According to the global report, 2.26 million new cases of breast cancer were diagnosed and 0.68 million died from the disease in 2020. Breast cancer continues to threaten maternal health by ranking first in morbidity and mortality. The disease is known for its complex clinical manifestations, morphological and molecular biological characteristics, and therapeutic resistance. Therefore, finding a suitable detection method to predict the prognosis of breast cancer is imminent due to the disease’s high mortality rate and heterogeneity.

Long non-coding RNAs (lncRNAs) are defined as RNAs longer than 200 nucleotides that do not encode proteins. lncRNAs, accounting for approximately 80% of the human transcriptome, perform many biological functions depend-
The tumor microenvironment is a crucial variable to breast cancer progression.[6, 7] This results from the infiltration of the tumor microenvironment by several immune cells such as T cells, B cells, and lymphocytes infiltrating around the breast cancer and tumor stroma.[8] Emerging evidence has suggested that the dysregulation of these immune cells correlates to immunosuppression and progression in several malignant tumors. Hence, the utility of immunotherapy in the treatment of tumors has been very critical in recent times.[9, 10] Accordingly, some of the biomarkers associated with T cells, known as immune checkpoints like PD-1/PD-L1, have shown to be a novel strategy in breast cancer treatment.[11] To further understand the benefit and risks associated with these therapies, biomarkers that can predict the treatment response are urgently needed.

Over the past years, growing evidence shows that IncRNAs can regulate tumor immunity in immune cells and the immune microenvironment.[12] Accordingly, the relationship between IncRNAs and cancer immunity has received increasing attention. Some immune-related IncRNAs (irlncRNAs) are involved in tumor cell migration, invasion, epithelial-mesenchymal transformation, and metabolism. In breast cancer, IncRNA SNHG1 regulates the differentiation of Treg cells to promote the immune escape of breast cancer via regulating miR-448/IDO.[13] Furthermore, IncRNA HISLA from tumor-associated macrophages regulates aerobic glycolysis in breast cancer cells.[14] Moreover, IncRNA BCRT1 promotes breast cancer progression by targeting miR-1303/PTBP3 axis.[15] In addition, IncRNA GATA3-AS1 facilitates tumor progression and immune escape in triple-negative breast cancer through destabilization of GATA3 and stabilization of PD-L1.[16]

Signature focusing on the tumor immune infiltration show promising predictive and prognostic value in the diagnosis, evaluation, and treatment of cancer. Individual genes are generally used to analyze immune-related signature to predict the prognosis of breast cancer. Here, we utilized a novel model of irlncRNAs pairing and iteration, which predicted breast cancer prognosis irrespective of the specific expression levels.

**Methods**

**Data Source, Preprocessing and Differentially Expressed Analysis**

The RNA-sequencing data consisting of 1164 female breast samples were downloaded from The Cancer Genome Atlas (TCGA) website (https://portal.gdc.cancer.gov). The corresponding clinical characteristics such as age, survival information, and clinical stage, were also downloaded from TCGA. R4.1.0 software was used to normalize, process, and analyze the data. Perl (https://www.perl.org) was used to convert the Ensembl ID of genes into a matrix of gene symbols and merge the RNA-sequencing data files into a matrix file. Gene transfer format (GTF) files were downloaded from Ensembl (http://asia.ensembl.org) to distinguish mRNAs from IncRNAs for further analysis. A list of recognized immune-related genes was downloaded from the ImmPort database (http://www.immport.org) and was used to screen irlncRNAs by a co-expression strategy. The immune-related genes with correlation coefficients of more than 0.4 and p-value less than 0.001 were considered as irlncRNAs. All methods were performed in accordance with the relevant guidelines and regulations.

To identify the differentially expressed irlncRNAs (DEirlncRNAs), we used the “limma” software package in R for differential expression analysis. The cutoff conditions were set as: |log2 fold change (log2FC)| >2.0, false discovery rate (FDR) <0.05.

**Pairing DEirlncRNAs**

The DEirlncRNAs were cyclically and separately paired, and a 0-or-1 matrix was constructed assuming Z is equal to IncRNA X plus IncRNA Y; Z is defined as 1 if the expression level of IncRNA X is higher than IncRNA Y, otherwise Z is defined as 0. Then, the constructed 0-or-1 matrix was further screened. When the expression quantity of IncRNA pairs was 0 or 1, there was no relationship between pairs and prognosis. An effective match was considered when the pairs of IncRNAs with 0 or 1 expression exceeded 20%.

