Hypoxia-Related Long Non-Coding RNA Signature Predicts the Prognosis of Breast Cancer

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Research Article

Keywords: Breast cancer, Hypoxia, TCGA, lncRNAs

Posted Date: November 22nd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1084000/v1>

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Abstract

Background: Breast cancer(BC) is the most common tumour in women. Hypoxia stimulates metastasis in cancer and is linked to poor patient prognosis.

Methods: We screened prognostic-related lncRNAs(Long Non-Coding RNAs) from the Cancer Genome Atlas (TCGA) data and constructed a prognostic signature based on hypoxia-related lncRNAs in BC.

Results: We identified 21 differentially expressed lncRNAs associated with BC prognosis. Kaplan Meier survival analysis indicated a significantly worse prognosis for the high-risk group($P < 0.001$). Moreover, the ROC-curve (AUC) of the lncRNAs signature was 0.700, a performance superior to other traditional clinicopathological characteristics. Gene set enrichment analysis (GSEA) showed many immune and cancer-related pathways and in the low-risk group patients. Moreover, TCGA revealed that functions including activated protein C (APC)co-inhibition, Cinnamoyl CoA reductase(CCR),check-point pathways, cytolytic activity, human leukocyte antigen (HLA), inflammation-promotion, major histocompatibility complex(MHC) class1, para-inflammation, T cell co-inhibition, T cell co-stimulation, and Type I and II Interferons (IFN) responses were significantly different in the low-risk and high-risk groups. Immune checkpoint molecules such as ICOS, IDO1, TIGIT, CD200R1, CD28, PDCD1(PD-1), were also expressed differently between the two risk groups. The expression of m6A-related mRNA indicated that YTHDC1, RBM15, METTL3, and FTO were significantly between the high and low-risk groups. Additionally, immunotherapy in patients with BC from the low-risk group yielded a higher frequency of clinical responses to anti-PD-1/PD-L1 therapy or a combination of anti-PD-1/PD-L1 and anti-CTLA4 therapies. *Except for* lapatinib, the results also show that a high-risk score is related to a higher half-maximal inhibitory concentration (IC50) of chemotherapy drugs.

Conclusion: A novel hypoxia-related lncRNAs signature may serve as a prognostic model for BC.

Introduction

BC is a highly lethal tumour in women, recently with an increasing incidence^{1,2}. Approximately 1.7 million women are diagnosed with BC worldwide per year, and half a million women die from it each year^{3,4}. The occurrence and development of BC is a very complicated pathological process, and the accurate pathogenesis of BC is still not clear⁵. In recent years, treatments for BC patients, such as surgical resection, radiation therapy, chemotherapy, hormone therapy, and targeted therapy have greatly improved patient progression-free(PFS) and overall survival(OS)⁶⁻⁸. However, metastasis and recurrence are still critical clinical events in BC⁹. Early detection of cancer by successful diagnostic markers improves the survival rate of patients with BC¹⁰. Currently, there is a lack of effective biomarkers for the early diagnosis of cancers¹¹. However, in recent years, studies on the involvement of hypoxia in tumours have gradually begun to show that it is a hallmark of the tumour microenvironment. Tumors frequently develop under hypoxic conditions because of insufficient blood supply¹². Severe tumour hypoxia ultimately leads to tissue necrosis, but hypoxia also exerts strong selective pressure towards a more aggressive tumour

type^{13,14}. Moreover, it is also reflected in enhanced angiogenesis and vasculogenesis reflecting altered gene expression, with suppression of apoptosis, promotion of autophagy^{15,16}, stimulation of the epithelial-mesenchymal transition (EMT)^{17,18}, and changes in anabolic phenotype to core cellular metabolism^{19,20}. In addition, hypoxia is associated with increased DNA damage and reduced expression of various DNA repair proteins²¹⁻²³. Meanwhile, hypoxia also promotes tumour immune evasion via diverse mechanisms²⁴. All of these findings demonstrate that hypoxia in tumours is associated with a poor prognosis.

