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# Research article

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# Construction of molecular subtype model of osteosarcoma based on endoplasmic reticulum stress and tumor metastasis-related genes

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## ARTICLE INFO

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# ABSTRACT

*Introduction:* Osteosarcoma, the prevailing primary bone malignancy among children and adolescents, is frequently associated with treatment failure primarily due to its pronounced metastatic nature.

*Methods:* This study aimed to establish potential associations between hub genes and subtypes for the treatment of metastatic osteosarcoma. Differentially expressed genes were extracted from patients diagnosed with metastatic osteosarcoma and a control group of non-metastatic patients, using the publicly available gene expression profile (GSE21257). The intersection of these gene sets was determined by focusing on endoplasmic reticulum (ER) stress-related genes sourced from the GeneCards database. We conducted various analytical techniques, including functional and pathway enrichment analysis, WGCNA analysis, protein-protein interaction (PPI) network construction, and assessment of immune cell infiltration, using the intersecting genes. Through this analysis, we identified potential hub genes.

*Results:* Osteosarcoma subtype models were developed using molecular consensus clustering analysis, followed by an examination of the associations between each subtype and hub genes. A total of 138 potential differentially expressed genes related to endoplasmic reticulum (ER) stress were identified. These genes were further investigated using Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Gene Set Enrichment Analysis (GSEA) pathways. Additionally, the PPI interaction network revealed 38 interaction relationships among the top ten hub genes. The findings of the analysis revealed a strong correlation between the extent of immune cell infiltration and both osteosarcoma metastasis and the expression of hub genes. Notably, the differential expression of the top ten hub genes was observed in osteosarcoma clusters 1 and 4, signifying their significant association with the disease.

*Conclusion:* The identification of ten key genes linked to osteosarcoma metastasis and endoplasmic reticulum stress bears potential clinical significance. Additionally, exploring the molecular sub-type of osteosarcoma has the capacity to guide clinical treatment decisions, necessitating further investigations and subsequent clinical validations.

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## 1. Introduction

Osteosarcoma, a primary bone tumor prevalent among children and adolescents, exhibits an annual incidence of approximately 8–11/million [1]. This malignancy is characterized by its highly aggressive nature, heterogeneity, and propensity for metastasis [2], leading to a significant risk of disability and mortality following metastatic spread. Osteosarcoma commonly manifests in the vicinity of the metaphysis of long bones, including the distal femur, proximal tibia, and humerus [3]. The precise etiology, progression, and metastasis of osteosarcoma have not been thoroughly investigated [4,5]. However, the elevated prevalence among adolescents and its predilection for specific anatomical sites suggest a correlation with accelerated bone tissue growth [6]. Presently, the primary therapeutic approach for osteosarcoma entails preoperative chemotherapy, surgical excision, and postoperative chemotherapy. Despite extensive removal of macroscopic tumor tissue during surgical intervention, recurrence frequently occurs [7]. The implementation of neoadjuvant chemotherapy has led to a substantial enhancement in the 5-year survival rate among individuals diagnosed with osteosarcoma [8]. When neoadjuvant chemotherapy is combined with surgical resection, the 5-year survival rate for patients with non-metastatic osteosarcoma is approximately 60% [9]. However, the survival rate drops significantly to only 20–30% for patients with metastatic osteosarcoma [9–11]. The high incidence of metastasis in osteosarcoma is the primary determinant of the unfavorable prognosis [12]. Presently, there is no treatment available that significantly improves the 5-year survival rate for patients with metastatic osteosarcoma [13].

The endoplasmic reticulum (ER) plays a crucial role in the processes of protein synthesis, folding, and structural maturation. ER stress, which occurs as a result of genetic or environmental factors, disrupts the ER's ability to properly modify, fold, and secrete proteins, leading to the accumulation of misfolded proteins within organelles [14]. This phenomenon of ER stress is closely linked to the onset and progression of various diseases, including tumors, diabetes, and neurodegenerative disorders [15–17]. Unfavorable conditions within the tumor microenvironment, including nutritional deficiency, hypoxia, hypermetabolism, and oxidative stress, have been shown to disrupt protein folding by the endoplasmic reticulum (ER), leading to the persistent activation of "ER stress." This phenomenon enhances the tumorigenic, metastatic, and drug-resistant properties of malignant cells [14,16,18]. Osteosarcoma is characterized by extensive genetic variation, dysregulation of multiple signaling pathways, and genome instability. These factors are closely associated with the regulation of bone development, tumor microenvironment, genome homeostasis, cell cycle control, and cell signal transduction pathways [16]. The correlation between ER stress-related genes and osteosarcoma metastasis has been established [19], and the utilization of molecular subtype models is crucial for predicting tumor risk and evaluating prognosis [20–22]. Nevertheless, there is currently a lack of research on molecular subtype models specifically pertaining to ER stress and osteosarcoma metastasis.

