

The Pyroptosis-Related 9 LncRNA Signature and LncRNA-miRNA-mRNA Regulatory Network in Breast Cancer: A Comprehensive Analysis Based On TCGA Database

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Abstract

Background: Pyroptosis has been verified to participate in various malignancies. However, studies on pyroptosis-related lncRNAs in breast cancer and its effects on tumor immune micro-environment are still limited. Consequently, it was aimed in this study to construct a pyroptosis-related lncRNAs signature for prognostic prediction and explore the effect of the pyroptosis-related lncRNAs on tumor immune microenvironment through lncRNA-miRNA-mRNA regulatory network.

Methods: The pyroptosis-related differentially expressed genes (DEGs) were discovered using differential expression analysis. The differentially expressed lncRNAs (DELncRNAs) associated with DEGs were discovered using correlation analysis. The function of DEGs was analyzed using GO and KEGG analyses. The lncRNAs signature used as the prognostic model of breast cancer was constructed using univariate and multivariate Cox analysis, and the effectiveness was verified by K-M analysis and ROC curve. The risk score calculated using the prognostic model was proved as an independent factor by univariate Cox analysis, multivariate Cox analysis and PCA analysis, and used to predict patient prognosis through nomogram. The pathways enriched in High risk group and Low risk group were analyzed by GSEA. The differences in immune cell distribution (B cell memory, T cell CD4+, T cell CD8+ among others) were analyzed using ssGSEA. The immune function (type I/II IFN response among others), immune checkpoint (ADORA2A among others) and m6A-related protein expression (FTO among others) of High risk group and Low risk group were compared. The regulatory network of pyroptosis-related lncRNA-miRNA-mRNA was constructed and the core network was extracted. The functions of the target genes of miRNA associated with DELncRNAs were explored using GO and KEGG analysis.

Results: A 9 lncRNAs signature (LMNTD2-AS1, AL589765.4, AC079298.3, U62317.3, LINC02446, AL645608.7, HSD11B1-AS1, AC009119.1, AC087239.1) was constructed as the prognostic model of breast cancer. Significant differences were discovered in immune cell distribution, immune function, immune checkpoint and m6A-related protein expression between High risk group and Low risk group. The regulatory network of lncRNA-miRNA-mRNA was constructed and found to participate in the crosstalk among apoptosis, pyroptosis and necroptosis of breast cancer.

Conclusions: The 9 lncRNAs signature was valuable for predicting breast cancer prognosis, and the pyroptosis-related lncRNAs influenced tumor immune microenvironment of breast cancer through the lncRNA-miRNA-mRNA regulatory network.

Introduction

Breast cancer (BC) is a kind of life-threatening malignancy which primarily affects breast tissue and exhibits high tendency of pulmonary and osseous metastasis.

According to the latest data of global cancer burden published by World Health Organization International Agency for Research on Cancer (IARC) by 2020 year-end, breast cancer has become the world No.1 cancer with 2.26 million patients, surpassing the former No.1 lung cancer with 2.2 million patients. In spite of great development in BC diagnosis and therapy in recent years, the prognosis of BC is still unsatisfactory due to lack of obvious symptoms during early stage. Consequently, it is urgently needed to develop innovative and efficient methods for diagnosis, treatment and prognostic evaluation.

Pyroptosis, known as the inflammatory programmed cell death induced by Gasdermin, was initially observed by Friedlander in 1986 as lytic death of mouse macrophages accompanied with non-specific leakage of cell content induced by anthrax lethal toxin¹. Based on studies over the years, the definition of pyroptosis was finally amended by the Nomenclature Committee on Cell Death (NCCD) as the regulable cell death generated by plasma membrane pore formation induced by Gasdermin, which is frequently but not always completed by the activation of inflammatory Caspase². Induced by pathogens or damages, Caspase-1 is activated by canonical inflammasome pathway, or Caspase 4/5/11 is activated by non-canonical inflammasome pathway, which subsequently activate Gasdermin and leads to cell membrane perforation and cell death³.

It has been discovered that pyroptosis is closely related to tumor progression and metastasis⁴ as well as drug resistance⁵ through regulation on tumor immune microenvironment⁶. Orita et al reported that GSDMD was up-regulated in normal tissues and down-regulated or even silenced in colorectal cancer⁷, and Wang et al reported that the proliferation of gastric cancer cells promoted by down-regulation of GSDMD was possibly related to the acceleration of cell cycle S/G2 transition⁸. Yu et al reported the transition from autophagy to pyroptosis induced by doxorubicin in melanoma cells was accomplished through the activation of eEF2k, indicating the existence of crosstalk between prognosis and other kinds of cell death⁹.

As for studies in breast cancer, a few natural products were also shown to induce pyroptosis. Draganov et al reported that ivermectin activated Caspase-1 and induced pyroptosis of breast cancer cells through the over-expression of P2X4/ P2X7 and release of ATP¹⁰. Pizato et al discovered that docosahexaenoic acid (DHA) inhibited proliferation of breast cancer cells by inducing pyroptosis through NF- κ B/Caspase/GSDMD/IL-1 β ¹¹. In addition, pyroptosis was also discovered to exert an essential effect on drug resistance of breast cancer cells. IL-1 β , which is an important inflammatory material released during pyroptosis, was reported to induce tamoxifen resistance through down-regulation of ER- α ¹² and doxorubicin through up-regulation of BIRC3¹³, indicating that the pyroptosis is a potential target for increasing efficacy of regular chemotherapeutic drugs.

Long non-coding RNA (lncRNA) is a kind of regulatory non-coding RNA with length of more than 200nt. lncRNAs participate in various physiological processes including cell differentiation regulation, cell cycle regulation, epigenetic regulation and dosage compensation effect. lncRNAs also act as the pivotal part in tumorigenesis and the dysregulation of which is closely associated with various tumorigenesis process including proliferation, metastasis¹⁴, invasion¹⁵ and angiogenesis¹⁶. In recent years, a few related lncRNAs were discovered as dysregulated in breast cancer¹⁷, which lay theoretical basis for the application of lncRNAs in diagnosis, prognostic prediction and treatment of breast cancer. It was also reported that some lncRNAs participated in tumorigenesis through lncRNA-miRNA-mRNA regulatory network¹⁸. However, studies on lncRNAs which exert a regulatory effect on breast cancer through

pyroptosis and the corresponding LncRNA-miRNA-mRNA regulatory network are still limited. Consequently, in this study we compared the expression profile of samples from patients and healthy subjects included in TCGA, found pyroptosis-related LncRNAs significantly associated with clinical manifestations and established an effective clinical model for prognostic prediction. Moreover, we also explored the possible mechanism of pyroptosis-related LncRNAs regulation through tumor immune microenvironment, and constructed the pyroptosis-related LncRNA-miRNA-mRNA regulatory network in order to provide new insights into the application of pyroptosis-related LncRNAs in diagnosis, prognostic prediction and treatment of breast cancer.

Material And Methods

Collection of data

The RNA expression profiles of 1109 tumor samples and 113 normal samples were obtained from TCGA database. The information of patients with more than one invalid indexes were excluded from this study.

The identification of pyroptosis-related LncRNAs

In total 52 pyroptosis-related genes were collected from literature. Pearson correlation analysis was performed to determine the LncRNAs correlated to pyroptosis-related genes. The association between LncRNAs and pyroptosis-related genes was considered valid with a correlation coefficient $|R2| > 0.4$ and $p < 0.001$.