LncRNAs are a new type of potential biomarker and therapeutic target for cancer treatment^{25,26}. Apart from gene regulation, a recent study found that lncRNAs participate in tumour invasion and metastasis by regulating the EMT²⁷. For instance, the high expression of lncRNA p10247 is closely related to metastasis in breast cancer²⁸. There is increasing evidence that various of lncRNAs can be utilized to construct a predictive model of the prognosis of different cancers²⁹⁻³¹. Zhang et al. developed an effective hypoxia-related lncRNA assessment model for prognosis of clear cell renal carcinoma³². A study assembled 10 hypoxia-related lncRNAs by Zhang et al revealed that NOX4, CHST1 and COL8A1 were related to poor prognosis in human gastric cancer tissues³³. In a related study, Gong et al. chose 13 hypoxia-related gene expression signatures to evaluate the hypoxic status of BC, indicating that BCL11A and CPEB2 might act as a oncogene and tumour suppressor, respectively³⁴. But at the moment, there has been little research on hypoxia-related lncRNAs in BC. In the present study, we first identified 21 hypoxia-related lncRNAs and constructed a reliable prognostic model for further research in BC based on TCGA datasets using bioinformatics analysis. In addition, We then explored the role of hypoxia-related mRNA, immune responses and chemotherapy efficacy in patients with invasive breast carcinoma.

Results

Enrichment analysis of hypoxia-related genes

We identified 69 hypoxia-related DEGs (43 downregulated and 26 upregulated; Table S2). Biological Process (BP), Molecular Function (MF), and Cellular Component (CC) were then considered. Kyoto Encyclopedia of Genes and Genomes (KEGG) -based analysis revealed that the over-expressed genes were mainly involved in the hypoxia-inducible factor (HIF)-1 signaling pathway, Glycolysis/Gluconeogenesis, MAPK signaling pathway, Insulin resistance and others (Figure 2 and Table S3).

The hypoxia-related lncRNA prognostic signature

We identified 3695 hypoxia-related lncRNAs (Table S4). Univariate COX analysis confirmed 274 significant hypoxia-related lncRNAs, which were included in the multivariate analysis. Overall, 21 differently expressed lncRNAs (AC087239.1, AC015802.3, GORAB-AS1, LINC01235, USP30-AS1, LINC01614, LINC02446, AC121247.1, TFAP2A-AS1, LINC01929, KLHDC7B-

DT,LINC00702,AL133467.1,TDRKH-AS1,AC004585.1,SIDT1-AS1,AC012213.3,HSD11B1-AS1,MIR4435-2HG,AL645608.7 and LRRC8C-DT) were found to be independent prognosis predictors in BC patients (Table S5).

Survival results and multivariate analysis

Kaplan Meier survival analysis indicated a worse prognosis for the high-risk group ($P < 0.001$, Figure 3A). Meanwhile, for predicting the prognosis of BC patients (Figure 2B), the AUC of the lncRNA signature was 0.700, showing superior performance to other traditional clinicopathological characteristics (Figure 3B).

As shown in our risk survival status plot, we found the risk score in BC patients increased proportionally to the survival of patients. Meanwhile, the heatmap showed 5 positive correlations and 8 negative correlations among the novel lncRNAs identified in this study, which needs validation in further work (Figure 3D).

The AUCs for 3-, 5-, and 7-year survival of predictions for the 21 lncRNA signature were 0.695, 0.700 and 0.713, respectively (Figure 3C). According to COX analysis, age (HR: 1.05, 95CI: 1.03-1.07) and our risk signature (HR: 1.17, 95CI: 1.10-1.23) were independent prognostic factors for survival of BC patients, respectively (Figure 4A, 4B). We can see the relationship between novel lncRNAs and mRNA from Figure 4C. In addition, the heatmap showing clinicopathological manifestations and hypoxia-related lncRNA prognostic signatures is also shown (Figure 5). Based on the relationship between clinicopathological characteristics and the selected hypoxia-related lncRNA prognostic signature, the clinical utility of the nomogram was accurate and steady and could be adopted for clinic management for BC patients (Figure 6).

