Research Article

Necroptosis-Associated IncRNA Prognostic Model and Clustering Analysis: Prognosis Prediction and Tumor-Infiltrating Lymphocytes in Breast Cancer

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Received 28 January 2022; Revised 15 March 2022; Accepted 24 March 2022; Published 27 April 2022

Academic Editor: Qin Yuan

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Necroptosis plays an important role in tumor genesis and progression. This study aims to identify necroptosis-related lncRNAs (NR-lncRNAs) in breast cancer (BC), and their prognostic value and relationship with the tumor immune environment (TIE) through bioinformatics.

Methods. A total of 67 necroptosis-related genes (NRGs) are retrieved, and 13 prognostically relevant NR-lncRNAs are identified by co-expression and Univariate Cox regression analyses. After unsupervised clustering analysis, the patients are classified into three clusters, and their survival and immune infiltration are compared. Lasso regression analysis is conducted to construct a prognostic model using eight lncRNAs (USP30-AS1, AC097662.1, AC007686.3, AL133467.1, AP006284.1, NDUFA6-DT, LINC01871, AL135818.1). The model is validated by Kaplan-Meier survival analysis, Multivariate Cox regression analysis, and receiver-operating characteristic (ROC) curves. Correlation analysis is useful to identify associations between risk scores and clinicopathological features. GSEA, drug prediction, and immune checkpoints analysis are further used to differentiate between the risk groups. Results. The C3 cluster has longer overall survival (OS) and the highest immune score, indicative of an immunologically hot tumor that may be sensitive to immunotherapy. Furthermore, the OS is significantly higher in the low-risk group, even after dividing the patients into subgroups with different clinical characteristics. The area under the ROC curve (AUC) for 1-, 3-, and 5-year survival in the training set are 0.761, 0.734, and 0.664, respectively, which indicate the moderate predictive performance of the model. Conclusion. NR-lncRNAs can predict the prognosis of BC, distinguish between hot and cold tumors, and are potential predictive markers of the immunotherapy response.

1. Introduction

Breast cancer (BC) is the most prevalent malignancy in women and has surpassed lung cancer in terms of incidence worldwide [1]. The current therapeutic approaches have increased the 5-year survival rate of BC patients to 90% in developed countries, although the rate varies greatly by region [2]. Although long considered non-immunogenic or weakly immunogenic tumors, there is a renewed interest in developing immunotherapies against BC due to the encouraging outcomes of other tumors such as melanoma and non-small cell lung cancer. In fact, immune checkpoint inhibitors have been effective against BC in clinical practice. However, only a fraction of cancer patients benefit from immunotherapy due to considerable heterogeneity in treatment sensitivity and side effects [3]. Furthermore, there is a paucity of reliable biomarkers for monitoring the effects of immunotherapy. Thus, it is crucial to identify novel biomarkers to predict the efficacy of immunotherapy and devise individualized therapy plans for cancer patients [4].

Recent studies suggest that some pathological subgroups of breast tumors are rich in tumor-infiltrating lymphocytes (TILs), which serve as a reliable prognostic biomarker of BC. For every 10% increase in TILs, the risk of recurrence and death decreases by varying degrees [5–8]. In particular, the infiltration of cytotoxic CD8+ T lymphocytes, CD4+ T
lymphocytes, and tumor-associated macrophages (TAMs) predicts favorable outcomes [9, 10]. Since chemotherapeutic
drugs can enhance the anti-tumor immune response by
clearing the immunosuppressive cells or coaxing the tumor
cells to release neoantigens [11, 12], higher levels of TILs
can improve tumor response to immune checkpoint inhibi-
tors [4].

Necroptosis is a programmed form of necrosis that
occurs in a caspase-independent manner and partly via
the apoptosis pathways [13]. Apoptosis inhibition is one of the
mechanisms employed by tumor cells to acquire drug resis-
tance, which leads to chemotherapy failure [14]. Induction
of necroptosis is a potential therapeutic alternative given its
crucial role in tumorigenesis, metastasis, and anti-tumor
immunity [15]. Furthermore, the necroptosis signaling
factor RIPK3 is known to regulate the function of dendritic
cells (DCs) and natural killer cells (NKS), and Fas-associated
death domain (FADD) can restrain T cell-mediated necropto-
ic signaling [16–18]. Necroptosis also initiates an adaptive
immune response by liberating damage-associated molecular
patterns (DAMPs) from the dying tumor cells, prompting
the phagocytes to release pro-inflammatory cytokines [19,
20]. However, there are reports that necroptosis can promote
tumor progression by recruiting pro-tumorigenic inflamma-
tory cells [21]. Therefore, the exact role of necroptosis in
tumor progression and immune response needs further
investigation.