In this study, the author developed a molecular subtype model for osteosarcoma AMI. By analyzing the gene data from 14 metastatic osteosarcoma patients and 19 non-metastatic osteosarcoma patients, the study identified co-expressed differential genes associated with osteosarcoma metastasis and ER stress. Subsequently, a validation set comprising 10 key genes was established, confirming the robustness of the molecular subtype model for osteosarcoma. These key genes hold promise as potential targets for targeted therapeutic drugs and molecular markers for prognostic evaluation.

## 2. Materials and methods

#### 1 GEO data difference analysisC

We performed an analysis on the osteosarcoma dataset. GSE21257 comes from the Gene Expression Omnibus (GEO) database [23]. GSE21257 has 53 samples, including 14 samples of osteosarcoma metastases (metastases present at diagnosis) and 19 samples of non-metastases (no metastases). The chip platform is a GPL10295 Illumina human-6 v2. 0 expression beadchip (using nuIDs as identifiers).

We used the standardized data expression matrix downloaded from GEO to match, select, and delete gene data based on the samples. The selected samples were grouped according to metastasis and non-metastasis for subsequent analysis. For the preprocessed data set, we drew a boxplot using the R language's boxplot function to observe the data distribution. Expression difference *P*-values and expression fold change values were calculated using the R package limma (Version 3.42.2) [24]. We selected genes with a *P*-value <0.05 and |log2FC| > 0.263 (1.2-fold relationship) as significantly differentially expressed mRNAs. The R package heatmap function (Version 1.0.12) [25] was used to make differential heat maps and volcano maps for visualization.

# 2 Molecular subtype construction

We calculated the AMI molecular subtypes with the ConsensusClusterPlus package [26] and the Rtsne package [27]. The ggplot2 package was used for molecular subtype visualization. Correlations between hub genes and molecular subtypes were plotted with the gspubr package.

## 3 Enrichment analysis of intersection genes

The GeneCards database (https://www.genecards.org/) [28] provides annotated and predicted human genetic information. The database automatically integrated genetic data from approximately 150 network sources, including genomics, transcriptomics,



Fig. 1. Flow chart.



Fig. 2. Difference analysis (Pink is the metastatic group and green is the non-metastatic group): (A) Boxplot before GSE21257 data processing. (B) Boxplots after GSE21257 data processing. (C) PCA graph. (D)GSE21257 dataset differential genes volcano plot. Red is up-regulated differential genes, blue is down-regulated differential genes, and grey is non-significant genes. (E) GSE21257 datasetdifferential genes heat map. Blue is the metastatic group, pink is the non-metastatic group, green is low expression, and red is high expression. (F) Venn diagram of the intersection of endoplasmic reticulum stress-related genes and differential genes. 138 potential ER stress-related differentially expressed genes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2 Molecular Subtype Construction



Fig. 3. Molecular typing of osteosarcoma: (A) The cumulative distribution function (CDF) curve. (B) The CDF Delta area curve. (C) Heat map of molecular subtype sample clustering. (D) The spatial distribution of different clusters.

3 Enrichment analysis

proteomics, and genetics, as well as clinical and functional information.

In this analysis, ER stress-related genes were downloaded from the GeneCards database using "endoplasmic reticulum stress" as the search key. We took the intersection of differential genes and ER stress-related human genes to create a Venn diagram, which was constructed using the R-package Venn diagram function (Version 1.6.20) [29].

Gene Ontology (GO) [30] describes our understanding of the field of biology based on three aspects, molecular function (MF), cellular components (CC), and biological processes (BP). GO enrichment analysis is typically used to explore the enrichment degree of GO terms associated with differentially expressed genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) [31] is a utility database resource for understanding advanced functions and biological systems (such as cells, organisms, and ecosystems) based on molecular-level information, especially genome sequencing and other high-throughput experimental techniques generated from large molecular datasets. KEGG was established in 1995 by Kanehisa's laboratory at the Center for Bioinformatics at Kyoto University, Japan.