The identification and functional analyses of pyroptosis-related DEGs

The clinical indexes contained in the dataset included survival time, survival status, gender, age stage and TMN classification. The R package "limma" was used to screen DEGs from expression profile data and the criteria of pyroptosis DEGs was $|\log2FC| \geq 1$ and $FDR < 0.05$. Subsequently, the R package "ggplot2" was used to perform Gene Ontology (GO) functional analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of the obtained pyroptosis-related DEGs.

The establishment and verification of pyroptosis-related LncRNAs signature

Univariate Cox analysis of overall survival (OS) was performed on the pyroptosis-related DELncRNAs and multiple variate Cox analysis was performed on the obtained candidate DELncRNAs to construct the Cox regression model. The formula of risk score was defined as follows:

Risk score = (CoefficientLncRNA1 \times expression LncRNA1) + (CoefficientLncRNA2 \times expression LncRNA2) + ... + (CoefficientLncRNA n \times expression LncRNA n). Subsequently, the patients were divided into High risk group and Low risk group based on the median value of risk scores. Kaplan-Meier analysis was performed on overall survival time of the two groups. Univariate and multiple variate Cox analysis of clinical indexes (age, gender, stage, risk score) were performed and the results were shown by forest plot. The R package "time ROC" was used to assess the accuracy of pyroptosis-related LncRNAs signature in prognostic prediction. In addition, a nomogram was also drawn to predict the survival time on the basis of clinical manifestations including gender, TMN classification, stage, age and risk score.

Gene Set Enrichment Analysis (GSEA)

GSEA was performed using GSEA_4.1.0 version. The gene sets database for analysis was selected as c2.cp.kegg.v7.4.symbol.gmt(curated). The threshold value was set as $p\text{-value} < 0.05$ and $|\log2FC| \geq 1$.

Analyses of immune cells and gene expressions

TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, EPIC were used to analyse the difference of immune cell distribution between Low risk group and High risk group. The correlation between various immune cell types were analyzed on the basis of results of CIBERSORT. The differences of immune function between Low risk group and High risk group were compared on the basis of ssGSEA analysis using the R package "GSVA". The expressions of checkpoint-related genes (collected from literature) were compared between Low risk group and High risk group. The expressions of m6A-related genes (collected from literature) were compared between Low risk group and High risk group. The PCA analysis of Low risk group and High risk group was performed using the R package "ggplot2". The relationship between pyroptosis-related mRNA and pyroptosis-related LncRNA and the effects on patients was demonstrated using Sankey gram by means of the R package "ggalluvial".

The construction of LncRNA-miRNA-mRNA regulatory network

The targets miRNA of pyroptosis-related DELncRNAs were predicted using miRcode database (miRcode - transcriptome-wide microRNA target prediction including LncRNAs). Subsequently, the target genes of the predicted miRNAs were searched in three databases including miRDB (miRDB - MicroRNA Target Prediction Database), miRTarBase (miRTarBase: the experimentally validated microRNA-target interactions database (cuhk.edu.cn)), TargetScan (TargetScanHuman 7.1). Target genes which existed in at least two of the three databases were considered valid. GO and KEGG analyses of these target genes were performed and the network among the pyroptosis-related DELncRNAs, the target miRNAs of these LncRNAs and the target genes of these miRNAs was drawn using cytoscape 3.7.2. The hub genes of the network were discovered using the plug-in module of cytoscape hubba.

Statistical analyses

Chi-squared test was used to analyze the differences in clinical features. Independent prognostic factors were identified using univariate and multivariate Cox analyses. Time-dependent ROC curve analysis was used to evaluate the accuracy of prognostic model in predicting OS. The proportion of tumor-infiltrating

immune cells, the expressions of m6A genes and immune checkpoint genes were compared between the High risk and Low risk groups using Wilcoxon-test. The correlation between tumor-infiltrating immune cells was analysed using Spearman correlation analysis. R. version 4.0.7 was used to perform the statistical analyses in this study. The threshold value of statistical significance was set as $P < 0.05$.

Results

GO and KEGG analyses of the pyroptosis-related DEGs

In total, 14 pyroptosis-related DEGs (BAK1, BAX, ELANE, GSDMD, IL18, TP63, AIM2, GSDMC, IL6, NLRP6, NLRP7, NOD2, PYCARD, GZMA) and 97 pyroptosis-related DELncRNAs were discovered. BP of the the pyroptosis-related DEGs was enriched in cytokine production, regulation of interleukin-1 beta production, interleukin-1 beta production, regulation of interleukin-1 production, interleukin-1 production, response to liposaccharide, positive regulation interleukin-1 beta production, chemokine production, pyroptosis. CC of the the pyroptosis-related DEGs was enriched in inflammasome complex, secretory granule lumen, cytoplasmic vesicle lumen, vesicle lumen among others. MF of the the pyroptosis-related DEGs was enriched in heat shock protein binding, protease binding, cytokine receptor binding among others (Fig.1A). KEGG was principally enriched in NOD-like receptor signalling pathway, salmonella infection, influenza A, pathogenic Escherichia coli infection, cytosolic DNA-sensing pathway, tuberculosis,transcriptional misregulation in cancer, lipid and atherosclerosis, legionellosis among others (Fig.1B).

The construction of prognostic pyroptosis-related LncRNA signature

Univariate Cox analysis of overall survival (OS) was performed on the pyroptosis-related DELncRNAs and 21 LncRNAs (LMNTD2-AS1, AC137630.3, AC015802.3, AL133467.1, LINC02195, LINC02446, LINC01943, USP30-AS1, LIPE-AS1, LRR8C-DT, AC004585.1, AC009119.1, AL589765.4, AC079298.3, AC087239.1, KLHDC7B-DT, U62317.3, AC015819.1, AC109446.2, AL645608.7, HSD11B1-AS1) were found to exert an statistically significant effect on OS. Subsequently multiple variate Cox analysis was performed on these 21 candidate LncRNAs and 9 of them(LMNTD2-AS1, AL589765.4, AC079298.3, U62317.3, LINC02446, AL645608.7, HSD11B1-AS1, AC009119.1, AC087239.1) were finally used to construct the Cox model and the formula of risk score was as follows:

$$\text{Risk score} = (-0.0792 \times \text{expression LMNTD2-AS1}) + (0.1266 \times \text{expression AL589765.4}) + (0.4728 \times \text{expression AC079298.3}) + (-0.1732 \times \text{expression LINC02446}) + (-0.3569 \times \text{expression U62317.3}) + (0.0548 \times \text{expression AL645608.7}) + (-0.2019 \times \text{expression HSD11B1-AS1}) + (0.0501 \times \text{expression AC009119.1}) + (-0.3345 \times \text{expression AC087239.1}).$$

The verification of the effectiveness of the LncRNAs signature Kaplan-Meier curve was drawn to determine the effect of the LncRNAs signature on overall survival of patients. The overall survival of the High risk group was significantly higher than that of the Low risk group ($p < 0.001$) (Fig.2A), indicating the prognostic value of the signature in breast cancer. The risk plot of patients in High risk group and Low risk group was also drawn and the mortality increased along with the rising of risk score (Fig.2B). ROC curve was drawn to evaluate the sensitivity and specificity of the LncRNAs signature in breast cancer prediction. The AUC of the LncRNAs signature was 0.615, indicating a moderate specificity and sensitivity(Fig.2C). The AUC at 1 year, 2 years and 3 years were 0.615, 0.626, 0.613, respectively (Fig.2D).