The long non-coding RNAs (lncRNAs) regulate the
expression of genes involved in cell cycle control and signal
transduction pathways, thereby affecting tumor cell prolifera-
tion, apoptosis, metastasis, and invasion [22, 23]. In addition,
several lncRNAs have been identified that are related to the
functional regulation of TILs [24]. For instance, low expres-
sion of lncRNA BM466146 in BC cells allows more competi-
tive endogenous RNAs (ceRNAs) to bind to hsa-miR-224-
3p, which upregulates CXCL-13 and eventually activates the
cytotoxic CD8+ T cells [25]. Likewise, silencing the lncRNA
SNHG1 inhibits the differentiation of Treg cells and immune
escape of BC cells by promoting miR-448 expression and
reducing indoleamine 2, 3-dioxygenase (IDO) levels [26].
In this study, we identified the necroptosis-related lncRNAs
(NR-lncRNAs) in BC and compared the immune signatures
of patients classified based on NR-lncRNAs to screen ideal
candidates for immunotherapy and improve prognosis.

2. Materials and Methods

2.1. Data Extraction. Sixty-seven necroptosis-related genes
(NRGs) were obtained by reviewing relevant literature and
are listed in Appendix I [27]. The survival and transcripto-
omic (FPKM format) data of 1078 BC samples was retrieved
from the TCGA database (https://portal.gdc.cancer.gov/
projects/TCGA-BRCA). The data were extracted and col-
lated using Strawberry Perl.

2.2. Identification of NR-lncRNAs. The transcriptomic data
was divided into lncRNA and mRNA data using Strawberry
Perl software and annotation files. The expression matrix
data of the 67 NRGs were extracted using the limma R pack-
age with correlation factor =0.4 and p value=0.001 as the
thresholds. A total of 1470 NR-lncRNAs were identified
by co-expression analysis, organized into expression matri-
ces, and merged with the survival data. Univariate Cox
regression analysis was conducted using the survival R
package with p value=0.01 as the threshold, and 13 prog-
nostically relevant lncRNAs (PR-lncRNAs) were identified
and used to draw the corresponding forest plots. The differ-
entially expressed PR-lncRNAs between tumor and normal
samples were identified using the limma R package (*p <
0.05, **p < 0.01, ***p < 0.001).

2.3. Immune Infiltration Analysis. The potential subgroups
of BC based on PR-lncRNAs were identified using the Con-
sensusClusterPlus R package. The survivals of patients in the
different subgroups were compared using the Kaplan-Meier
method. The correlation between the expression of PR-
lncRNAs and that of PD-L1 and CTLA-4 was also analyzed.
The differential expression of PR-lncRNAs between sub-
groups and their correlation were analyzed using the limma
R package. Immune cell infiltration in the different sub-
groups was evaluated using the CIBERSORT algorithm,
and the ESTIMATE algorithm was used to calculate the
Immune, Stromal, and ESTIMATE scores of each sample
to assess tumor purity.

2.4. Construction and Validation of NR-lncRNAs Prognostic
Model. The samples were divided into the training set and
the validation set at a 1:1 ratio using the caret R package.
The clinical characteristics of patients in the two data sets
were compared by the chi-square test. The PR-lncRNAs
were selected for Lasso regression analysis to avoid overfit-
ting and remove closely related genes. In contrast, the minor
penalty term (λ) was chosen using cross-validation. The
optimal model for predictive performance was then selected
using the glmnet R package, and constructed based on eight
PR-lncRNAs. The equation for the risk score is shown below:

$$\text{Risk score} = \sum_{i=1}^{n} \text{coef}_i * x_i,$$  

where coef_i represents the risk coefficient and x_i represents
the expression of each lncRNA. Patients were classified into
high-risk and low-risk groups based on the median risk
score. The R packages survival, survminer, and timeROC
were used for survival analysis and model evaluation.
Kaplan-Meier survival curves and ROC curves for 1-, 3-
and 5-year survival were plotted for the training and valida-
tion sets.