The analysis used in this study was based on the use of the R package clusterProfiler package (version 3.14.3) [32] to perform GO function/pathway enrichment analysis on the intersection genes and the significance threshold was set to  $P \le 0.05$ . Bubble plots were created for visualization using the R package ggplot2 (Version 3.3.3) [33]. The pathway network diagram was drawn to visualize the pathway relationship using the cytoscape plug-in ClueGo [34].

We selected and downloaded the c2.cp.v7.2.symbols.gmt gene set data from the GSEA (http://www.gsea-msigdb.org/gsea/index. jsp) [35] database as the reference gene set and performed GSEA enrichment analysis on the two sets of data with the R clusterProfiler package. The GSEA statistical process was used to calculate the enrichment score, estimate the significance of the enrichment score, correct for multiple hypothesis testing, and select the enrichment results with P < 0.05 to draw the GSEA enrichment map.



Fig. 4. Enrichment analysis: (A) GOBP enrichment bubble chart of intersection genes. The vertical axis is the BP name, the horizontal axis is the number of enriched genes, and the dot size is the ratio of the number of enriched genes to the total number of uploaded genes. The larger the ratio, the larger the dots. The redder the dot color, the more significant the P value. (B) GOCC enrichment bubble chart of intersection genes. The vertical axis is the CC name, the horizontal axis is the number of enriched genes, and the size of the dots is the ratio of the number of enriched genes to the total number of uploaded genes. The larger the ratio, the larger the dots. The redder the dot color, the more significant the P value. (C) GOMF enrichment bubble chart of intersection genes. The vertical axis is the MF name, the horizontal axis is the number of enriched genes, and the size of the dots is the ratio of the number of enriched genes to the total number of uploaded genes. The larger the ratio, the larger the dots. The redder the dot color, the more significant the P value. (D) PathwaysEnrichment bubble chart of intersection genes. The vertical axis is the pathways name, the horizontal axis is the number of enriched genes, and the size of the dots is the ratio of the number of enriched genes to the total number of uploaded genes. The larger the ratio, the larger the dots. The redder the dot color, the more significant the P value. (E) GO enrichment histogram of intersection genes. The horizontal axis is the number of genes, and the vertical axis is the GO item. (F) KEGG network diagram. Different colors represent different types of KEGG pathways, and bold fonts represent pathways. The more significant the P value, the larger the dots, and the two-point line represents the correlation between functions. (G) TOP5 KEGG GSEA diagram. Enrichment Score polyline section. The horizontal axis is the sorted gene, and the vertical axis is the corresponding ES. The peak in the line graph is the Enrichment score of this genes set, and the genes before the peak are the core genes under the genes set. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

- 4 WGCNA analysis
- 4 WGCNA analysis

The gene co-expression network was constructed using the WGCNA R package [36]. By calculating the Pearson correlation coefficient between two genes, we used the expression data to create a similarity matrix and chose an appropriate soft threshold  $\beta$  to make the constructed network more compatible with the standard of a scale-free network. We transformed the adjacency matrix into a topological overlap matrix TOM and used hierarchical clustering to generate a hierarchical clustering tree of genes. The correlations between genes and clinical information were calculated, and the significant associations of modules with ER-related DEGs were analyzed.