The results of univariate and multivariate Cox regression analyses indicated that besides age and stage, the LncRNAs signature also acted as an independent prognostic factor of overall survival in breast cancer patients (Fig.3A, Fig.3B). The PCA analysis based on the 4 LncRNAs of the prognostic signature presented obvious separation between the data from High risk group and Low risk group(Fig.3C). The nomogram used to predict the survival time on the basis of clinical manifestations including gender, TMN classification, stage, age and risk score was shown in Fig.3D.

Gene set enrichment analysis (GSEA)

The expression profile data of patients in High risk group and Low risk group was used to perform gene set enrichment analysis (GSEA) in order to elucidate the differentiated pathways and functions associated with distinct survivals and prognosis. The pathways enriched in High risk group mainly included: KEGG_O_GLYCAN_BIOSYNTHESIS, KEGG_WNT_SIGNALING_PATHWAY, KEGG_ADHERENS_JUNCTION, KEGG_ECM_RECEPTOR_INTERACTION, KEGG_FOCAL_/_ Fig.4A).

The pathways enriched in Low risk group mainly included:

KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY, KEGG_FC_EPSILON_RI_SIGNALING_PATHWAY, KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION, KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION, KEGG_CELL_ADHESION_MOLECULES_CAMS, KEGG_CHEMOKINE_SIGNALING_PATHWAY(Fig.4B).

The immune cell classification of patients in High risk group and Low risk group was analyzed using 7 algorithms including TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, EPIC (Fig.5A). The barplot of immune cell distribution by CIBERSORT was shown in Fig.5B. The correlation between immune cell types was drawn on the basis of CIBERSORT results (Fig.5C).

The immune functions of patients in High risk group and Low risk group were analyzed through ssGSEA. The immune functions including Type_I_IFN_Response, Type_II_IFN_Response, APC_co_stimulation, APC_co_inhibition, Cytolytic_activity, Check-point, T_cell_co-stimulation, MHC_class_I, parainflammation, T_cell_inhibition, Inflammation_promoting, HLA, CCR were significantly different between patients in High risk group and Low risk group (Fig.6A). The expression levels of check-point-related genes were analyzed and all verified as significantly different between patients in High risk group and Low risk group (Fig.6B). The expression levels of m6A-related genes were analyzed and 8 genes were verified as significantly different between patients in High risk group and Low risk group, including FTO, METTL3, HNRNPC, ZC3H13, YTHDC2, YTHDC1, METTL14, YTHDF2 (Fig.6C).

The interaction between pyroptosis-related DEGs and pyroptosis-related DELncRNAs.

The regulatory relationship between pyroptosis-related DEGs and pyroptosis-related DELncRNAs in High risk group and Low risk group was described using Sankey gram (Fig.7).

The pyroptosis-related LncRNA-mRNA-miRNA regulatory network

The pyroptosis-related LncRNA-miRNA-target regulating network was constructed using cytoscape 3.7.2 (Fig.8A) and the hub genes were discovered using the plug-in module of cytoscape hubba (Fig.8B). GO and KEGG analyses of these target genes of the miRNAs associated with DELncRNAs were also performed.

According to the GO analysis results, the target genes of the miRNAs associated with DELncRNAs were mainly enriched in cytokine receptor binding, phospholipid binding, cysteine-type endopeptidase activity involved in apoptosis, tumor necrosis factor receptor superfamily binding among others (Fig.8C).

According to the KEGG analysis results, the target genes of miRNAs corresponding to the pyroptosis-related DELncRNAs were mainly enriched in necroptosis, lipid and atherosclerosis, pathways of neurodegeneration diseases, TNF signalling pathway, miRNAs in cancer, apoptosis and various infections, such as pathogenic *Escherichia coli* infection, human immunodeficiency virus 1 infection and especially, Coronavirus disease – COVID 19 among others (Fig.8D).

Discussions

Defined as a kind of inflammatory programmed cell death induced by gasdermin in 2015, pyroptosis has been revealed to participate in various tumors such as melanoma¹⁹, colorectal cancer²⁰, hepatocellular carcinoma²¹, lung cancer²², cervical cancer²³, leukemia²⁴ and breast cancer²⁵. In breast cancer, the over-expression of GSDMB which belongs to the gasdermin family and acts as the executor of pyroptosis, is associated with poorer prognosis and tumorous progression²⁶. In addition, the inflammasome/IL-1 pathway in pyroptosis exerted an essential role in the progression and invasion of breast cancer through regulation of tumor immune microenvironment²⁷. Lnc RNA and miRNA also participated in tumorigenesis and progression of breast cancer through mutual regulation, which has not been completely clarified at present.

In this study, 14 pyroptosis-related genes differentially expressed between tumor and normal samples and 213 LncRNAs associated with the pyroptosis-related DEGs were discovered using expression profile data of breast cancer patients in TCGA database. On the basis of these DELncRNAs, a novel and efficient signature of breast cancer containing 9 pyroptosis-related DELncRNAs was identified using multivariate Cox analysis and the risk score was verified as an independent prognostic factor through multivariate Cox analysis. The different pathways enriched in either high risk group or low risk group based on the signature were analyzed. Immune responses including immune function, immune checkpoint, m6A-related proteins, distribution of tumor infiltrating immune cells, correlation between various immune cells were also analyzed. The regulatory network of pyroptosis-related LncRNA-miRNA-mRNA was constructed and the target genes were found to participate in the crosstalk among apoptosis, pyroptosis and necroptosis of breast cancer.

The pyroptosis-related DEGs discovered in this study included BAK1, BAX, ELANE, GSDMD, IL18, TP63, AIM2, GSDMC, IL6, NLRP6, NLRP7, NOD2, PYCARD, GZMA. According to the GO analysis, the functions of DEGs were mostly involved in the regulation of cytokine (IL-1, IL-1 β) and chemokine production, response to LPS, pyroptosis-related intracellular construction (inflammasome, secretory granule and cytosolic granule) and molecular function related to cell death. It has been known that IL-1 β and pyroptosis-related inflammasome exerted a pivotal role in various pathological process of breast cancer, such as cell multiplication and migration, tumor angiogenesis and invasion, which contributed to breast cancer recurrence²⁸. In addition, IL-1 β was found to stimulate the expression of CCL2 in tumor cells and macrophages, and as a chemokine, CCL2 recruited macrophages and facilitated the metastasis of breast cancer²⁹. Our results in GO analysis were consistent with these previous findings.

According to the KEGG analysis, the pathways in which the DEGs participated mostly included NOD-like receptor signalling pathway, various bacterial and virus infection, cytosolic DNA-sensing pathway and transcriptional misregulation in cancer. NOD-like receptors (NLRs) acted as the sensors for the assembly of canonical inflammasomes, which induced canonical pyroptosis accompanied with the release of IL-1 β and IL-18 and GSDMD cleavage³⁰. Inflammasomes are related to both infections and non-infectious diseases. Cytosolic pattern recognition receptors (PRRs) were activated by danger-associated molecular patterns (DAMPs), such as abnormal nucleic acids, and pathogen-associated molecular patterns (PAMPs), such as liposaccharide³¹. The corresponding downstream signalling pathway was activated, leading to the production and release of type I interferons and pro-inflammatory cytokines, the assembly of inflammasomes and finally pyroptosis³². Our KEGG analysis results in this study were consistent with these previous findings.