**Construction of the Prognostic Model**

First, the survival information was combined with DEirlncRNAs pairs after which a univariate Cox regression analysis was conducted to select survival-related irlncRNAs pairs with p<0.01 as filter criteria. Then we divided the samples randomly and equally into a training set (n=518) and a validation set (n=517), combining the two sets was the total set. In the training set, the Lasso regression analysis was performed with cross validation to select the irlncRNAs pairs most correlated with prognosis. These final DEirln-
cRNAs pairs were used for the construction of the prognostic model. The following formula was used to calculate the risk score with the constructed prognostic model for all the cases: \[ \text{RiskScore} = \text{Exp1} \times \text{Coef1} + \text{Exp2} \times \text{Coef2} + \ldots + \text{Expn} \times \text{Coefn} \] \( \text{Exp} \) represents the expression level of each irLncRNA pair, and \( \text{Coef} \) represents the coefficient of each DEirLncRNA pair.

**Application and Validation of the Prognostic Model**

The receiver operating characteristic (ROC) curve was used to evaluate the predicted values of the model and the "survival ROC" package of R was used to calculate the area under the curve (AUC). The 1-, 5-, and 10-year ROC curves of the training set, the validation set, and the total set were plotted. According to the median risk score of the training set, the breast cancer patients were divided into the high- and low-risk groups within the three sets. The Kaplan-Meier log-rank analysis was used to compare the differences in survival between the two groups among the three sets using the “survival” package. The univariate and multivariate Cox regression analyses were performed to validate the relationship between the model and clinicopathological characteristics. Risk curves and point maps were used to observe the survival of patients. The Wilcoxon signed-rank test was used to analyze the differences in the risk score among groups with different clinicopathological characteristics. In addition to the above, R packages also included “survminer”, “pHeatmap”, “ggupbr”, and “complexHeatmap”.

**Exploration of Tumor-Infiltrating Immune Cells on Risk Score**

Some well-known methods including XCELL, TIMER, QUANTISEQ, MCPcounter, EPIC, CIBERSORT-ABS, and CIBERSORT were utilized to calculate the content of tumor-infiltrating immune cells of the cases downloaded from the TCGA website. Spearman correlation analysis was performed to evaluate the relationship between risk score and tumor-infiltrating immune cells. Wilcoxon signed-rank test was used to compare the differences in tumor-infiltrating immune cells content between high- and low-risk groups of the model. The results were shown as boxplots. These results were analyzed using the R “ggplot2” package, and \( p<0.05 \) was considered statistically significant.

**Investigation of Clinical Performance on Risk Score**

To investigate the clinical performance of the model, differential expression of immune checkpoints between high- and low-risk groups were compared. The results were shown in violin plots which were performed by “limma” and “ggpubr” packages of R. Furthermore, we calculated the half-maximal inhibitory concentration (IC50) of common chemotherapeutic drugs for breast cancer from the TCGA. According to the national comprehensive cancer network (NCCN), chemical drugs such as methotrexate, doxorubicin, gemcitabine, and paclitaxel are commonly used in the treatment of breast cancer. Wilcoxon signed-rank test was used to analyze the differences in the IC50 for the above-mentioned between the high-risk and low-risk groups. The results were shown as boxplots by using “pRrophetic” and “ggplot2” packages of R.

**Collection of Clinical Samples**

Fifteen female breast cancer patients from The First Affiliated Hospital of Anhui Medical University in the year of 2021 who had underwent modified radical mastectomy without preoperative therapy were collected in this study. Fresh breast cancer tissue samples were obtained and snap-frozen in liquid nitrogen immediately. The clinicopathological characteristics including patient age, grade, tumor size, lymph node status and the expression of ER, PR and Ki67 were collected. All patients included in this study signed informed consent and this study was approved by the Institutional Review Board of The First Affiliated Hospital of Anhui Medical University, Hefei, China.