5 PPI Interaction Network Construction

#### Table 1

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CO-2001243         bP         negative regulation of intrinsic appoints signaling tatiway         2.05E-09           CO-2002293         BP         regulation of oxiditive stress-induced cell death         3.94E-09           CO-2003201         BP         regulation of oxiditive stress-induced cell death         3.27E-10           CO-2003134         CC         COPII-coated ER to Golgi transport vesicle         3.27E-13           CO-0003135         CC         codard vesicle         1.27E-13           CO-0003135         CC         intrinsic component of endoplasmic reticulum membrane         8.59E-09           CO-0003127         CC         intrinsic component of endoplasmic reticulum membrane         1.46E-08           CO-0003127         CC         endoptive vesicle         2.90E-08           CO-0003127         CC         endoptive vesicle membrane         2.92E-07           CO-00030506         CC         Golgi-associated vesicle membrane         2.82E-07           CO-00030505         CC         pagocytic vesicle membrane         2.82E-07           CO-00030517         CC         folgi-associated vesicle membrane         2.82E-07           CO-0003053         CC         nedoplasmic reticulum         2.81E-06           CO-0003054         CC         norobip damic reticulum         2.82E-07	GO:0043555	BP	regulation of translation in response to stress	2.41E-09
CO20022933         pr         reactive oxygen species metabolic process         2.784-09           CO20049732         PP         positive regulation of rotacit rests-induced cell death         3.246-09           CO20049733         CC         CO2PI-coated ER to Golg it ransport vesicle         2.028-15           CO2005798         CC         Co2PI-coated ER to Golg it ransport vesicle         2.028-16           CO20030176         CC         intrinsic component of endoplasmic reticulum membrane         8.59E-09           CO20030177         CC         intrinsic component of endoplasmic reticulum membrane         1.98E-08           CO20030176         CC         endorytic vesicle         2.90E-08           CO20030176         CC         endorytic vesicle membrane         2.90E-08           CO20030176         CC         endorytic vesicle membrane         2.90E-08           CO200301660         CC         endorytic vesicle membrane         2.82E-07           CO200302665         CC         phagocytic vesicle         3.80E-07           CO20030172         CC         cough endoplasmic reticulum         2.82E-07           CO20030127         CC         cough endoplasmic reticulum         2.82E-07           CO20030127         CC         cough endoplasmic reticulum         2.82E-07 <td< td=""><td>GO:2001243</td><td>BP</td><td>negative regulation of intrinsic apoptotic signaling pathway</td><td>2.63E-09</td></td<>	GO:2001243	BP	negative regulation of intrinsic apoptotic signaling pathway	2.63E-09
COL1903201prregulation to cutative sites-induced cen deam5.74E-09COC0045732BPpositive regulation of process6.27E-09COC0050796CCCCColg it cassociated vesicle1.27E-13COC0050716CCintegral component of endoplasmic reticulum membrane8.59E-09COC0050716CCintegral component of endoplasmic reticulum membrane1.48E-08COC0050176CCendoplasmic reticulum membrane1.96E-08COC0050179CCendoplasmic reticulum nembrane2.47E-07COC0050705CCendoplasmic reticulum membrane2.47E-07COC0050705CCEndoplasmic reticulum nembrane2.47E-07COC0050705CCEndoplasmic reticulum2.21E-06COC0050705CCendoplasmic reticulum2.21E-06COC0050705CCendoplasmic reticulum2.21E-06COC0050705CCendoplasmic reticulum2.21E-06COC0050705CCendoplasmic reticulum2.21E-06COC0050711CCcolpla estate2.95E-06COC0050711CCoruelear envelope4.38E-05COC0050305CCnuclear envelope1.32E-05COC0050711CCoruelear envelope1.32E-05COC0050727MFendopetidas reticulum lumen1.35E-05COC0050737MFendopetidas estate activity involved in apoptotic process8.94E-07CO0005077MFendopetidas estativity involved in apoptotic proces8.94E-05COC0005077MF	GO:0072593	BP	reactive oxygen species metabolic process	2.75E-09