Gene set enrichment analyses

The majority of the novel hypoxia-related lncRNAs in the prognostic signature in the low risk group was based on GSEA identified immune pathways such as asthma, autoimmune thyroid disease, T/B cell receptor signaling, FC gamma R-mediated phagocytosis, graft versus host disease, hematopoietic cell lineage, systemic lupus erythematosus, the intestinal immune network for IgA production, and primary immunodeficiency (Figure 7 and Table S6).

Immunity and gene expression

Cell type Identification By Estimating Relative Subsets Of RNA Transcripts (CIBERSORT), ESTIMATE, Microenvironment Cell Populations-Counter method (MCP counter), single-sample gene set enrichment analysis (ssGSEA) and Tumor Immunoassay Resource (TIMER) algorithms were used to generate a heatmap of immune responses (Figure 8). Correlation analysis among immune cell subpopulations based on ssGSEA of TCGA-BRCA data indicated that T cell functions including APC co-inhibition, CCR, checkpoint expression, cytolytic activity, HLA, inflammation-promoting, MHC class1, para-inflammation, T cell co-inhibition, T cell co-stimulation, and Type I and II IFN responses were all

significantly different between the low-risk and high-risk groups (Figure 9A). Moreover, given the importance of checkpoint-based immunotherapy, we further explored the differences in immune checkpoints between the two groups. Significant differences in the expression of ICOS, IDO1, TIGIT, CD200R1, CD28, PDCD1 (PD-1) and other immune checkpoints between the two groups of patients are depicted in Figure 9B. The expression of m6A-related mRNA between the high and low-risk groups indicated that the expression of YTHDC1, RBM15, METTL3, and FTO was significant (Figure 10). Furthermore, to determine the capacity of the hypoxia-related lncRNA prognostic signature to predict the response to anti-PD-1/PD-L1 therapy, we used immunophenogram analysis to investigate the correlation between the immunopheno-score (IPS) and low/high risk groups. We found that in the CTLA4_{negative/positive} + PD-1_{negative/positive} type, the low risk group exhibited higher IPS than the high risk group ($P=0.0001$) (Figure 9C). These results illustrate that patients from the low risk group showed a higher positive response to anti-PD-1/PD-L1 therapy or a combination of anti-PD-1/PD-L1 and anti-CTLA4 therapies.

Correlation analysis between the risk model and chemotherapy outcome

We also explored the relationship between the risk signature and the efficacy of common chemotherapeutic agents in the treatment of patients with BC based on the TCGA-BRCA project. We found that the high-risk score is related to a higher IC50 of chemotherapy drugs such as paclitaxel ($p=0.01$), camptothecin ($p<0.001$), docetaxel ($p<0.05$), epothilone B ($p<0.05$), AZD2281 (Olaparib) ($P<0.001$), gefitinib ($p<0.001$), gemcitabine ($P=0.001$), rapamycin ($p=0.001$), midostaurin ($p=0.01$) and tipifarnib ($p<0.001$), while the IC50 of lapatinib was found to be lower ($p<0.001$). These findings indicate that the model can be used as a potential predictor of chemotherapeutic drug sensitivity (Figure 11).

Discussion

Increasing evidence is indicating that tumour hypoxia may promote invasive cancer growth and metastasis⁴⁵⁻⁴⁷. Besides standard prognostic factors such as tumour stage, nodal status and tumour grade, tumour hypoxia in some cancers has been recommended as a prognostic factor for patient outcome⁴⁸. In the present study, we identified novel hypoxia-related prognostic lncRNA signatures based on the TCGA-BRCA project. Subsequently, we have also explored the roles of immune infiltrating cells in the tumour microenvironment and the immune checkpoint inhibitors in patients with breast cancer. In addition, we provide evidence for potential biomarker and therapeutic targets in hypoxia signaling pathways.