Through univariate Cox analysis, 21 pyroptosis-related LncRNAs (LMNTD2-AS, AC137630.3, AL589765.4, AC015802.3, AC079298.3, AC087239.1, AL133467.1, LINC02195, KLHDC7B-DT, U62317.3, LINC02446, AC015819.1, AC109446.2, LINC01943, AL645608.7, USP30-AS1, LIPE-AS1, LRRC8C-DT, AC004585.1, HSD11B1-AS1, AC009119.1) were considered to exert a significant effect on overall survival, in which 9 (LMNTD2-AS1, AL589765.4, AC079298.3, U62317.3, LINC02446, AL645608.7, HSD11B1-AS1, AC009119.1, AC087239.1) were finally chosen to build the prognostic model through multiple variate Cox analysis. Among these LncRNAs, U62317.3 which targeted GZMB and IRF1 was reported to exert an important effect on tongue cancer through the miR let7 family³³. LINC02446 which targeted CASP1, CASP4, GZMB, IL18, IRF1, AIM2, NLRP1, GZMA was found to inhibit the metastasis of bladder cancer cells³⁴ and melanoma³⁵, and act as a prognostic signature in bladder cancer³⁶. HSD11B1-AS1 which targeted NLRP1 and NLRP3 was reported to participate in corticoid metabolism and relevant physiological function³⁷. AC079298.3 targeted NLR4, which assembled inflammasome in responding to bacterial products and exerted an essential role in pyroptosis and inflammation-induced tumorigenesis³⁸. AL589765.4, AL645608.7 and AC087239.1 targeted BAX, which was traditionally considered as a key protein of apoptosis³⁹ and recently reported to participate in the induction of pyroptosis through reactive oxygen species (ROS) activated by iron⁴⁰. Besides BAX, AC087239.1 also targeted GSDMB, CHMP2A, CHMP3, CHMP4A, CHMP6, GSDMD, GPX4, PYCARD. After being cleaved by GZMA from cytotoxic lymphocytes, GSDMB triggered pyroptosis of target cells⁴¹. GSDMD which was cleaved by inflammatory caspases induced

cell pyroptosis by forming membrane pores⁴². PYCARD took part in inflammasome activation⁴³ and the up-regulation of PYCARD independently predicted unfavorable prognosis of glioma⁴⁴. CHMP2A, CHMP3, CHMP4A, CHMP6 belong to the chromatin-modifying protein/charged multivesicular body protein (CHMP) family, which participated in the formation of endocytic multivesicular bodies (MVBs) and cell cycle progression. CHMP2A was found to be stably and universally expressed in buccal-mucosa tumors⁴⁵, and significantly up-regulated in progressive Renal cell carcinoma (RCC)⁴⁶. CHMP3 as part of endosomal sorting complex required for transport (ESCRT)⁴⁷ exerted an important role in epithelial-mesenchymal transition (EMT), which was associated with tumor progression and metastasis⁴⁸. CHMP4A was found to be associated with hypoxia inducible factor 1⁴⁹, and up-regulated significantly in early esophageal cancer⁵⁰. CHMP6 regulated endosomal cargo sorting⁵¹ and mediated the recycling of Ras to plasma membrane and promoted the signalling pathway of growth factor⁵².

Based on the risk scores calculated with the above-mentioned eight LncRNAs signature, patients were divided into High risk group and Low risk group. The risk score was verified as an independent prognostic factor by multiple variate Cox analysis. According to the Kaplan-Meier analysis, the overall survival time of High risk group was significantly different from that of Low risk group ($p < 0.001$), indicating the validity of the 8 LncRNAs signature in predicting prognosis of breast cancer patients. Furthermore, the specificity and sensitivity were determined using ROC curve and shown by DCA curve. The T stage distribution of patients in High risk group was significantly different from that in Low risk group, indicating that the eight LncRNAs and their target proteins were possibly involved in the expansion of tumor volume and affected range of tissues.

The pathways enriched in High risk group were mainly involved in glycan biosynthesis, wnt signalling pathway, adherens junction, ECM receptor interaction, focal adhesion, TGF- β signaling pathway. Adherens junction-related proteins such as vimentin and E-cadherin exerted an essential role in malignant progression of cancer through Epithelial-Mesenchymal Transition (EMT)⁵³. Focal adhesion kinase genes were up-regulated in cancer and used as targets in cancer therapies⁵⁴. The ECM receptor interaction was found to participate in the proliferation and invasion of kidney cancer cells⁵⁵. It was also reported that TGF- β suppressed pyroptosis in triple-negative breast cancer⁵⁶, and wnt/ β -catenin participated in the metastasis of breast cancer⁵⁷.

The pathways enriched in Low risk group were mainly involved in cytotoxicity mediated by natural killer cells, Fc epsilon RI signaling pathway, antigen processing and presentation, interaction between cytokine and cytokine receptor, cell adhesion molecules (CAMs), chemokine signaling pathway. The above-mentioned immune reactions were all known as important influence factors of tumor progression and metastasis. The enrichment results indicated that infiltrating immune cells and tumor immune micro-environment exerted a pivotal role in restraint of tumor expansion and invasion, and was associated with the prognosis of breast cancer patients.

The relative quantity of infiltrating immune cells of High risk group and Low risk group was evaluated using 7 algorithms including TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL and EPIC. According to the results, B cells, CD4+ T cells, CD8+ T cells, T cell follicular helper, regulatory T cells (Tregs), activated NK cells, monocytes, macrophage M1 cells, immune scores and microenvironment scores were enriched in Low risk group, indicating the contributions of above-mentioned immune cells to tumor suppression. Meanwhile, macrophage M2 cells, activated myeloid dendritic cells, mast cells, neutrophils, endothelial cells, cancer-associated fibroblasts, granulocyte-monocyte progenitor were enriched in High risk group, indicating the promoting effect exerted by these cells on progression and metastasis of breast cancer. Among these immune cells, macrophage M2 cells had been known to promote the progression and metastasis of carcinomas⁵⁸, while the roles played by mast cells in tumor immune microenvironment were quite controversial. On one hand, mast cells were once considered as a marker of favourable prognosis⁵⁹. While on the other hand, it was confirmed as responsible to chemotherapy resistance⁶⁰, inflammation and angiogenesis⁶¹ by recent studies, which supported the findings of immune cell classification in this study. The dual role of mast cells in breast cancer needed further investigation. Furthermore, the quantity of myeloid dendritic cells was reported as higher in breast cancer patients than healthy people⁶². Neutrophils⁶³, endothelial cells⁶⁴, cancer-associated fibroblasts⁶⁵ were reported as markers of unfavorable tumor prognosis, which exhibited consistence with our results, indicating that risk scores calculated with the 9 LncRNAs signature acted as a satisfactory criterion to separate the tumor immune microenvironment and prognosis of breast cancer patients.

Immune checkpoint pathways normally maintain self-tolerance and avoid excessive immune responses to microbial infection. However, under pathological situation, immune checkpoint can be used by cancer to escape from immune surveillance, and thus become an innovative target of chemotherapies⁶⁶. In this study, the immune checkpoint-related proteins of High risk group were significantly different from those of Low risk group, indicating that the division of breast cancer patients by risk score calculated using the 9 LncRNAs signature adequately presented the differences in tumor immune micro-environment.