CO:001372DPDistitVe regulation of protein catabolic process6.276-99CO:00031134CCCO PUI-coated ER to Gigi transport vesicle2.02E-10GO:0003135CCcoated vesicle2.02E-10GO:0003176CCintegral component of endoplasmic reticulum membrane8.59E-09GO:0003177CCintegral component of endoplasmic reticulum membrane1.96E-08GO:0003179CCendoplasmic reticulum membrane2.90E-08GO:0003030CCendopci vesicle membrane2.47E-07GO:00030660CCGo Gigi transport vesicle membrane2.82E-07GO:00030660CCendopci vesicle membrane2.82E-07GO:00030660CCendoplasmic reticulum3.80E-07GO:00030660CCendoplasmic reticulum2.21E-06GO:00030660CCendoplasmic reticulum2.21E-06GO:0003071CCrough endoplasmic reticulum2.21E-06GO:0003071CCrough endoplasmic reticulum2.35E-07GO:0003071CCcollasmic reticulum2.35E-06GO:0005788CCendoplasmic reticulum fumen1.15E-05GO:0005788CCendoplasmic reticulum fumen1.32E-05GO:0001267MFcysteine-type endopertidase regulator activity involved in apoptotic process8.04E-07GO:0001687MFcysteine-type endopertidase regulator activity involved in apoptotic process3.82E-05GO:00016903MFcysteine-type endopertidase regulator activity involved in apoptotic process3.82E-05 <td>GO:1903201</td> <td>DP</td> <td>regulation of oxidative stress-induced cell death</td> <td>5.94E-09</td>	GO:1903201	DP	regulation of oxidative stress-induced cell death	5.94E-09
COLOUGNIANCCCOLOUR (LABLE) IN 10 Uoing Lineagin Visible3.200-13COLOURS (C)Goldy-LABLE IN 10 Uoing Lineagin Visible2.202-10COLOURS (C)integral component of endoplasmic reticulum membrane1.48E-08COLOURS (C)integral component of endoplasmic reticulum membrane1.48E-08COLOURS (C)endopcinic reticulum Golgi intermediate compartment1.96E-08COLOURS (C)endopcinic visicle membrane2.90E-08COLOURS (C)Golgi-associated vesicle membrane2.47E-07COLOURS (C)ER to Golgi transport vesicle membrane2.52E-07COLOURS (C)CCendopcitic vesicle3.80E-07COLOURS (C)CCER to Golgi transport vesicle membrane2.61E-06COLOURS (C)CCCOLOURS (C)2.95E-06COLOURS (C)CCCOLOURS (C)2.95E-06COLOURS (C)endoplasmic reticulum2.45E-07COLOURS (C)companic reticulum fumen2.45E-06COLOURS (C)companic reticulum fumen2.45E-06COLOURS (C)endoplasmic reticulum fumen1.35E-05COLOURS (C)<	GO:0045732	BP	COPIL cost of FD to Color transport verials	0.2/E-09
CO.000796C.Outge-Bastchitet Vesite1.271-73CO.00030176CCintegral component of endoplasmic reticulum membrane8.59E-09GO.00030177CCintrinsic component of endoplasmic reticulum membrane1.48E-08GO.00030793CCendoplasmic reticulum Gastchiter1.96E-08GO.0003076CCendoplasmic reticulum Gastchiter2.90E-08GO.00030760CCGolgi-associated vesicle membrane2.47E-407GO.00030658CCtransport vesicle membrane2.82E-407GO.00030666CCendocytic vesicle membrane2.82E-407GO.00030670CCplagotty vesicle membrane2.82E-407GO.0003066CCendocytic vesicle2.82E-407GO.0003071CCrough endoplasmic reticulum2.1E-06GO.00030721CCCOPII vesicle coat2.95E-06GO.0005731CCmitochondrial outer membrane4.59E-06GO.0003774CCor unclear envelope4.84E-06GO.0003778CCor unclear envelope1.38E-05GO.0003788CCor under envelope1.32E-05GO.0003798CCor uter membrane1.32E-05GO.0003798MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO.0003798MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO.000377MFchaperone binding3.35E-05GO.0003028MFoxidoreductase activity, acting on the aldehyde or oxo group of donors	GO.0030134		Colri acconited versiole	3.20E-13
COCOUNTSCCContext restrict2.222-10GOUD031227CCintrinsic component of endoplasmic reticulum membrane1.48E-08GOUD05737CCendoplasmic reticulumGolgi intermediate compartment1.96E-08GOUD05737CCendoptisk reticulumGolgi intermediate compartment1.96E-08GOUD05737CCendoptisk reticulumGolgi intermediate compartment2.47E-07GOUD05060CCGolgi-associated vesicle membrane2.47E-07GOUD12507CCER to Golgi transport vesicle membrane2.82E-07GOUD05335CCphagocytic vesicle membrane2.82E-07GOUD05335CCphagocytic vesicle membrane2.82E-07GOUD05335CCphagocytic vesicle combrane2.82E-07GOUD05371CCrough endoplasmic reticulum2.61E-06GOUD05371CCnuclear envelope4.84E-06GOUD05635CCendoplasmic reticulum Iumen1.15E-05GOUD05635CCendoplasmic reticulum Iumen1.32E-05GOUD05635CCendoplasmic reticulum Iumen1.32E-05GOUD05635CCendoplasmic reticulum Iumen1.32E-05GOUD05635CCendoplasmic reticulum Iumen1.32E-05GOUD05635MFcyticule activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06GOUD01620MFcyticule activity, acting on the aldehyde or oxo group of donors1.43E-05GOUD016203MFcyticule activity, acting on the aldehyde or oxo group of donors1.49	GO:0003798	CC	costed vericle	1.2/E-13 2.02F 10