**RNA Extraction and Quantitative Real-Time PCR Analysis (qRT-PCR)**

Total RNA of the tissue samples was extracted using TRIzol Reagent (Thermo Fisher Scientific, China) and reverse-transcribed into complementary DNA (cDNA) using the Evo M-MLV RT Premix (Accurate Biology, China) according to the manufacturer's instructions. QRT-PCR was performed using a SYBR Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China) and repeated 3 times. The detailed primer sequences of 18 IncRNAs in this study were shown in Table S1.

**Statistical Analysis**

For qRT-PCR, the mean cycle thresholds (CT) value was used to reflect lncRNA expression level and obtained the value of the IncRNA pairs. When assuming \( Z \) is equal to IncRNA X plus IncRNA Y; \( Z \) is defined as 1 if the expression level of IncRNA X is higher than IncRNA Y, otherwise \( Z \) is defined as 0. The risk score of each case was calculated by using the formula shown in “Construction of the prognostic model” section. All statistical analyses were performed using SPSS 22.0. Spearman's correlation coefficient was used to assess the correlation between clinicopathological parameters and risk score. Student t test was performed to compare values obtained from two groups. When \( p<0.05 \) were considered significant.
Results

Identification of DEirIncRNAs

The flow chart of the study was shown in Figure 1. Firstly, we downloaded transcriptome profiling consisting of 1053 breast cancer samples and 111 normal samples from TCGA website and then annotated according to GTF files from Ensembl. Next, we obtained the irIncRNAs by co-expression of immune-related genes from the ImmPort database. A total of 1041 irIncRNAs were identified (Table S2), and 55 were distinguished as DEirIncRNAs. Among the 55 distinguished DEirIncRNAs, 39 were upregulated while 16 were downregulated (Fig. 2a and b, Table S3).

Construction of DEirIncRNAs Pairs and Prognostic Model

According to the iteration loop and the 0 or 1 matrix, we obtained 1172 valid DEirIncRNAs pairs. Following, the DEirIncRNAs pairs were merged with the survival information. Through Cox regression analysis, we obtained 20 DEirIncRNAs pairs that were correlated with survival (Fig. 2c). Furthermore, 15 DEirIncRNAs pairs were included in the model after Lasso regression analysis (Fig. 2d and e, Table 1).

In order to verify the accuracy of the prognostic model, we drew the ROC curve and calculated AUC. We analyzed the model in the training set, and the 1-year AUC was 0.838, the 5-year AUC was 0.688, and the 10-year AUC was 0.788 (Fig. 3a). We validated the model in the validation set, and ob-
served that all the AUC values were more than 0.680 (1-year AUC = 0.760, 5-year AUC = 0.719, 10-year AUC = 0.683, Fig. 3b). Similar trend was obtained for the total set (1-year AUC = 0.797, 5-year AUC = 0.700, 10-year AUC = 0.736, Fig. 3c). According to the median value of risk score in the training set, all the samples were divided into the high-risk group and the low-risk group. We then performed a survival analysis of the training, the validation, and the total sets using Kaplan-Meier curves. As shown in figure 3d-f, patients in the high-risk group had worse overall survival than those in the low-risk group (p<0.001).

Assessment of the Correlation Between the Prognostic Model and Clinicopathological Characteristics

The risk curves and scatter plots were used to display the risk score and the survival outcome of each breast cancer patient in the training, the validation, and the total sets. The results showed that the mortality in the low-risk group was lower than in the high-risk group (Fig. 4a-c).

To evaluate whether the prognostic model of the DEirlnRNAs pairs was an independent prognostic factor for breast cancer, univariate and multivariate Cox regression analyses were conducted. In the training set, the hazard ratio (HR) of risk score and 95% CI were 1.733 and 1.419-2.116 in univariate Cox regression analysis (p<0.001), and 1.714 and 1.338-2.197 in multivariate Cox regression analysis (p<0.001) respectively (Fig. 4d). This suggested that the prognostic model of DEirlnRNAs pairs were independent prognostic factors in patients with breast cancer. In the validation set, the prognostic model also showed statistical