As the most universal pattern of post-transcriptional modification of LncRNAs and eukaryotic mRNA, N⁶-Methyladenosine (m⁶A) exerts its roles such as cancer progression and metastasis principally by recruiting eIF3 and m⁶A-relevant proteins with YTH domain⁶⁷. m⁶A proteins have been developed as targets of chemotherapies and biomarkers for prognosis prediction⁶⁸. In this study, the expressions of FTO, ZC3H13, METTL3, METTL14, YTHDC1, YTHDC2 were found as significantly different between patients in High risk group and Low risk group. It was reported that FTO promoted the progression and invasion of breast cancer through regulating the m⁶A levels of target genes⁶⁹. ZC3H13⁷⁰ and METTL14⁷¹ acted as writers in m⁶A pathways and were reported as tumor-suppressor genes in breast cancer, while in this study ZC3H13 was up-regulated in High risk group and associated with unsatisfactory prognosis, which seemed ambiguous and needs further investigation. In this study, METTL3 expression was significantly lower in High risk group than Low risk group, which was consistent with the findings of Y Shi et al that the metastasis of triple-negative breast cancer was promoted by reduced expression of METTL3⁷². Known as writers of m⁶A pathways, YTHDC1⁷³/ YTHDC2⁷⁴ inhibited tumor proliferation and migration and acted as protective genes. In this study, YHDC1/2 were both up-regulated in patients of High risk group than those of Low risk group and inconsistent with previous findings, which needed further study.

LncRNAs and miRNAs are both important regulatory factors of pyroptosis. Consequently, the pyroptosis-related LncRNA-miRNA-mRNA regulatory network was constructed using cytoscape 3.7.2 and the hub genes were discovered using the plug-in module of cytoscape hubba. GO and KEGG analyses of these target genes of the miRNAs associated with DELncRNAs were also performed.

Among the hub genes of the regulatory network, MIAT exhibited the highest score. MIAT participated promoted cancer progression, migration and metastasis⁷⁵, and was significantly up-regulated in breast cancer tissues and correlated with diameter, molecular classification, axillary lymph node metastases and TNM stages of tumors⁷⁶. hsa-miR-24-3p predicted as the potential target of MIAT was reported to be a tumor-suppressor miRNA and negatively correlated with TMN stage⁷⁷. hsa-miR-17-5p was also predicted as the potential target of MIAT and reported previously to promote cancer through regulation on cytotoxic T lymphocytes and NK cells⁷⁸. hsa-miR-129-5p was reported to be up-regulated in colorectal cancer tissues and positively correlated with patient overall survival⁷⁹. WDFY3-AS2 was also reported as a potential prognostic factor of triple negative breast cancer⁸⁰. The relationship between these nodes in the network still needed further studies.

In comparison with pyroptosis-related DEGs, the GO analysis results of the miRNA target genes contained cysteine-type endopeptidase activity, DNA-binding transcription activator activity, death receptor binding and TNF receptor binding, indicating the inherent correlation between pyroptosis and apoptosis. As for the KEGG analysis results of the miRNA target genes, besides the common pathways of various cancers, microbial infections and apoptosis, the miRNA target genes were also enriched in non-infective diseases such as neurodegeneration, rheumatoid arthritis and non-alcoholic fatty liver disease; cancer-related pathways such as miRNAs in cancer, proteoglycans in cancer, viral carcinogenesis, PD-L1 signaling pathway; necroptosis and related pathways such as TNF- α and Toll-like signaling pathway; and immune-related pathways such as NK cell-mediated toxicity, T cell receptor signaling pathway and Th17 cell differentiation. The results indicated that the target genes of pyroptosis-related LncRNA-miRNA-mRNA regulatory network was universally interrelated with various infectious, non-infectious diseases and relevant immune responses through possible crosstalk among apoptosis, pyroptosis and necroptosis.

Conclusions

As a kind of programmed cell death discovered in recent years, pyroptosis has been reported to exert an essential role in tumorigenesis, progression and metastasis, and provides an innovative target for development of cancer therapy. However, the specific mechanism of regulatory LncRNAs in pyroptosis of breast cancer has not yet been fully clarified. In this study, a 9-LncRNAs signature was constructed to predict the prognosis of breast cancer patients, and the regulatory network of LncRNA-miRNA-mRNA in pyroptosis was constructed. The miRNAs regulated by the pyroptosis-related LncRNAs possibly mediated crosstalk among pyroptosis, apoptosis and necroptosis through their target genes. Our work provided an innovative insight into the development of cancer diagnosis tools and therapies. More efforts are needed to verify the conclusion of this work with experimental and clinical approaches.

Declarations

Ethics approval and consent to participate

The data used in this study is open to public in TCGA database (Home | NCI Genomic Data Commons (cancer.gov))

Consent to publication

Not applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declared no competing interests.

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Author's contributions

Ye Tian designed the methodology, performed data analysis and visualization and completed the original writing.

Yanan Zhang was responsible to data curation and manuscript editing.

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Figures

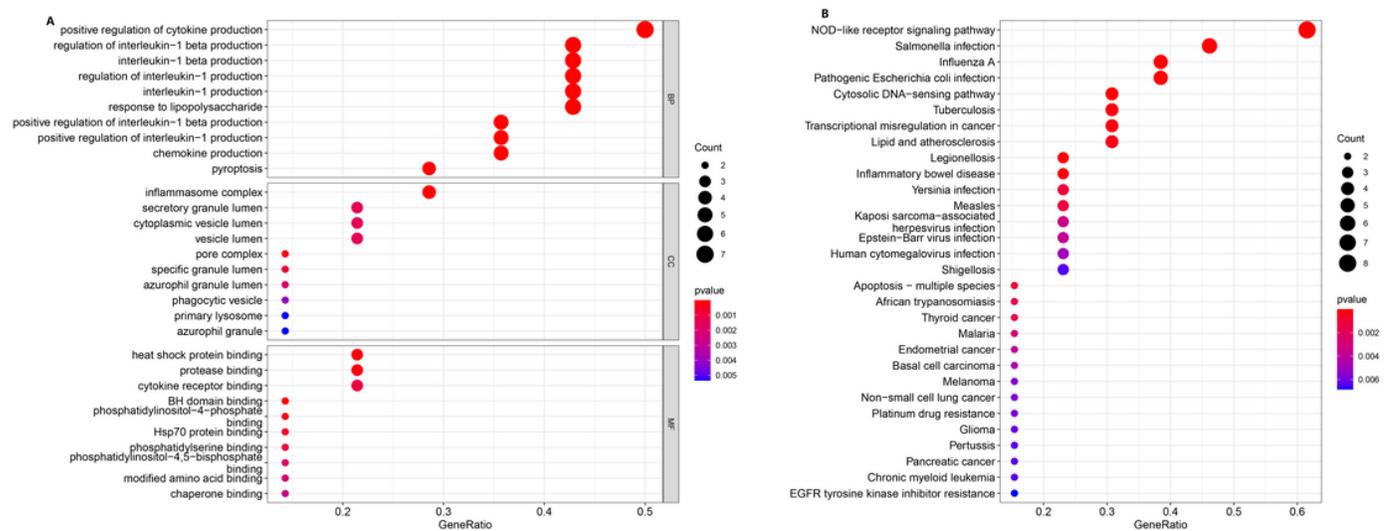


Figure 1

GO and KEGG analyses of pyroptosis-related DEGs using R packages “ggplot2” and “org.Hs.eg.db”(adjust p value <0.05) The R package “org.Hs.eg.db” was used to convert gene symbols into entrezIDs by mean of mget function. The X-axis represented the generation which was defined as the quantity of genes enriched in a GO function or a KEGG pathway /the quantity of total involved genes. The Y-axis represented every specific function or pathway enriched in the involved genes. Larger diameter of circle indicated higher quantity of genes enriched in each item, and color of circle closer to red indicated lower adjust p value. BP is the abbreviation of biological process. MF is the abbreviation of molecular functions. CC is the abbreviation of cellular components. A GO analysis. B KEGG analysis.