CO-00017/0CCIntegrat component of endoplasmic reticulum membrane1.48E-08CO-0001277CCendoplasmic reticulum-Gogi intermediate compartment1.96E-08CO-0003090CCendoplasmic reticulum-Gogi intermediate compartment2.90E-08CO-0003060CCGolgi-associated vesicle membrane2.47E-07CO-0003060CCER to Golgi transport vesicle membrane2.52E-07CO-00030666CCendoptic vesicle3.80E-07CO-00030666CCendoptic vesicle membrane2.52E-07CO-00030667CCpragocytic vesicle3.80E-07CO-0005731CCrough endoplasmic reticulum2.21E-06CO-0005732CCorgan endoplasmic reticulum2.52E-07CO-0005731CCrough endoplasmic reticulum2.52E-07CO-0005741CCmitochondrial outer membrane2.52E-06CO-0005741CCmitochondrial outer membrane2.52E-06CO-0005741CCmitochondrial outer membrane1.32E-05CO-0005748CCorganelle outer membrane1.33E-05CO-0005778CCorganelle outer membrane1.33E-05CO-0005778MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NAD as acceptor6.96E-06CO-0005770MFchaperone binding3.75E-05CO-0005777MFchaperone binding3.75E-05CO-0005778MFchaperone binding3.75E-05CO-0005778MFchaperone binding3.75E-05CO-	GO:0030135 GO:0030176	CC	integral component of endoplasmic reticulum membrane	2.02E-10 8 50E 00
CO:000573CCIntrinst Comported Organia1+06-04CO:0005733CCendocytic vesicle2,90E-08CO:0030660CCGolgi ransport vesicle membrane1.08E-07CO:0030658CCtransport vesicle membrane2,47E-07CO:0030666CCendocytic vesicle membrane2,82E-07CO:0030666CCendocytic vesicle membrane2,82E-07CO:0030666CCendocytic vesicle membrane2,82E-07CO:0030666CCendocytic vesicle membrane2,82E-07CO:0005791CCrough endoplasmic reticulum2,21E-06CO:0005791CCrough endoplasmic reticulum2,21E-06CO:0005791CCmitchondrial outer membrane2,58E-06CO:0005731CCmitchondrial outer membrane4,59E-06CO:0005635CCendoplasmic reticulum1.15E-05CO:0005635CCendoplasmic reticulum lumen1.15E-05CO:0005734CCorganelle outer membrane1.23E-05CO:0005735MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07CO:0005735MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07CO:0005735MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07CO:0005735MFcysteine-type endopeptidase regulator activity involved in apoptotic process3.35E-05CO:0005136MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process <td>GO:0030170 GO:0031227</td> <td>CC</td> <td>integral component of endoplasmic reticulum membrane</td> <td>0.39E-09</td>	GO:0030170 GO:0031227	CC	integral component of endoplasmic reticulum membrane	0.39E-09
CO-0000753CCEndoptasmin Fettrumurdogi merimeune Compariment1.500-08CO-00001593CCGolgi-associated vesicle membrane1.08E-07GO-00030660CCGolgi-associated vesicle membrane2.47E-07GO-0012507CCER to Golgi transport vesicle membrane2.42E-07GO-00030666CCendocytic vesicle3.80E-07GO-00030573CCpragocytic vesicle membrane2.42E-07GO-0005791CCrough endoplasmic reticulum2.21E-06GO-0005791CCrough endoplasmic reticulum2.52E-07GO-0005791CCrough endoplasmic reticulum2.52E-06GO-0005791CCnutchondrial outer membrane4.59E-06GO-0005793CCnutchar envelope4.84E-06GO-0005788CCendoplasmic reticulum lumen1.15E-05GO-0003126CCorganelle outer membrane1.32E-05GO-0003127CCorganelle outer membrane1.32E-05GO-0003128MFcysteine-type endopeptidase regulator activity involved in apottoic process8.04E-07GO-00051087MFchaperone binding1.32E-05GO-00051087MFchaperone binding1.32E-05GO-0003218MFchaperone binding3.38E-05GO-0003218MFchaperone binding3.38E-05GO-0003218MFchaperone binding3.38E-05GO-0003218MFchaperone binding3.38E-05GO-0003177MFheat shock protein binding3.38E-05<	GO:0031227	CC	and misic component of endoplasmic reticulum memorane	1.46E-08