**Table 1. The prognostic model of 15 differentially expressed irlnRNAs pairs for breast cancer**

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Coef</th>
</tr>
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<tr>
<td>AP005131.7</td>
<td>LINC00511</td>
</tr>
<tr>
<td>AP005131.7</td>
<td>AC009093.1</td>
</tr>
<tr>
<td>U62317.4</td>
<td>AP000251.1</td>
</tr>
<tr>
<td>AC011247.1</td>
<td>ATP2A1-A51</td>
</tr>
<tr>
<td>C6orf99</td>
<td>LINC01087</td>
</tr>
<tr>
<td>C6orf99</td>
<td>U62317.1</td>
</tr>
<tr>
<td>C6orf99</td>
<td>AC020663.2</td>
</tr>
<tr>
<td>C6orf99</td>
<td>LINC02544</td>
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<tr>
<td>LINC01929</td>
<td>AC020663.2</td>
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<td>AP005131.2</td>
</tr>
<tr>
<td>U62317.1</td>
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<tr>
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<td>AP005233.2</td>
</tr>
<tr>
<td>LINC00511</td>
<td>LINC01152</td>
</tr>
<tr>
<td>AL356417.2</td>
<td>AP005131.2</td>
</tr>
<tr>
<td>AC009093.1</td>
<td>AP005131.2</td>
</tr>
</tbody>
</table>

**Figure 2.** Construction of a prognostic model using differentially expressed irlnRNAs (DEirlnRNAs) pairs. (a) The DEirlnRNAs showed by heatmap. (b) The DEirlnRNAs showed by volcano, red dots represented the upregulated while green dots represented the downregulated. (c) Forest plots showing HR of DEirlnRNAs pairs obtained by multivariate Cox regression analysis. (d) and (e) Lasso regression analysis to identify DEirlnRNAs most correlated with overall survival.
differences by univariate Cox regression analysis \( p<0.001, \) \( HR = 1.728, \) 95% CI [1.334-2.239]) and multivariate Cox regression analysis \( p<0.001, \) \( HR = 1.816, \) 95% CI [1.369-2.408], Fig. 4e). Similar results were obtained in the total set by univariate Cox regression analysis \( p<0.001, \) \( HR = 1.722, \) 95% CI [1.471-2.016]) and multivariate Cox regression analysis \( p<0.001, \) \( HR = 1.697, \) 95% CI [1.416 -2.035], Fig. 4f).

Furthermore, we compared the differences in clinicopathological characteristics between the low- and high-risk group of all the samples (Fig. 5a). As shown by the strip chart and scatter plots, ER, PR, age, TNM stage, T stage, M stage, and N stage were significantly related to the risk score (Fig. 5b-h).

**Estimation of Tumor-Infiltrating Immune Cells and Immune Checkpoints with the Prognostic Model**

Because lncRNAs play an important role in tumor immunity, we investigated the relationship between the model and the tumor immune microenvironment using all the breast cancer samples. As shown in Figure 6a and b, the immune score and stroma score were higher in the low-risk group than in the high-risk group \( p<0.05 \). We compared various tumor-infiltrating immune cells between the high-risk and low-risk groups, the results are listed in Figure S1. In addition, we integrated the relationships between various immune cells within the risk group performed by Spearman correlation analysis into a bubble chart. The results showed that the risk score was more positively associated with M2 macrophages, cancer-associated fibroblasts and neutrophils, whereas they were negatively associated with T cells, CD8+ T cells, NK cells, B cells, and M1 macrophages (Fig. 6c, Table S4).

**Immunotherapy is a novel and effective treatment method for breast cancer.** We analyzed differences in immune checkpoints common in breast cancer between high- and low-risk groups. The expression of PCDC1 (PD-1), CD274 (PD-L1), CTLA4, and CDK4 were significantly lower in the high-risk group (Fig. 7a-d).

**Analysis of the Correlation Between the Prognostic Model and Chemotherapeutic Drugs**

The prediction of how effective a chemotherapy drug can guide the selection of clinical drugs. We assessed the correlation between the prognostic model and the efficacy of common chemotherapeutics used in the treatment of breast cancer. A higher IC50 of chemotherapeutic agents, such as methotrexate, doxorubicin, and gemcitabine were associated with the high-risk group \( p<0.05, \) Fig. g. While paclitaxel had no significance with the model \( p>0.05, \) Fig. 7h). The data indicated that the model might predict the treatment response to chemotherapy agents.
Figure 4. The survival outcome of breast cancer patients. Risk curves and scatter plot showed the survival outcome of each breast cancer patient in the training (a), validation (b) and total sets (c). The green and red dots respectively represent survival and death. The univariate and multivariate Cox regression analyses for evaluating the independent prognostic value of the prognostic model and clinicopathologic parameters in the training (d), validation (e) and total sets (f). P<0.05 was considered statistically significant.