In the present work, we identified 69 hypoxia-related DEGs. KEGG pathway analysis has further clarified that these genes mainly participated in the hypoxia-inducible factor (HIF)-1 signaling pathway, Glycolysis/Gluconeogenesis, MAPK signaling pathway, insulin resistance and other signaling pathways. Guo et al. reported that HIFs target a wide range of genes which function to control a variety of signaling

pathways⁴⁹; HIFs are expected to become new targets for hepatocellular carcinoma therapy. A recent study revealed that HIFs are the major sensors of low oxygen involved in viral replication and virion assembly⁵⁰. In the present study, 21 differently expressed lncRNAs can be regarded as independent prognosis factors for BC patients. A recent study found that LINC01235 was upregulated in gastric cancer (GC) tissues and cells, and microRNA (miR)-6852-5p might be a key gene involved in the regulation of TWIST2 by LINC01235. The LINC01235-TWIST2 positive feedback loop mainly affected the migration and invasion of GC cells⁵¹. Similar studies showed that Immune-Related lncRNA USP30-AS1 was found to be over-expressed in cervical cancer, melanoma, glioblastoma multiforme and bladder cancer, associated with longer OS⁵²⁻⁵⁵. High LINC01614 expression was also markedly associated with the epithelial-mesenchymal transition (EMT), influencing metastasis and the prognosis of several cancers⁵⁶. lncRNA LINC02446 suppresses the proliferation and metastasis of bladder cancer cells by binding with EIF3G and regulating the mTOR signaling pathway⁵⁷. Results from a related study suggest that TFAP2A-AS1 acts as a tumour suppressor in BC via the miR-933/SMAD2 axis⁵⁸. lncLINC01929 accelerates the progression of oral squamous cell carcinoma by targeting the miR-137-3p/FOXC1 axis and is associated with survival in the TCGA dataset⁵⁹⁻⁶¹. The high expression of KLHDC7B-DT aggravates pancreatic ductal adenocarcinoma development via inducing cross-talk between cancer cells and macrophages⁶². Immune-associated lncRNA AL133467.1, was identified as a predictive factor with significant prognostic value in ovarian carcinoma, corresponding to long survival⁶³. Similar to our study, Lai et al. reported that they had utilized AC004585.1 and two risk parameters (tumour subtype and TNM stage) as independent indicators to develop a novel prognostic model in breast cancer patients⁶⁴. Studies reported that lncRNA MIR4435-2HG can promote hepatocellular carcinoma proliferation and metastasis through the miR-22-3p/YWHAZ axis⁶⁵ and promote the progression of head and neck squamous cell carcinoma by regulating the miR-383-5p/RBM3 axis⁶⁶. Another study found that the downregulation of MIR4435-2HG suppresses breast cancer progression via the Wnt/beta-catenin signaling pathway⁶⁷. Additionally, we first identified other lncRNAs (AC087239.1, AC015802.3, GORAB-AS1, AC121247.1, LINC00702, TDRKH-AS1, SIDT1-AS1, AC012213.3, HSD11B1-AS1, AL645608.7 and LRRC8C-DT) as independent prognostic factors in BC patients. The risk model in the present study consists of 21 hypoxia-related genes representing a convenient detection tool in the clinic.

Recently, immune checkpoint inhibitors (ICIs) have attracted attention in cancer immunotherapy. Tumours can influence immune checkpoint networks to protect themselves from the immune system. However, few studies have explored the relationship between ICIs and tumour hypoxia. It has been reported that hypoxia can affect immune checkpoints, causing an up-regulation of PD-L1 and increased expression of CTLA⁶⁸. Our results document higher expression of hypoxia-related biomarkers (eg. PDCD1, CD276, CD274) among patients in the low-risk group. Interestingly, in our further immunotherapy analysis, the low-risk group received higher scores in all four types of immunophenoscore. This may indicate that patients with low-risk scores are more suitable candidates for immunotherapy.

In this study, we have established for the first time a model to develop and validate a hypoxia risk score based on 21 hypoxia-associated genes. This model served as an independent prognostic factor for BC patients and reflected the overall intensity of the immune response in the BC microenvironment. In summary, we developed and validated a hypoxia risk score model based on a novel hypoxia-related gene signature revealing the relationship between hypoxia and the tumour immune microenvironment. Nevertheless, further investigations in a larger cohort of patients are needed to verify the accuracy of our model.