The independent prognostic value of the model was con-
ﬁrmed by Univariate and Multivariate Cox regression ana-
lyses, and the survival rates in different subgroups classified
on the basis of age and tumor stages were compared. The differ-
ences in risk scores between the above subgroups, and the
differential expression of the eight PR-lncRNAs between
high- and low-risk groups were analyzed using R packages
limma and ggpubr.
Figure 1: Extraction and unsupervised consensus clustering analysis of 13 prognosis-related lncRNAs. (a). Forest plot of 13 prognosis-related lncRNAs based on Univariate Cox regression analysis. (b). Heat map showing the expression of 13 prognosis-related lncRNAs between tumor and normal samples. (c). Consensus matrix $k=3$. (d). Kaplan-Meier survival curves for C1, C2, and C3 clusters.
2.5. Comparison of High- and Low-Risk Groups. The correlation between immune cells and risk score was analyzed using R packages limma, ggplot2, ggpubr, and ggExtra. A total of 47 immune checkpoints were obtained by scanning relevant literature (in Appendix 2). Differential expression analysis of immune checkpoint genes between risk subgroups was performed using the limma package (*p < 0.05, **p < 0.01, ***p < 0.001). The pRRophetic package was used to

Figure 2: Correlation of prognosis-related lncRNAs with the tumor immune landscape. Correlation of 13 prognosis-related lncRNAs with (a) PD-L1 expression, (b) CTLA-4 expression, (c) Immune scores, (d) Stroma scores, (e) ESTIMATE scores, and (f) Immune cell infiltration in the C1, C2, and C3 clusters.
predict the IC50 of anti-tumor drugs to identify responsive subgroups. GSEA 4.1.0 was used to compare gene set functions between the high- and low-risk groups with $p < 0.05$ and FDR $< 0.25$ as the criteria.

3. Results

3.1. Identification of Prognostically Relevant NR-lncRNAs in BC. We extracted 67 NRGs from the mRNA expression matrix of BC samples from the Cancer Genome Atlas and identified 1470 NR-lncRNAs through co-expression analysis. Furthermore, 13 of these NR-lncRNAs were prognostically significant, including the pro-oncogenic AC097662.1, AC005034.5, and AC007319.1 (HR>1), and the anti-oncogenic USP30-AS1, AC007686.3, AL513190.1, AL133467.1, COL4A2-AS1, AP006284.1, AC136475.2, NDUFA6-AS1, LINC01871, and AL135818.1 (HR<1; $p < 0.01$) (Figure 1(a)). Each of the above lncRNAs was

![Figure 3: Construction of risk model. (a) and (b) Eight lncRNAs identified by the Lasso regression analysis. (c) and (d) Risk scores of patients in the (c) training and (d) validation sets. (e) and (f) Survival status of patients in the (e) training and (f) validation sets. (g) and (h) Expression levels of 8 lncRNAs in the high- and low-risk groups in (g) training and (h) validation sets.](image-url)
differentially expressed between the tumor and normal samples (Figure 1(b), Figure S1).

3.2. The PR-lncRNAs of BC Correlate with Immune Checkpoints and TILs. The patients were regrouped into three clusters on the basis of the PR-lncRNAs, of which C1 showed lower overall survival (OS) rates than that of C2 or C3, indicating that the PR-lncRNAs are strongly associated with the prognosis of BC (Figures 1(c) and 1(d)). In addition, all PR-lncRNAs except AC097662.1 and COL4A2-AS1 showed significant differences in their expression level between at least two patient clusters (Figure S2, S3). We further analyzed the correlation between the PR-lncRNAs and immune checkpoints PD-L1 and CTLA-4. PD-L1 was positively correlated with USP30-AS1, AL513190.1, AL133467.1, AC007319.1, LINC01871, and AL135818.1, and negatively with AP006284.1. CTLA-4 showed positive correlation with USP30-AS1, AC097662.1, AC007686.3, AL133467.1, AP006284.1, NDUFA6-DT, LINC01871, and AL135818.1, and negative correlation with AP006284.1 (Figures 2(a) and 2(b)).

The C3 cluster had the highest Immune and ESTIMATE scores, followed by C2 and C1 (Figures 2(c)–2(e)). Furthermore, the anti-tumor immune cell populations such as CD4+ activated memory T cells, CD8+ T cells, follicular helper T cells, memory B cells, M1 macrophages, and NK cells were more abundant in the C2 and C3 clusters. In contrast, the infiltration of the CD4+ resting memory T cells, naive B cells, M0 and M2 macrophages, resting mast cells, and resting NK cells was either higher in the C1 cluster or similar among all clusters. Monocytes and neutrophils were equally abundant in all three clusters (Figure 2(f), Figure S4).