Figure 5. Evaluation of clinicopathological characteristics by the prognostic model. (a) The strip chart showed the differences in clinicopathological characteristics between high- and low-risk groups. (b-h) Scatter plots showed the differences in risk score among different clinical feature groupings. *p<0.05, **p<0.01, ***p<0.001.
Validation of the Prognostic Model Using Clinical Samples by qRT-PCR

To further determine the accuracy of this model, we collected 15 breast cancer fresh samples and carried out qRT-PCR experiments, finally obtained the risk score for each patient based on the coefficients of DEirlncRNAs pairs (Table 2). We grouped patient age, grade, T stage, N stage, ER/PR and KI67 index, then compared the differences in risk score between different groups. There was no statistical significance between the different groups (Fig. 8a-f). Afterward, we analyzed the correlation between risk score with either age or KI67 index, the results showed both statistical significance (p<0.05, Fig. 8g and h). The results were almost in line with our previous data.

Discussion

Breast cancer is the most commonly diagnosed cancer among women worldwide. Although the 5-year survival rate of stage II breast cancer is about 93% and 72% for stage III, breast cancer is still the leading cause of cancer death. Therefore, besides the traditional clinical risk factors, additional biomarkers to predict the prognosis and treatment of breast cancer are needed as well.

In recent years, the utility of immunotherapy for the treatment of breast cancer patients has widely gained attention. Although previous studies have used immune-related lncRNAs to predict breast cancer prognosis, the signature in those studies was constructed using a single gene. Herein, our study presents the first report using a differentially expressed irlncRNAs pairs model to predict the treatment and prognosis of breast cancer. The two-lncRNA pairs were superior to a single gene, that it is not dependent on the expression levels of each gene, but the relative expression of the two genes. For different detection systems, data correction can be done without.

To construct the model, we downloaded breast cancer transcription data from TCGA database and obtained DEirlncRNAs. The DEirlncRNAs pairs were identified using an improved method of cyclically and singly pairing along with a 0 or 1 matrix. We further performed univariate regression analysis to select survival-related DEirlncRNAs pairs. To better verify the accuracy of the model, we randomly and equally divided the samples into the training set and the validation set. The Lasso regression analysis was used to screen the DEirlncRNAs pairs for the prog-
nostic model construction in the training set. According to the median value of risk score in the training set, all the patients, whether in the training set or the validation set, were divided into high-risk and low-risk groups. Next, we calculated the AUC value from the ROC curve to validate the prognostic model. Finally, we evaluated the accuracy of the model by analyzing the differences in various clinical factors including survival, clinicopathological features, tumor-infiltrating immune cells, and immune checkpoints.

In our signature, 15 DEirlncRNAs pairs consisting of 18

Table 2. Risk score and clinicopathological characteristics of breast cancer patients

<table>
<thead>
<tr>
<th>Samples</th>
<th>Risk score</th>
<th>Age(y)*</th>
<th>Grade</th>
<th>T</th>
<th>N</th>
<th>ER</th>
<th>PR</th>
<th>KI67(%)#</th>
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<td>-</td>
<td>+</td>
<td>+</td>
<td>10</td>
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<td>2</td>
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<td>+</td>
<td>25</td>
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<tr>
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<td>1</td>
<td>\</td>
<td>+</td>
<td>+</td>
<td>12</td>
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<td>+</td>
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<td>2</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>-</td>
<td>50</td>
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</table>