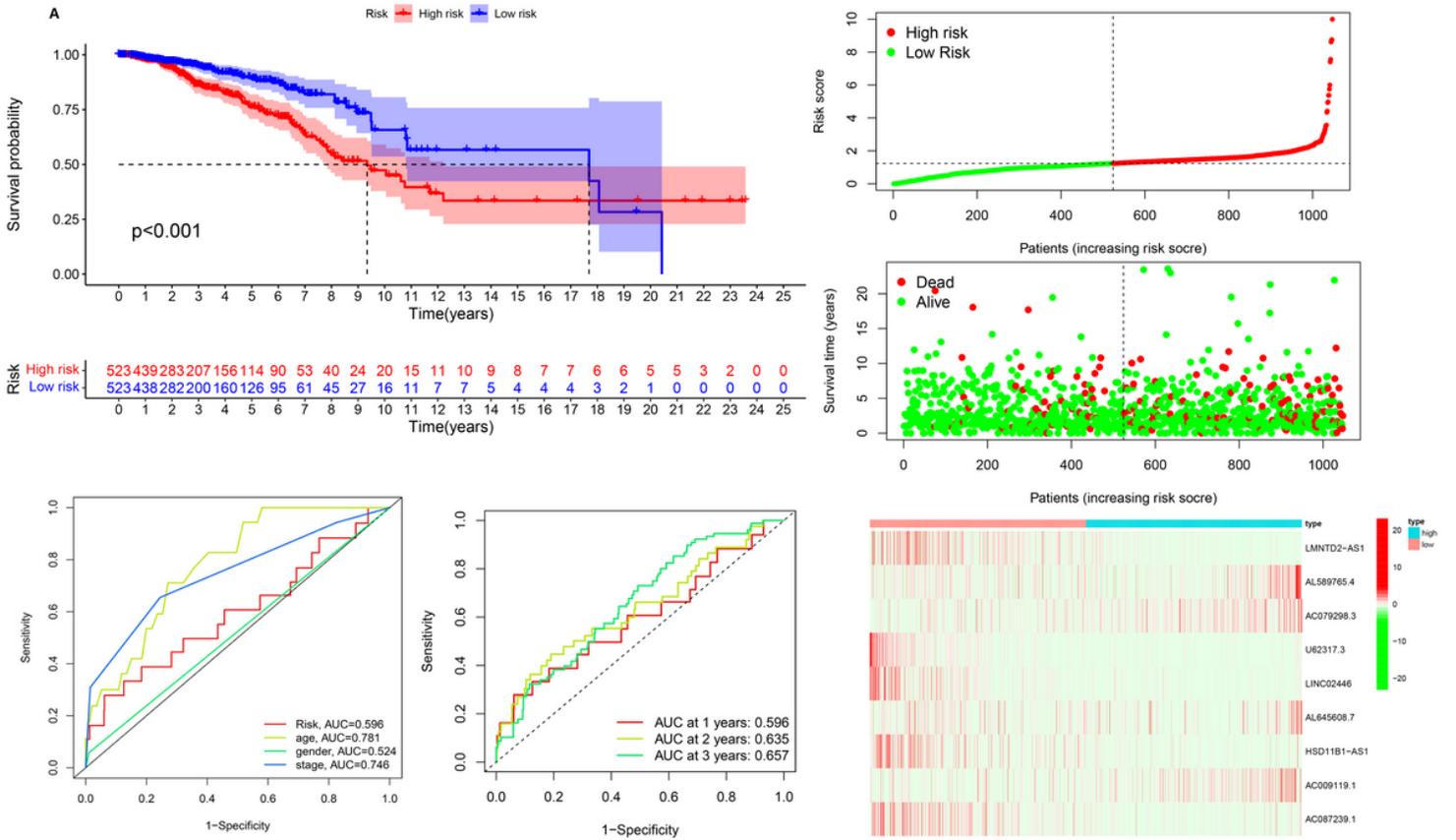


Figure 2

Evaluation on the validity of the pyroptosis-related 9 LncRNAs signature A K-M analysis revealed that the overall survival of High risk group (red) was significantly lower than that of Low risk group (blue) ($p < 0.001$). B The upper figure described the classification of patients on the basis of risk scores calculated with the signature formula; the middle figure indicated that the mortality was increased along with the rising risk scores; the bottom figure represented the differential expressions of the 9 LncRNAs in High risk group and Low risk group. C The AUC of the signature was 0.589 at the first year, which was valid for the prediction of breast cancer prognosis. D The AUC values at the 1st, 2nd and 3rd year.