COUNDED         C.         Cologi-associated vesicle membrane         2.06-00           COUNDAGED         C.         Golgi-associated vesicle membrane         2.47E-07           GOUNDAGES         C.         transport vesicle membrane         2.82E-07           GOUNDAGES         C.         endocytic vesicle membrane         2.82E-07           GOUNDAGES         C.         phagocytic vesicle         3.80E-07           GOUNDAGES         C.         robge endoplasmic reticulum         2.82E-07           GOUNDAGES         C.         robge endoplasmic reticulum         2.95E-06           GOUNDAGES         C.         nuclear envelope         4.84E-06           GOUNDAGES         C.         organelle outer membrane         1.32E-05           GOUNDAGES         C.         organelle outer membrane         1.33E-05           GOUNDAGES         MF         cystene-type endopeptidase regulator activity involved in apoptotic process         8.04E-07           GOUNDAGES         MF         chaperone binding	GO:0003733	CC	endoprisine reticuluit-Goigi intermediate compartment	2 90E-08
Construct	GO:0030660	CC	Golgi-associated vesicle membrane	2.90E-03
GOUDDADDCCER to Golgi transport vesicle membrane2.52E-07GOUD12507CCendocytic vesicle membrane2.82E-07GOUD030666CCendocytic vesicle membrane2.82E-07GOUD05791CCrough endoplasmic reticulum2.21E-06GOUD05791CCfcolin1-1-rich granule lumen2.61E-06GOUD05791CCCOPII vesicle coat2.95E-06GOUD05791CCmitochondrial outer membrane4.59E-06GOUD05635CCnuclear envelope4.84E-06GOUD05635CCendoplasmic reticulum lumen1.15E-05GOUD05635CCouter membrane1.23E-05GOUD05635CCouter membrane1.23E-05GOUD01866CCorganelle outer membrane1.33E-05GOUD01867MFcyteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07GOUD01620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06GOUD01602MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.98E-05GOUD01603MFoxidoreductase activity, acting on the aldehyde or oxo group of donors3.83E-05GOUD015185MFcholesterol binding3.75E-05GOUD01503MFcotesterol binding3.75E-05GOUD01503MFcotesterol binding3.89E-05GOUD015148MFantioxidant activity5.26E-05GOUD015140MFpertidas regulator activity involved in apoptot	GO:0030658	CC	transport vesicle membrane	2 47F-07
ColonationColonation2.82E-07CO003066CCendocytic vesicle membrane2.82E-07GO:0045335CCphagocytic vesicle3.80E-07GO:005791CCrough endoplasmic reticulum2.21E-06GO:0030127CCficolin-1-rich granule lumen2.61E-06GO:0005635CCnuclear envelope4.84E-06GO:0005788CCendoplasmic reticulum lumen1.15E-05GO:0005788CCouter membrane1.25E-05GO:0005788CCouter membrane1.33E-05GO:0005786CCouter membrane1.33E-05GO:0005787MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07GO:0016020MFoxidoreductase activity, acting on the aldehyde or xog group of donors, NAD or NADP as acceptor6.96E-06GO:0015087MFchaperone binding1.43E-05GO:0015083MFoxidoreductase activity, acting on the aldehyde or xog group of donors1.96E-05GO:0015083MFoxidoreductase activity, acting on the aldehyde or xog group of donors3.83E-05GO:0015083MFchaleerone binding3.75E-05GO:0015083MFcholesterol binding3.23E-05GO:0015083MFcholesterol binding3.23E-05GO:0015083MFcholesterol binding3.23E-05GO:00151445MFcholesterol binding5.23E-05GO:00151787MFmisfolded protein binding5.23E-05GO:00151082MFantit	GO:0012507	CC	EB to Golgi transport vesicle membrane	2.52E-07
GO:0045335CCphagocytic vesicle3.80E-07GO:0045335CCrough endoplasmic reticulum2.21E-06GO:0005791CCrough endoplasmic reticulum2.61E-06GO:0030127CCCCGO:011-1-tink granule lumen2.61E-06GO:0005741CCmitochondrial outer membrane4.59E-06GO:0005755CCnuclear envelope4.84E-06GO:0005788CCendoplasmic reticulum lumen1.15E-05GO:0015786CCouter membrane1.23E-05GO:0016620MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07GO:001620MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process8.04E-05GO:001642277MFpeptide binding1.43E-05GO:0015485MFcholesterol binding3.89E-05GO:0015485MFcholesterol binding3.89E-05GO:0015485MFcholesterol binding3.89E-05GO:0051787MFmisfolded protein binding3.89E-05GO:0051787MFmisfolded protein binding5.23E-05GO:0051787MFmisfolded protein binding5.23E-05GO:0051802	GO:0030666	CC	endocytic vesicle membrane	2.82E-07