*: Age was correlated to risk score (R = 0.517, p<0.05). #: K167 was correlated to risk score (R = 0.532, p<0.05). -: negative; +: positive.
DEirlncRNAs were used to construct the model. Some of the DEirlncRNAs identified in our study have been reported to play an important role in malignant tumors. For example, LINC00511 may contribute to breast cancer tumorigenesis, proliferation, migration and invasion, and stemness.[21, 22] The tumor-promoting functions of LINC00511 have also been reported in gastric cancer,[23, 24] hepatocellular carcinoma,[25, 26] colorectal cancer[27, 28] and bladder cancer.[29, 30] LINC01087 could represent a novel, specific and promising biomarker not only for the diagnosis and prognosis of luminal subtypes and triple-negative breast cancers but also as a predictive biomarker of pharmacological interventions.[31] It has also been reported that the overexpression of LINC01087 in breast cancer can promote the invasion and migration of breast cancer cells.[32] LINC02544 may promote proliferation, invasion, and migration of breast cancer cells after neoadjuvant chemotherapy.[33] LINC01152 could induce tumorigenesis in glioblastoma via the Notch signaling pathway[34] and promotes cell proliferation and survival in hepatocellular carcinoma.[35] Amelia et al. found that there were many IncRNAs dysregulation in non-small cell lung carcinoma, in which LINC01929 was upregulated.[36] LINC01929 functioned as a tumor-promoting IncRNA in oral squamous cell carcinoma via the miR-137-3p/FOXC1 axis.[37] In addition, some DEirlncRNAs such as AL356417.2, AP005233.2, ATP2A1-AS1, C6orf99, U62317.1, and U62317.4 appear only in bioassay and have not been experimentally confirmed. Other DEirlncRNAs, AC009093.1, AC011247.1, AC020663.2, AP000251.1, AP005131.2, AP005131.7, and ZNF350-AS1 were revealed for the first time.

Over the years, the role of immune cells in breast cancer has been increasingly discovered. To investigate the relationship between risk score and tumor-infiltrating immune cells, we used seven common acceptable methods including XCELL, TIMER, QUANTISEQ, MCPcounter, EPIC, CIBERSORT-ABS, and CIBERSORT to estimate the infiltration of immune cells in breast cancer samples. Due to the differences and complexity among the various algorithms, the results were not compared with each other. Our results showed that the risk score was more positively related to cancer-promoting immune cells like M2 macrophages and cancer-associated fibroblast, while negatively related to anti-cancer immune cells like T cells, CD8+ T cells, NK cells, B cells, and M1 macrophages. In addition to immune cells, we also analyzed the relationship between risk score and immune checkpoints common to breast cancer. Immune checkpoints, especially those associated with T cells, have
been used in clinical treatment. The first immunotherapies to the immunomodulatory receptor CTLA4 and blockade of the immunoinhibitory receptor PD-1 in cancer immunotherapy have created a paradigm of cancer therapy.[11, 38] Based on our results, PCDC1 (PD-1), CD274 (PD-L1), and CTLA4 were highly expressed in the low-risk group. Our findings suggested that the prognostic model can predict the efficacy of immunotherapy in clinical settings.

Chemotherapy is a common treatment for breast cancer and hence we analyzed the relationship between risk score and IC50 of 4 common chemotherapy drugs used in breast cancer treatment. It was observed that methotrexate, doxorubicin, and gemcitabine were associated with the high-risk group. This finding showed that the prognostic model could indicate the chemotherapeutics sensitivity. In addition, we collected breast cancer samples and detected the relative expression levels of 18 lncRNAs by qRT-PCR. Based on the contrasting CT values, 1 or 0 was obtained for each lncRNA pair, then 1 or 0 was multiplied by the coefficients to obtain the risk score for each patient. The risk score were then used for subsequent analysis. According to the model results, the risk score was statistically significant with patient age, TNM stage, T stage, M stage, N stage, ER, PR and expression. Although in our samples, the risk values were not statistically significant in the age, T stage, N stage, ER, PR and KI67 index subgroups, the trend was the same as in the model. Small sample covering only 15 cases may be the reason. Additionally, we analyzed the correlation of risk values with either patient age or KI67 index, and the results showed statistically significant. These results indicated that the established risk-predicting model may have potential value for predicting patient outcomes of breast cancer. Further investigation to validate the model needs to be carried with large sample set and patient follow up.

Conclusion

We constructed a prognostic model, which based on fifteen pairs of lncRNAs, predicting both prognosis of breast cancer and efficacy of immunotherapy and chemotherapy as well.

Disclosures

Ethics Committee Approval: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Review Board of The First Affiliated Hospital of Anhui Medical University, Hefei, China (No. 5101389).

Peer-review: Externally peer-reviewed.

Conflict of Interest: None declared.

Funding: This study was supported by Hefei Municipal Natural Science Foundation (2022037) and The Natural Science Fund for Colleges and Universities in Anhui Province (KJ2021ZD0018).


References