3.3. Construction and Validation of Risk Model. Lasso regression analysis was performed with the 13 PR-lncRNAs, and USP30-AS1, AC097662.1, AC007686.3, AL133467.1, AP006284.1, NDUFA6-DT, LINC01871, and AL135818.1 were used to construct the prognostic model (Figures 3(a) and 3(b)). The patients were divided into the training and validation sets, which were comparable since the clinico-pathological features did not show significant differences (Table 1). The risk score, survival status, and expression of the 13 PR-lncRNAs in both data sets are shown in Figure 3 (training set - a, c, e, and g; validation - d, f, and h). The risk score was calculated as follows: USP30-AS1 expression * (-0.0051) +

Table 1: Clinical features of training and validation sets.

| Variables | Training set (n=540) | Validation set (n=538) | p-value |
|-----------|----------------------|------------------------|---------|
| Age       |                      |                        |         |
| < =60     | 295                  | 299                    | 0.8017  |
| >60       | 245                  | 239                    |         |
| Gender    |                      |                        |         |
| Female    | 540                  | 538                    | 0.9514  |
| Stage     |                      |                        |         |
| I         | 88                   | 93                     | 0.895   |
| II        | 312                  | 299                    |         |
| III       | 119                  | 125                    |         |
| IV        | 10                   | 9                      |         |
| Unknown   | 11                   | 12                     |         |
| T stage   |                      |                        | 0.059   |
| T1        | 142                  | 136                    |         |
| T2        | 324                  | 297                    |         |
| T3        | 54                   | 83                     |         |
| T4        | 20                   | 19                     |         |
| Unknown   | 0                    | 3                      |         |
| N stage   |                      |                        | 0.6715  |
| N0        | 260                  | 250                    |         |
| N1        | 170                  | 184                    |         |
| N2        | 64                   | 55                     |         |
| N3        | 36                   | 39                     |         |
| Unknown   | 10                   | 10                     |         |
| M stage   |                      |                        | 0.6735  |
| M0        | 449                  | 448                    |         |
| M1        | 12                   | 9                      |         |
| Unknown   | 79                   | 81                     |         |

Table 1: Clinical features of training and validation sets.
Figure 4: Continued.
AC097662.1 expression * (1.4470) + AC007686.3 expression *
(-0.4771) + AL133467.1 expression * (-0.0146) + AP006284.1 expression * (-0.0188) + NDUA6-DT expression * (-1.6889) + LINC01871 expression * (-0.0852) + AL135818.1 expression *
(-1.846). Kaplan-Meier survival analysis of both training and vali-
dation sets revealed that the high-risk group had signifi-
cantly worse OS than the low-risk group (Figures 4(a) and
4(b)). Furthermore, the area under the ROC curve (AUC) of
1-, 3-, and 5-year OS for the training set was 0.761,
0.734, and 0.664, and that for the validation set was 0.653,
0.667, and 0.623, respectively (Figures 4(g–l)), which
indicated the predictive value of the risk score. Multivariate
Cox regression analysis further showed that the risk score
was an independent predictor of worse survival as opposed
to age and tumor stage. Therefore, the risk score-based
model may be more reliable than clinicopathological factors
for predicting the patient prognosis (Figures 4(c–f)). We
also compared the OS of the high- and low-risk groups
that were divided into subgroups of age (<60 years and >60
years) and tumor stage (I-II, III-IV, T1-2, T3-4, N0, N1,
M0, and M1), and found that the high-risk group had
shorter OS regardless of the age and tumor stage
(Figures 5(a)–5(j))). Taken together, the lncRNA-based risk
model can be applied to elderly and young, as well as early
to advanced BC patients.

