978	0.950 – 1.006	0.117	-	-	-
Menstrual status	1.071	0.250 – 4.591	0.926	-	-	-
AHNAK	1.0004	1.0000 – 1.0007	0.0486	1.0005	1.0000 – 1.0011	0.0319
AKAP12	1.0058	1.0005 – 1.0110	0.0311	1.0082	1.0013 – 1.0152	0.0195
CIDEA	1.0012	1.0003 – 1.0020	0.0077	1.0011	1.0002 – 1.0019	0.0164
ADIPOQ	1.0262	1.0018 – 1.0512	0.0350	1.0329	1.0003 – 1.0665	0.0477

Ki-67, AHNAK, AKAP12, CIDEA, and ADIPOQ are continuous variables.

2.4 *Establishment and validation of an efficacy model of NAC in breast cancer*

2.4.1 *Construction of the nomogram efficacy prediction model*

AHNAK, CIDEA, ADIPOQ, and AKAP12 were used as factors in the efficacy *prediction model, and a nomogram was constructed with R software (Figure 6). AHNAK, AKAP12, CIDEA, and ADIPOQ were used as continuous variables (10 was used as the unit because the expression levels were high). As shown in Figure 6, the left column is the name of the corresponding line, "Points" records the scores of each predictive factor, all points of each factor can be summed to the "Total points" axis, and an imaginary line from the "Total points" axis to the "Prediction" axis can be drawn. Then, we can obtain the non-pCR rate of NAC in breast cancer. For example, we mark the gene expression quantity of AHNAK, CIDEA, ADIPOQ, and AKAP12 on their respective lines and then draw an imaginary line from the gene expression quantity to the Points axis for each gene. After that, we can add up all points of AHNAK, CIDEA, ADIPOQ, and AKAP12 to obtain the total points of the patient.*

Last, we draw an imaginary line from the Total Points axis to the Prediction axis. This way, we can predict the possibility of non-pCR before patients receive NAC.

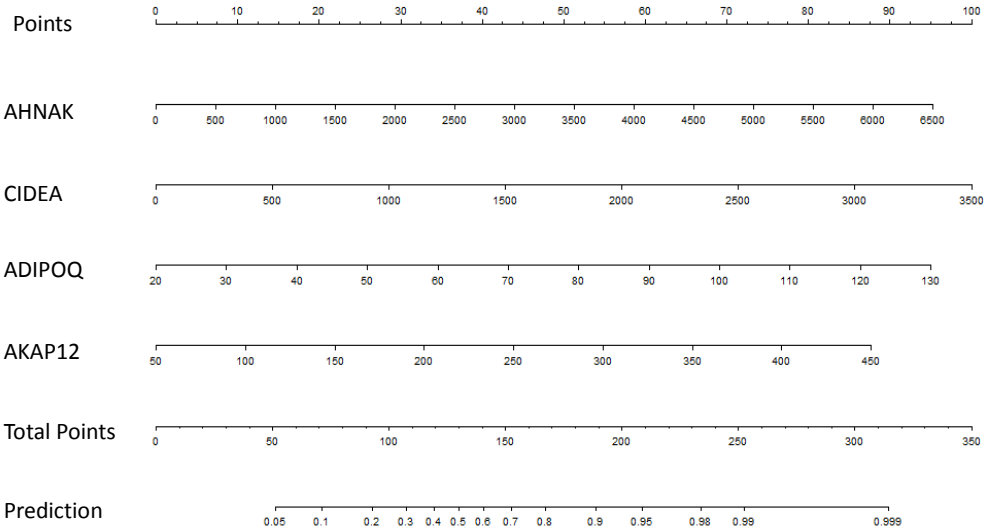

Figure 6 Nomogram prediction efficacy in breast cancer treated with NAC based on the expression of AHNAK, CIDEA, ADIPOQ, and AKAP12.

2.4.2 Validation of the predictive ability of the model

The predictive ability of the model was evaluated using predictive correlation and the area under the ROC curve (AUC). After calculation, the AUC was 0.8249 (95% CI: 0.7227–0.9271) (Figure 7-A). Bootstrap values were calculated from 1000 bootstrap repetitions to obtain a corrected AUC (c-index 0.7761). As shown in Figure 7-B, the Hosmer–Lemeshow test suggested that the model had high coherence (P=0.4945). This revealed that the prediction model based on the expression of the 4 genes displayed *good predictive value for the treatment outcome of NAC in breast cancer.*