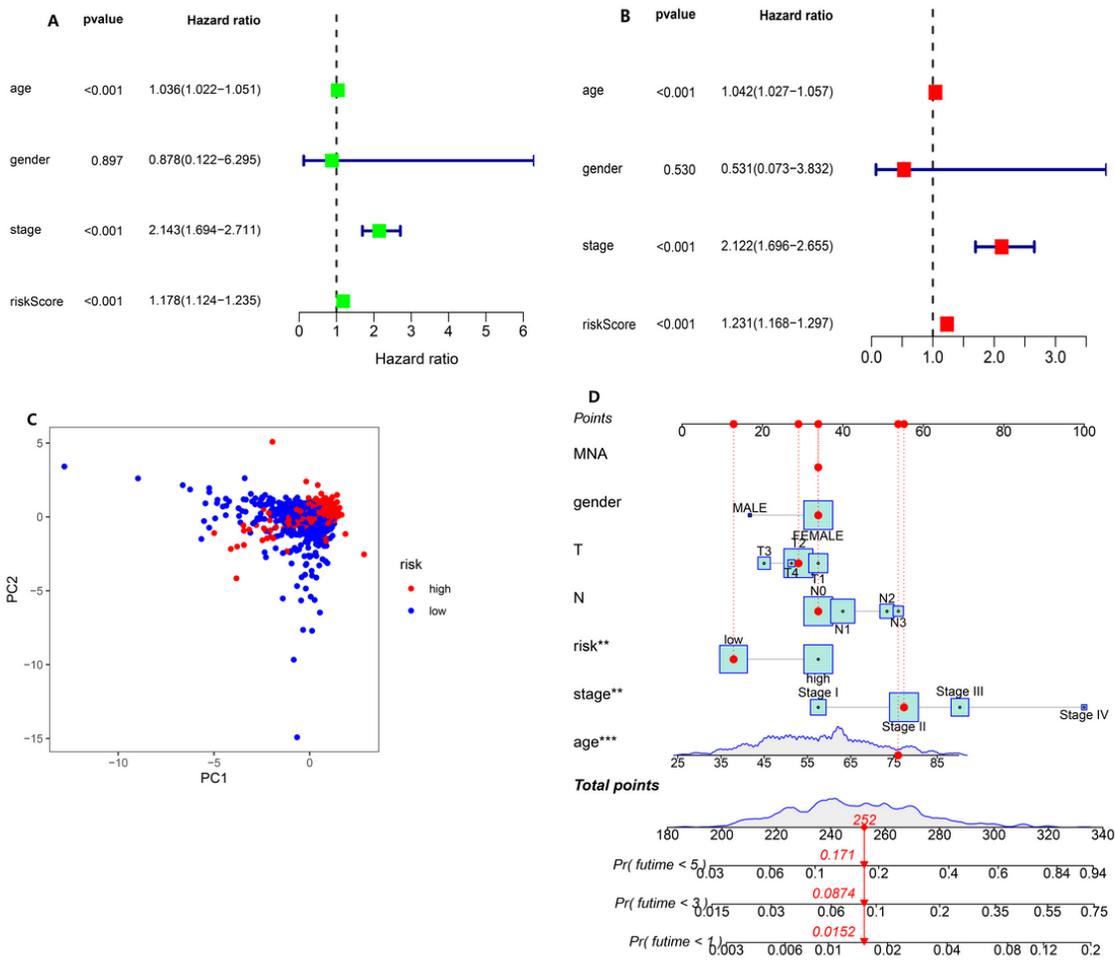


Figure 3

Evaluation on the prognostic value of the pyroptosis-related 9 lncRNAs signature using univariate and multivariate Cox proportional hazard ratio. PCA analysis and nomogram for prognostic prediction. A The risk score was verified as an individual factor associated with overall survival by univariate Cox regression analysis. Green: hazard ratio in univariate Cox regression analysis. B The risk score was verified as an independent prognostic factor of survival by univariate Cox regression analysis (Hazard ratio = 1.231; 95%CI: 1.091-1.158). Red: hazard ratio in multivariate Cox regression analysis. C Grouping of patients based on PCA analysis of the expressions of pyroptosis-related 9 lncRNAs. D The nomogram for prognostic prediction of breast cancer.

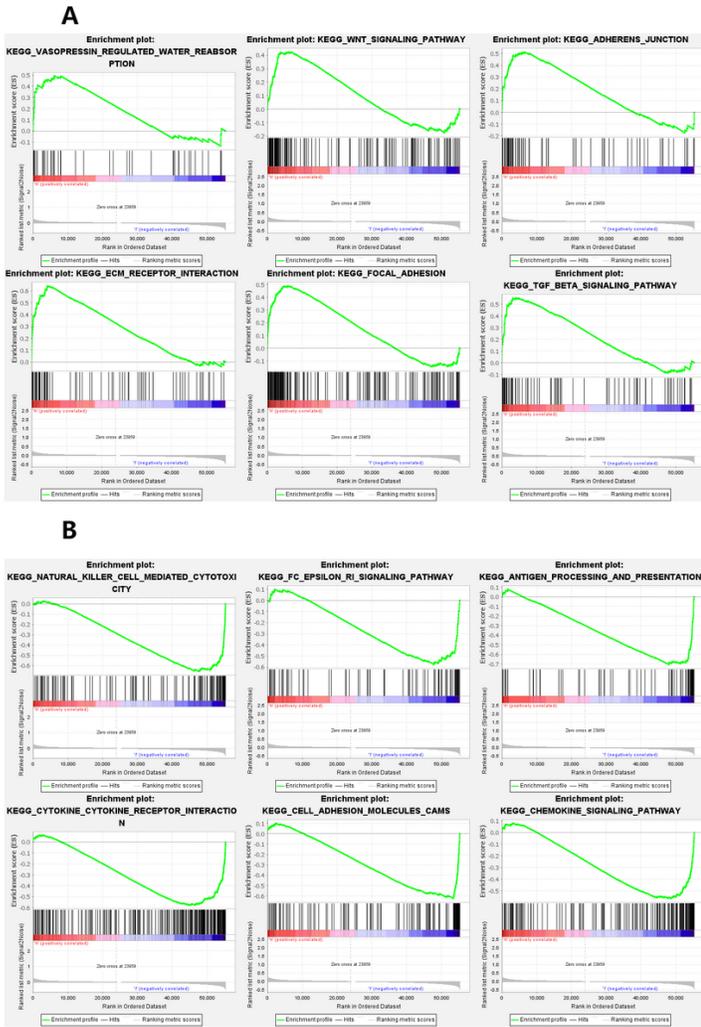


Figure 4

Gene sets enriched in High risk group and Low risk group (Top 6) A Enrichment in High risk group B Enrichment in Low risk group

Fig.5A

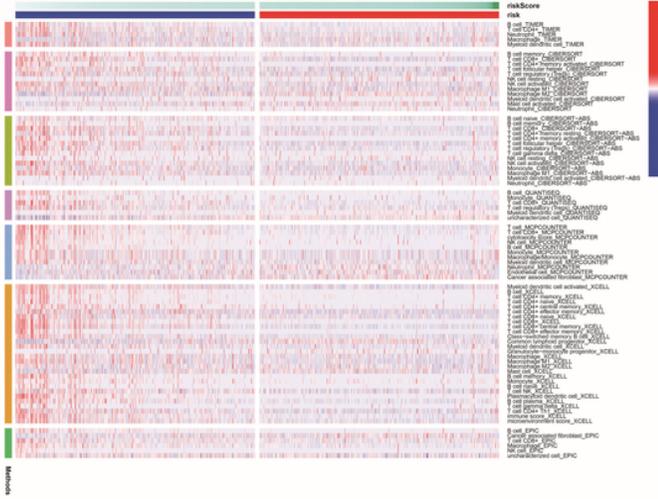


Fig.5C

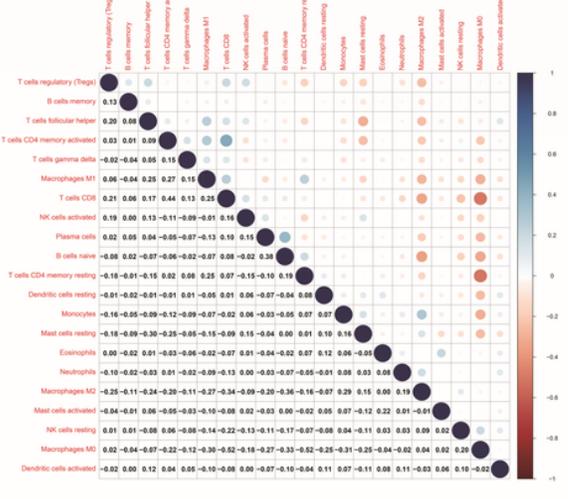


Fig.5B

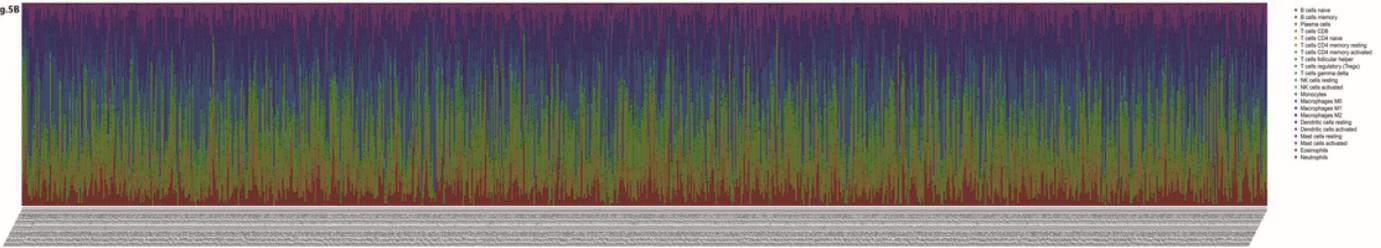


Figure 5

The immune cell distribution and correlation between various immune cell types A The immune cell classification in High risk group and Low risk group analyzed using TIMER, CIBERSORT, CIBERSORT-ABS, QUANTISEQ, MCPOUNTER, XCELL, EPIC B The correlation between immune cell types based on CIBERSORT result C The barplot of immune cell types analyzed by CIBERSORT from TCGA breast cancer patients