3.4. The Necroptosis Risk Score Correlated with Tumor
Immunie Status. The risk score was significantly different
between the M0 vs. M1, C1 vs. C2 or C3, and high- vs.
low-immune infiltration groups. Patients without distant
metastases, in clusters C2 and C3, or with high-immune
scores usually presented lower risk scores and better survival
prognoses (Figures 6(a)–6(g)). Furthermore, AC097662.1
was highly expressed in the high-risk group, while the other
lncRNAs showed the opposite trend corresponding to their
risk coefficients in the prognostic model (Figure 6(h)). These
findings indicate that necroptosis is associated with the
tumor immune status and distant metastasis. The predomi-
nant tumor-infiltrating immune cells in the low-risk group
were naïve B cells, monocytes, activated NK cells, plasma
cells, CD4+ activated memory T cells, and CD8+ T cells.
In contrast, high infiltration of M0 and M2 macrophages
and neutrophils was observed in the high-risk group
(Figures 7(a)–7(j)). The immune cells enriched in the low-
risk group were essentially the same as observed for the C2
or C3 clusters, whereas that in the high-risk group corre-
sponded to the immune cell profile of the C1 cluster. Taken
together, the risk score can effectively predict the infiltrating
immune cells in the breast tumor microenvironment.

3.5. The Necroptosis Risk Score Can Predict Response to
Immune Checkpoint Inhibitors. We next compared the
expression of immunotherapy-related genes and the IC50
of therapeutic agents between the two risk groups. Immune
checkpoints including PD-L1 (CD274), CD28 (CTLA-4
homolog), and CTLA-4, which are closely associated with
BC immunotherapy, were enriched in the low-risk group
(Figure 7(k)), indicating that these patients may be more
sensitive to immunotherapy. In addition, therapeutic agents
except A.443654, A.770041, AZD.0530, Bicalutamide,
BMS.708163, BMS.754807, BX.795, CMK, Erlotinib, GNF.2
JN,26854165, KIN001.135, and Lapatinib had lower IC50
values in the low-risk group (Figure S5, S6). To summarize,
patients in the C2 and C3 clusters with higher immunogeni-
icity, higher sensitivity to immunotherapy, and lower risk
score had better survival prognoses, whereas the C1 cluster
exhibited the opposite trend. Therefore, the necroptosis-
based risk score can be used to distinguish between the
immunologically “cold” and “hot” breast tumors to screen
for patients that may benefit from immunotherapy.

3.6. GSEA between High- and Low-Risk Groups. GSEA
showed that the gene sets associated with apoptosis, immune
function, lipid oxidation metabolism, and chemokine signal-
ing, including apoptosis, T cell receptor signaling, B cell
receptor signaling, natural killer cell-mediated cytotoxicity,
antigen processing and presentation, chemokine signaling,
Fc epsilon RI signaling, Fc gamma receptor-mediated
phagocytosis, glycerophospholipid metabolism, alpha linole-
ic acid metabolism, linoleic acid metabolism, arachidonic
acid metabolism, and chemokine signaling, were signifi-
cantly enriched in the low-risk group (Figures 8(a)–8(m)).
In the high-risk group, the p53 signaling pathway and
necroptosis regulation-related gene sets were enriched,
namely, the p53 signaling and ubiquitin-mediated

Figure 4: Validation of risk model. (a) and (b) Kaplan-Meier survival curves of high- and low-risk groups in the (a) training and (b) validation sets. (c) and (d) Univariate Cox regression analysis for risk score and clinicopathological features in the (c) training and (d) validation sets. (e) and (f) Multivariate Cox regression analysis of the risk score in (e) training and (f) validation sets. (g)–(l) The AUC of 1-, 3-, and 5-year OS in the (g)–(i) training and (j)–(l) validation sets.
Figure 5: Kaplan-Meier survival curves of the high- and low-risk patients in the <60 years, >60 years, Stage I-II, Stage III-IV, T1-2, T3-4, N0, N1, M0, and M1 subgroups.
Figure 6: Continued.
proteolysis (Figures 8(n) and 8(o)). The enrichment in gene sets of immune-related functions in the low-risk group was consistent with abundant immune cell infiltration in the tumor microenvironment.

4. Discussion

4.1. Unsupervised Consensus Clustering Analysis. BC is highly heterogeneous in terms of tumor morphology, prognosis, and treatment response. Although receiving maximum existing therapeutic regimens, there are 20% of patients still die, and 85% of the patients do not respond to conventional chemotherapy. BC is currently classified into pathological subtypes based on estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor 2 (HERT2), which cannot predict the response of individual patients to precision treatment regimens [28, 29]. To this end, we developed a model to predict the immunotherapy response of breast tumors based on necroptosis-related lncRNAs. Unsupervised consensus clustering analysis identified 13 PR-lncRNAs, and the patients were accordingly divided into three clusters with distinct necroptosis profiles. The C2 and C3 clusters showed higher survival rates than the C1 cluster, which led us to analyze their correlation with immune characteristics and model risk scores.