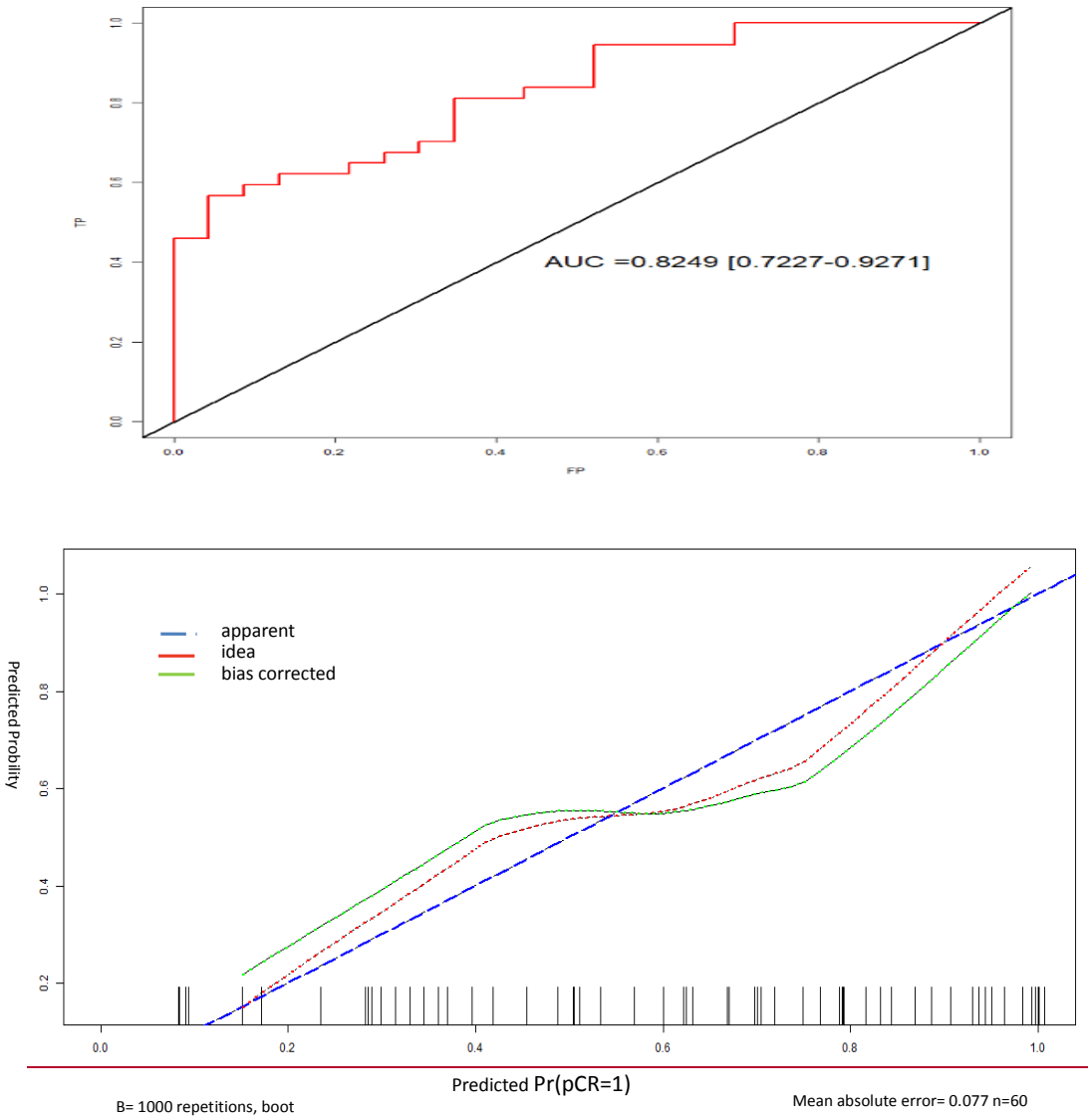

Figure 7 AUC (A) and calibration curves (B) of the prediction model

Discussion

Chemoresistance is one of the primary causes of treatment failure in breast cancer, leading to cancer metastasis and recurrence in 30% of breast cancer patients. Approximately 30% to 50% of patients will achieve pCR after NAC, and those who are assessed as showing pCR have better long-term outcomes [5,6]. Chemoresistance has a close and complex connection with gene expression, and cancer heterogeneity makes it more difficult to predict. Some subcellular populations pose a greater tendency to

evolve to resistant agents under treatment selection pressure [11]. Cancer heterogeneity also means there are differences in gene expression and chemotherapeutic sensitivity; therefore, it is vital to refine molecular typing and advance targeted treatment in breast cancer [12].

RNA-seq is a great technology for genome-wide analyses of transcriptome information at the single-nucleotide level, in addition to the quantification of gene expression [13]. RNA-seq can identify alternative splice sites, detect novel transcripts and tumour heterogeneity [14], and identify drug resistance biomarkers [15, 16]. With these bioinformatics approaches, scholars have found that drug resistance genes exist in breast cancer before NAC, and chemotherapy promotes the evolution of resistance genes and then causes resistance clinical outcomes [17–20]. Thus, the detection of breast tumour samples before NAC may provide reliable information on chemotherapy efficacy-associated genes.

Our study using RNA-seq at the level of gene expression revealed that AHNAK, CIDEA, ADIPOQ, and AKAP12 are associated with drug (anthracycline and taxanes) resistance in breast cancer during NAC treatment.