Conclusion

In conclusion, our study constructed a novel prognostic model to predict the outcomes of BC patients and provide indications for novel targets for the management of BC.

Methods

Data collection

RNA-sequence data of 1208 individuals were extracted from the TCGA-BRCA database (112 normal and 1096 tumours). In this study, we downloaded the hypoxia-related gene set from Gene Set Enrichment (hallmark-hypoxia), a web-based consortium with data on 200 genes upregulated in response to low oxygen levels³⁵. Overall, we identified 200 hypoxia-related genes (Table S1). Using a screening approach based on Pearson's correlation analysis (threshold of $|R2| > 0.4$; $P < 0.001$), we were able to identify 274 hypoxia-related lncRNAs. The clinical information on the breast cancer patients such as gender, age, stage, grade, TMN, survival time and survival status were also accessed. The criterion of $|\text{Log2FC}| \geq 1$, $\text{FDR} < 0.05$ was used to select the significantly differentially-expressed hypoxia-related lncRNAs. First, we separately analyzed upregulated and downregulated genes among hypoxia-related differentially-expressed genes (DEGs). Then, Gene Ontology (GO) enrichment analysis was used to explore the biological pathways associated with the DEGs. Moreover, the biological processes (BP), cellular components (CC) and molecular functions (MF) were analyzed utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using R software, ggplot2 package. The details are described in Figure 1.

Development of the hypoxia-related lncRNA prognostic signature

Hypoxia-related genes were extracted from the Molecular Signature Database (MSigDB, <http://www.gsea-msigdb.org/>). We used univariate and multivariate Cox regression analyses to construct the hypoxia-related lncRNA signature, stratified based on risk score ($\text{Coefficient lncRNA1} \times \text{expression of lncRNA1}$) + ($\text{Coefficient lncRNA2} \times \text{expression of lncRNA2}$) + ... + ($\text{Coefficient lncRNA} \times \text{expression lncRNA}$). The risk score was calculated for each sample, which were then divided into a high-risk and a low-risk group according to whether they were above or below the median.

The predictive nomogram

Gene set enrichment analyses (GSEA) were performed using the GSEA software to define the lncRNA signatures, which we then searched for in the TCGA-BRCA project. $P < 0.05$ and false discovery rate (FDR) $q\text{-value} < 0.25$ were considered statistically significant. The nomogram was then constructed based on the results of multiple survival analyses according to the method described previously for predicting 1, 3, and 5-year OS of BC patients.

Immunity analysis and gene expression

We used currently accepted methods, including TIMER^{36,37}, CIBERSORT^{36,37}, XCELL^{38,39}, QUANTISEQ^{40,41}, MCPcounter⁴² and EPIC⁴³, to assess cellular components or cellular immune responses and to compare high risk and low-risk groups based on hypoxia-related lncRNA signatures. A heatmap display was used to depict differences in the immune response through different algorithms. Apart from identifying immune function, ssGSEA was used to quantify the tumour-infiltrating immune cell subgroups. Potential immune checkpoints were also compared between high risk and low-risk groups.

Clinical significance of the Risk Score model for treatment of invasive breast cancer

We adopted the IC50 value for the chemotherapeutic drugs commonly used in the TCGA project to evaluate the model for the clinical treatment of BC. According to AJCC guidelines, chemotherapeutic drugs such as doxorubicin, docetaxel, paclitaxel, cisplatin, vinorelbine, gemcitabine, lapatinib and palbociclib were used for treating the BC patients. Differences in IC50 between high-risk and low-risk groups were compared by the Wilcoxon signed-rank test and R's pRRophetic and ggplot2 were used to construct a block diagram.