4.2. Construction and Validation of Risk Model. We further screened eight lncRNAs through Univariate Cox and Lasso regression analyses to construct a prognostic model, of which some have been previously associated with tumor development or prognosis. For instance, AL133467.1 is associated with a favorable immune landscape in ovarian tumors, which correlates with a good prognosis [30]. In our study, AL133467.1 was found to be beneficial for the survival of BC patients. LINC01871 is an established protective factor in BC and is associated with autophagy, ferroptosis, and tumor stem cells [31–33]. This was consistent with our findings, which led us to hypothesize that the NRGs co-expressed with LINC01871 may promote cancer cell death by necroptosis. USP30-AS1 is an intra-mitochondrial lncRNA that inhibits mitophagy and promotes mitochondrial dysfunction and oncogenic progression [34]. In addition, USP30-AS1 increases the oncogenicity of cervical cancer cells by upregulating PTP4A1 through the USP30-AS1/miR-299-3p/PTP4A1 network [35]. Likewise, USP30-AS1 was identified as an oncogene in BC, and could be a potential therapeutic target. The risk score of the lncRNA-based model was an independent predictor of poor prognosis after adjusting for age, tumor stage, and TNM stage. Furthermore, ROC curves proved the predictive performance of the model, and the survival analysis of high- and low-risk groups demarcated on the basis of age and tumor stage indicated that the model is broadly applicable to the BC patient population.

4.3. Association of Risk Score with Clinicopathological Factors, Clusters, and Immune Infiltration. We observed that the risk scores were lower in M0 versus M1 stage, although no study so far has reported necroptosis-associated lncRNAs as a risk factor for distant metastases in BC. However, similar risk scores of the older patients or those at higher tumor stages compared to their respective counterparts did not show significant differences.
Figure 7: Continued.
translate to similar survival prognoses, indicating that the combination of risk scores and clinicopathological features can better predict the prognosis of BC patients. The risk scores for C2 and C3 clusters were similar and significantly lower than that of C1 cluster, which was consistent with their respective survival prognoses. Patients with high immune scores, which corresponded to higher levels of TILs, had lower risk scores and a favorable prognosis. These findings are consistent with the current mainstream view on the role of TILs. Patients in the C2 and C3 clusters, and those with high immune scores were classified into the low-risk group, whereas the C1 cluster and low-immune scores comprised the high-risk group.

4.4. Cluster and Immune Infiltration Analysis. Studies show that increased infiltration of immune cells into tumor masses and high expression levels of PD-1/PD-L1 and CTLA-4 correlates with a favorable prognosis of BC [36, 37]. This suggests that IncRNAs associated with PD-L1 or CTLA-4 expression may be relevant to the immunotherapeutic response in BC, although there have been no reports so far. Consistent with our findings, the C3 cluster had significantly higher immune and ESTIMATE scores than C1. Interestingly, C2 had significantly lower immune, stromal, and ESTIMATE scores compared to C1, which did not correlate with the better prognosis in the former. The infiltration of memory B cells, CD8+ T cells, CD4+ T cells, follicular helper T cells, DCs, NK cells, and M1 macrophages was associated with anti-tumor effects, whereas Tregs and M2 macrophages promote tumor growth [38–40]. The anti-tumor immune cell types were significantly more enriched, whereas the M2 macrophages were less in the C3 cluster patients. Although C2 had fewer CD4 T cells compared to C1, it had a greater abundance of CD8+ T cells, NK cells, and plasma cells, which may explain the better survival prognosis in spite of a lower immune score. The γ/δ T cells have both anti- and pro-tumorigenic functions [41] and were more abundant in the C3 clusters, although the role of this subset in BC has not been fully elucidated. A recent study showed that N1-type neutrophils inhibit tumor growth, whereas the N2-type neutrophils have a pro-cancer effect [42]. Infiltrating neutrophils were overall lower in the BC samples and did not differ between the clusters. Further studies are needed to explore the immune environment of breast tumors.