AHNAK is a protein-coding gene. As a giant scaffold protein (approximately 700 kDa) [21], AHNAK is a key protein involved in the migration and invasion of breast cancer cells. Some researchers have reported that overexpression of AHNAK may result in doxorubicin resistance in breast cancer cell lines. For example, Tanja Davis reported that AHNAK knockdown prevented doxorubicin-induced modulation of cleaved caspase 7 protein expression and cell cycle arrest, while overexpression of AHNAK

induced S-phase arrest through decreased cleaved caspase 7 and cleaved PARP levels. This finding indicates that AHNAK plays an important role in the chemotherapeutic response of breast cancer cells and implies that AHNAK has potential for predicting the treatment response in breast cancer [22]. Our research revealed that the high expression of AHNAK increased the risk of non-pCR in breast cancer treated with NAC, which is consistent with the findings of former reports. However, exactly how AHNAK results in NAC resistance in breast cancer still needs to be elucidated.

A-kinase anchor protein 12 (AKAP12) is also a protein-coding gene. Its coding protein is associated with cell growth and is a scaffold protein in signal transduction. Some drug resistance research associated with cancer indicated that the gene fusion of ESR1-AKAP12 induces osimertinib (a kind of target drug) resistance in EGFR-mutated lung cancer[23]. Moreover, scholars have supported that AKAP12 renders prostate cancer cells resistant to docetaxel [24]. Our work identified AKAP12 as associated with breast cancer chemoresistance, and further study is needed.

Cell death-inducing DNA fragment factor 45-like effector A (CIDEA) and adiponectin (ADIPOQ) are key regulators of lipid metabolism and sugar metabolism and are closely related to metabolic diseases [25]. CIDEA and ADIPOQ are highly expressed in breast tissue and adipose tissue, and both of them could promote apoptosis of tumour cell lines and might inhibit tumour growth [26]. However, research supports that CIDEA participates in the interaction of tumour and nontumour mouse mammary epithelial cells, ultimately accelerating tumour progression [27]. In addition, a relevant point worth mentioning is that the adiponectin-AdipoR1 axis was defined as a predictor

of tyrosine kinase inhibitor resistance in metastatic renal cell carcinoma [28]. Our results indicated that the 2 genes are hazardous in breast cancer chemoresistance, and notably, the 2 genes have similar functions in apoptosis and metabolism. Regrettably, whether CIDEA and ADIPOQ induce chemoresistance via an interaction remains to be clarified. It is still unknown how CIDEA and ADIPOQ expression leads to poor clinical outcomes in non-pCR breast cancer patients.

With the development of precision medicine, gene models have gained an important role in cancer treatment. Currently, some gene models have been widely recognized internationally, such as BRCA1/2 detection, oncotype DX Breast Recurrence Score, MammaPrint and others [29]. However, these gene models are mainly used to assess whether low-risk patients forego chemotherapy. Although new follow-up data support that MammaPrint has the potential to predict the efficacy of NAC in breast cancer, more specific clinical evidence should be provided. BluePrint is an 80-gene test for the molecular retyping of breast cancer classified by protein function based on the I-SPY2 trial. For breast cancer patients who are assessed as having a high gene risk of recurrence by MammaPrint, their molecular typing can be redefined using BluePrint. BluePrint helps select the most suitable patients from NAC treatment and improves the pCR rate [30]. Although BluePrint provides data to construct a predictor model for selecting the most suitable breast cancer patients for NAC, its underlying data mainly originate from European and American populations, and more Chinese gene models need to be explored.

Our work revealed that the high expression of AHNAK, CIDEA, ADIPOQ, and

AKAP12 induces chemoresistance in breast cancer and reduces the pCR rate. Based on the expression of these 4 genes, the constructed efficacy prediction model is expected to be a new tool for selecting the optimal population of breast cancer patients for treatment with neoadjuvant chemotherapy. Limited by the sample size, we failed to perform further explorations. Next, we will collect samples and perform some experiments to verify our findings.

In short, we made a conclusion that high expression of AHNAK, CIDEA, ADIPOQ and AKAP12 indicates poor treatment response in breast cancer patients treated with NAC. The efficacy prediction model based on these results is expected to be a new method to select the optimal population of breast cancer patients for NAC.

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Not applicable

Authors' contributions

(I) Conception and design: LU Mei, GUO Rong, YANG Zhuangqing; (II) Administrative support: YANG Zhuangqing, NIE Jianyun; (III) Provision of study materials or patients: ZOU Jieya, YANG Xiaojuan, WANG Ji; (IV) Collection and assembly of data: ZHANG Qian, DENG Xuepeng, TAO Jianfen ; (V) Data analysis and interpretation: LU Mei, GUO Rong; (VI) Manuscript writing: LU Mei; (VII) Final approval of manuscript: All authors.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

Ethics approval was granted by the hospital research ethics committee (Ethics Approval Number KYLX202134) and all of 60 patients were volunteer for the study.

Consent for publication

The authors consent for publication in the Journal.

Competing interests

The authors declare that they have no conflicts of interest.

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Declarations：

All of authors reached a consensus for publication our paper

Our data were available for further research

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