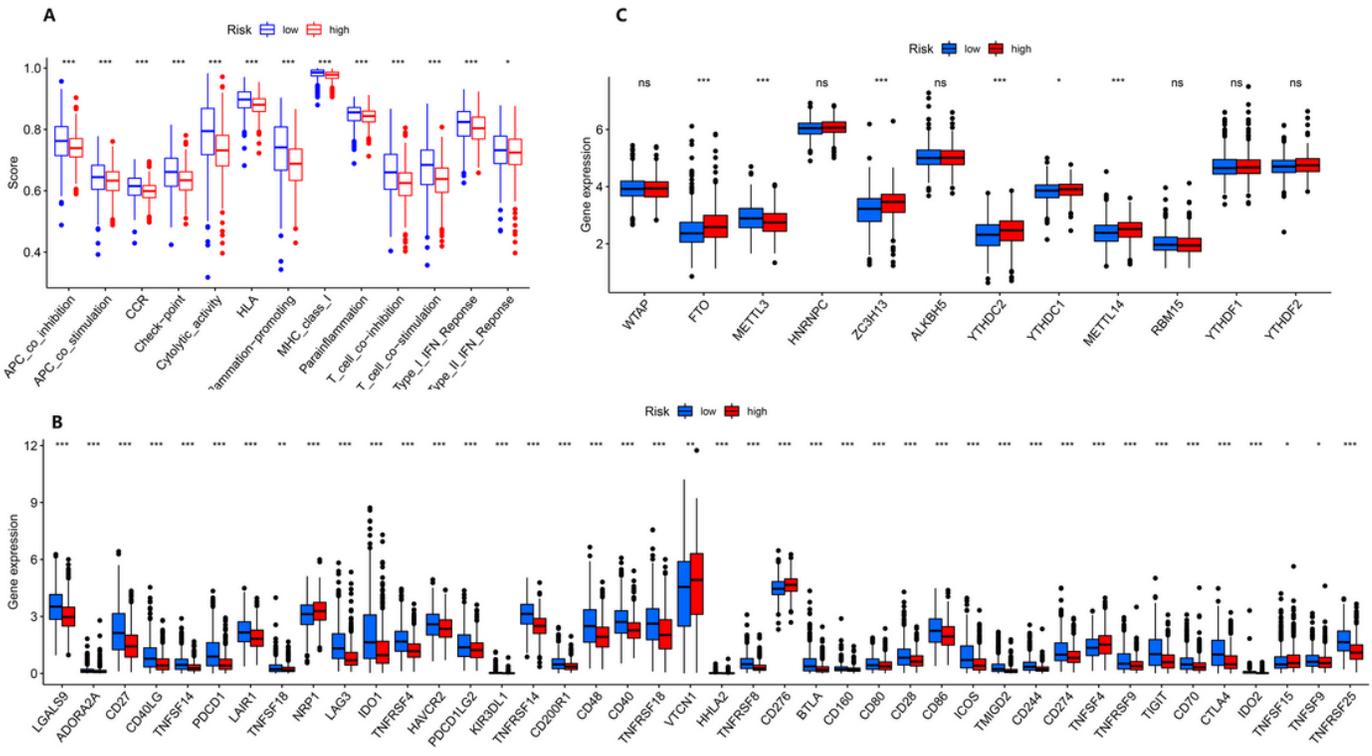


Figure 6

The differences in immune function correlated with immune cell classification on the basis of ssGSEA, checkpoint-related proteins and m6A-related proteins between High risk group (red) and Low risk group (blue) A Immune functions B Checkpoint-related proteins C m6A-related proteins

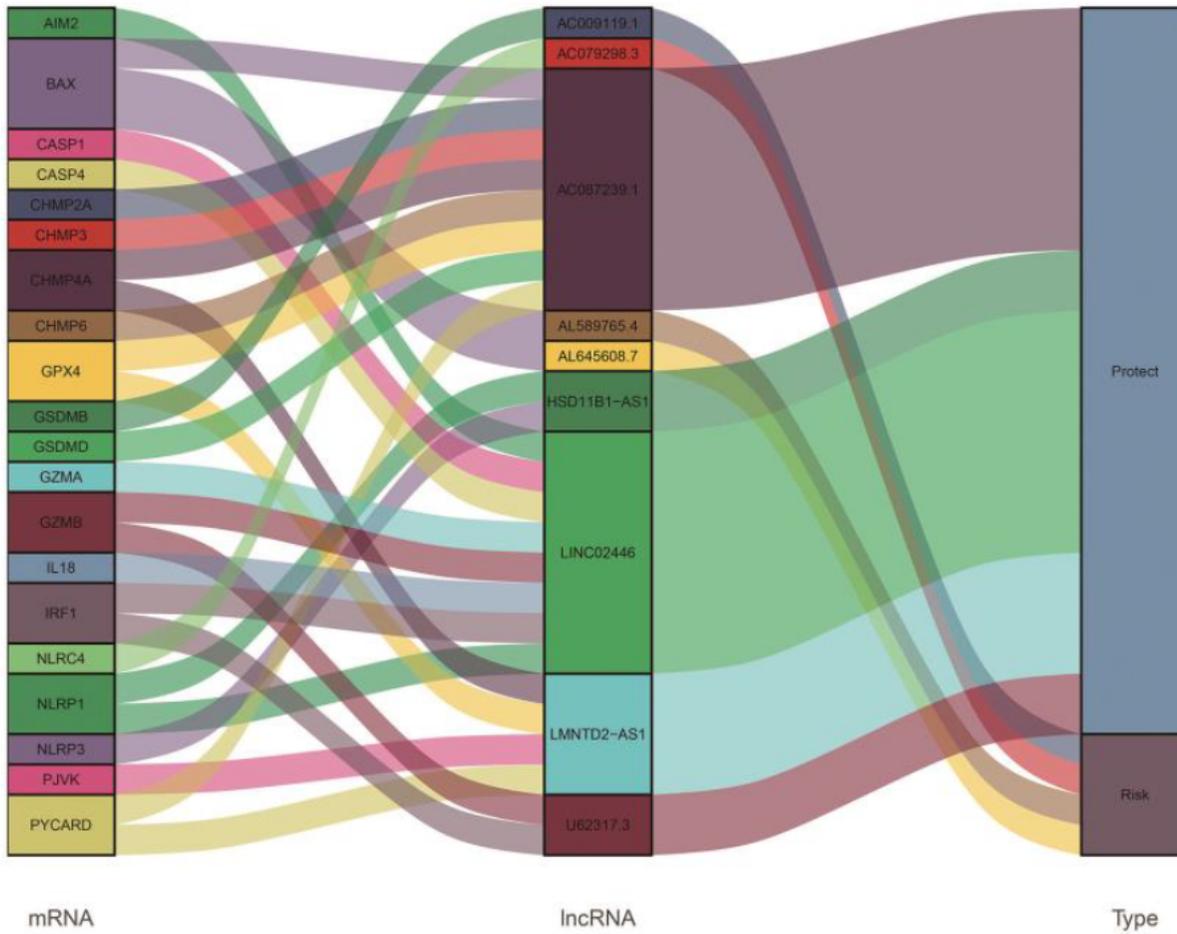


Figure 7

Sankey plots demonstrated the relationship between 9 pyroptosis-related LncRNAs and their target genes.