4.5. Immune Responsiveness of BC Patients. The low-risk group and the C3 and C2 clusters had similar immune cell infiltration. Immune checkpoint analysis further suggested that the low-risk group may be more sensitive to immunotherapy, and the results of drug sensitivity analysis can help screen potential therapeutic agents for low-risk patients. In
Figure 8: Continued.
Figure 8: Enrichment plots of apoptosis, immune associated, lipid oxidation metabolism, and chemokine signaling gene sets from GSEA. (a) KEGG_APOPTOSIS, (b) KEGG_B_CELL_RECEPTOR_SIGNALING_PATHWAY, (c) KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY, (d) KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY, (e) KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION, (f) KEGG_CHEMOKINE_SIGNALING_PATHWAY, (g) KEGG_FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS, (h) KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY, (i) KEGG_GLYCEROPHOSPHOLIPID_METABOLISM, (j) KEGG_ALPHA_LINOLENIC_ACID_METABOLISM, (k) KEGG_LINOLEIC_ACID_METABOLISM, (l) KEGG_ARACHIDONIC_ACID_METABOLISM, (m) KEGG_CHEMOKINE_SIGNALING_PATHWAY, (n) KEGG_P53_SIGNALING_PATHWAY, (o) KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS.
summary, the C1 cluster and high-risk group correspond to immunologically “cold” tumors, whereas the “hot” tumors in C3, C2, and the low-risk group may be more sensitive to immunotherapy. In addition, the NR-lncRNAs are potential predictive markers of immune efficacy, and may help predict the immune efficacy of patients and improve individualized treatment.

4.6. GSEA for Risk Groups. Seo J et al. found that the E3 ubiquitin ligase negatively regulates the necroptosis proteins RIPK1 and RIPK3 through ubiquitination and lysosome-dependent degradation, which in turn inhibits necroptosis [43]. Furthermore, p53 regulates the NRF-miR-873 network, which inhibits the translation of RIPK1 and RIPK3 [44]. It is possible that E3 ubiquitin ligases have a similar function in BC and would therefore be a potential therapeutic target. Further studies are needed to explore its mechanism of action.

Some studies suggest that necroptosis of tumor cells recruits TILs and enhances the immune effect of it after releasing response by promoting the release of inflammatory mediators such as calreticulin, HMGB1, ATP, IL-6, and IL-33, which strengthens the immunotherapeutic effect by converting the cold tumors to hot [45]. However, little is known regarding necroptosis induction by lymphocytes and its bearing on the response to immunotherapy [46]. Lipid oxidation function was abundant in the low-risk group, which raises the possibility that the intra-tumoral cytotoxic lymphocytes increase the level of oxidized lipids in tumor cells, resulting in necroptosis and increased response to immunotherapy. Taken together, necroptosis and immunotherapy may act synergistically against tumor growth, which would be an interesting hypothesis.

5. Conclusion

The combination of necroptosis sensitizers and immune checkpoint inhibitors can improve the efficacy of immunotherapy in tumors with low necroptosis potential. However, further studies are needed to determine the degree of induction and necroptosis. Our findings regarding the cross-talk between necroptosis, tumor immune landscape, and prognosis may help in the design of individualized immunotherapy protocols to improve the survival outcomes of BC patients.

5.1. Limitations of our Study. Our data was retrieved from public databases and did not cover all cases in the relevant regions. Besides, the predictive performance of the model in the validation set was not completely satisfactory. Therefore, our findings have to be confirmed further through functional assays.

Data Availability

The data used during the study are available at the TCGA (https://tcga-data.nci.nih.gov/tcga/) and the code is available from the corresponding author by request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors’ Contributions

X.C. proposed the concept and design of the research and provided suggestions for revision of the manuscript. S.T. analyzed the data, interpreted the results, wrote and revised the manuscript, and finally approved the version. K.T. participated in the discussion of the results, the revision, and editing of the manuscript during the rework process. All authors read and approved the final paper.

Acknowledgments

This work was supported by the Guangxi Natural Science Foundation Program (No. 2018GXNSFAA281036).

Supplementary Materials

Figure S1: Differential expression analysis of 13 IncRNAs between tumor and normal samples. Figure S2: Heat map of 13 IncRNAs and clinicopathological factors. Figure S3: Differential expression analysis of 13 IncRNAs among C1, C2 and C3. Figure S4: Immune cells infiltration in C1, C2, and C3. Figure S5, S6: IC50 of anti-cancer drugs in high- and low-risk groups. Appendix 1: Sixty-seven necroptosis-associated IncRNAs. Appendix 2: Forty-seven immune checkpoint genes. (Supplementary Materials)

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