Statistical analysis

Bioconductor packages in R software (version 4.1.0) were used for data analysis. Normally distributed variables were analyzed using the unpaired student's t-test and non-normally distributed values were analyzed using the Wilcoxon test. The differently expressed lncRNAs were identified by a Benjamini-Hochberg-based algorithm. GSVA, an R-package, was used to compare the ssGSEA-normalized BRCA DEGs with a genome. Decision curve analysis (DCA) was carried out and receiver operating characteristic (ROC) curves were constructed to evaluate the clinical fitness and accuracy of the nomogram, respectively⁴⁴. Logistic regression analysis was used to evaluate the relationship between hypoxia-related lncRNAs and clinicopathological manifestations by heatmapping. Survival of BC patients based on the hypoxia-related lncRNA signatures was assessed using the Kaplan–Meier method. The criterion for each analysis for statistical significance was set at $P \leq 0.05$.

Abbreviations

BC	Breast cancer
TCGA	The Cancer Genome Atlas
lncRNAs	Long Non-Coding RNAs
AUC	ROC-curve
GSEA	Gene set enrichment analysis
APC	activated protein C
CCR	Cinnamoyl CoA reductase
MHC	major histocompatibility complex
IFN	Interferons
IC50	half-maximal inhibitory concentration
PFS	progression-free
OS	overall survival
EMT	epithelial-mesenchymal transition
BP	Biological Process
MF	Molecular Function
CC	Cellular Component
KEGG	Kyoto Encyclopedia of Genes and Genomes
HIF	hypoxia-inducible factor
MCP-Counter	Microenvironment Cell Populations-Counter method
ssGSEA) ;	single-sample gene set enrichment analysis
TIMER	Tumor Immunoassay Resource
IPS	immunopheno-score
EMT	epithelial-mesenchymal transition
ICIs	immune checkpoint inhibitors
ssGSEA) ;	single-sample gene set enrichment analysis
TIMER	Tumor Immunoassay Resource
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Declarations

Acknowledgements

We thank Shuang Shen for help with some of the statistical analyses. We would like to express their gratitude to EditSprings (<https://www.editsprings.cn/>) for the expert linguistic services provided.

Funding

The work was supported by Clinical Project Foundation from Department of Science and Technology of Guizhou Province(LC[2021]004), the General Project Foundation of Science and Technology Bureau of Zunyi City(HZ (2019) 133,HZ (2019) 175).

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

Consent for Publication

All authors consent for publication.

Author contributions

J.L.H, S.S., and C.C. designed the study, offered critical input, and co-wrote the manuscript. D.O.,R.Q conducted the experiments, wrote the manuscript, drew and prepared figures and analyzed the data. S.S.,Y.M.G analyzed samples using R software. D.O. supervised the study.

Competing interests

The authors declare no competing interests.

Ethics approval and consent to participate

Not applicable.

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Figures

Figure 1

Flowchart for bioinformatics analysis of publicly available data from TCGA databases.

Figure 2

GO and KEGG analyses for hypoxia-related differentially expressed genes. (A) GO and (B) KEGG.

Figure 3

Hypoxia-related lncRNAs signature based on TCGA. (A) Kaplan-Meier curves result, (B). The AUC values of the risk factors, (C)The AUC of the for the prediction of 3, 5,7-year survival rate of BC and (D) Risk survival status plot.

Figure 4

Univariate and multivariate COX analysis for the expression of Hypoxia-related lncRNAs. (A). univariate, (B). multivariate, (C). The relationship between thenovel lncRNA and mRNA expression.

Figure 5

Heatmap for ferroptosis-related lncRNAs prognostic signature and clinicopathological manifestations.

Figure 6

A nomogram for both clinic-pathological factors and prognostic hypoxia-related lncRNAs.

Figure 7

Gene set enrichment analysis (GSEA) enrichment map for hypoxia-related lncRNAs.

Figure 8

Heatmap for immune responses among high and low risk groups.

Figure 9

(A). Correlation analysis between immune-related lncRNAs and immune cell subpopulations.(B). Expression of immune checkpoints among high and low risk groups.(C).Immunophenogram analysis to investigate the correlation between the immunopheno-score (IPS) and low/high risk groups.

Figure 10

The expression of m6A-related genes between high and low risk group.

Figure 11

Correlation analysis between the risk model and chemotherapy outcome

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