GO:0005791CCrough endoplasmic reticulum2.21E-06GO:0005791CCfrodin1-trich granule lumen2.61E-06GO:0005711CCOPII vesicle coat2.95E-06GO:0005731CCmitochondrial outer membrane4.59E-06GO:0005788CCnuclear envelope4.84E-06GO:0005788CCorganelle outer membrane1.23E-05GO:0005788CCorganelle outer membrane1.33E-05GO:0019867CCouter membrane1.33E-05GO:0019867CCouter membrane6.96E-06GO:001019867MFcysteine-type endopeptidase regulator activity involved in apototic process8.04E-07GO:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06GO:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.98E-05GO:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO:0015035MFcholesterol binding2.13E-05GO:001503MFoxidoreductase activity, acting on the aldehyde or oxo group of donors3.88E-05GO:001503MFcholesterol binding3.75E-05GO:0015185MFcholesterol binding3.23E-05GO:0015185MFcholesterol binding5.23E-05GO:00151767MFmisfolded protein binding5.23E-05GO:00151780MFpeptidase regulator activity5.26E-05GO:00151780MF	GO:0045335	CC	phagocytic vesicle	3.80E-07
GO:1904813CCfoolin-1-rich granule lumen2.61E-06GO:00030127CCCOPII vesicle coat2.95E-06GO:0005741CCmitochondrial outer membrane4.59E-06GO:000535CCnuclear envelope4.84E-06GO:0005788CCendoplasmic reticulum lumen1.15E-05GO:0019667CCorganelle outer membrane1.33E-05GO:0016620MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07GO:001697MFchaperone binding1.43E-05GO:001697MFchaperone binding1.43E-05GO:001697MFchaperone binding1.43E-05GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.38E-05GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors3.89E-05GO:0016903MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05GO:0016903MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.89E-05GO:00172MFheat shock protein binding5.28E-05GO:0015180MFpeptidae binding5.28E-05GO:0016200MFantioxidant activity5.46E-05GO:0016210 <td>GO:0005791</td> <td>CC</td> <td>rough endoplasmic reticulum</td> <td>2.21E-06</td>	GO:0005791	CC	rough endoplasmic reticulum	2.21E-06
G0:0030127CCCOPII vesicle coat2.95E-06G0:0005741CCmitochondrial outer membrane4.59E-06G0:0005635CCnuclear envelope4.84E-06G0:0005788CCendoplasmic reticulum lumen1.15E-05G0:0019667CCouter membrane1.23E-05G0:0019867CCouter membrane1.33E-05G0:001620MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07G0:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06G0:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.38E-05G0:001620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05G0:0016303MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05G0:0016403MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:001372MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:001372MFheat shock protein binding3.28E-05G0:0051787MFmisfolded protein binding5.23E-05G0:0051787MFpeptidas inhibitor activity5.26E-05G0:0051787MFmisfolded protein binding5.23E-05G0:005182MFunfolded protein binding5.28E-05G0:005182MFsterol transporter activity5.26E-05 <td< td=""><td>GO:1904813</td><td>CC</td><td>ficolin-1-rich granule lumen</td><td>2.61E-06</td></td<>	GO:1904813	CC	ficolin-1-rich granule lumen	2.61E-06
G0:0005731CCmitochondrial outer membrane4.59E-06G0:0005635CCnuclear envelope4.84E-06G0:0005788CCendoplasmic reticulum lumen1.15E-05G0:0019867CCorganelle outer membrane1.23E-051G0:0019867CCouter membrane1.33E-05G0:0019867CCouter membrane1.33E-05G0:001602MFcysteine-type endopeptidase regulator activity involved in apoptotic process6.96E-06G0:001620MFcysteine-type endopeptidase regulator activity involved in apoptotic process6.96E-06G0:001603MFchaperone binding1.43E-05G0:0016903MFpetide binding1.38E-05G0:0016903MFpetide binding2.13E-05G0:0015485MFamide binding2.13E-05G0:0015485MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:0031072MFmisfolded protein binding3.89E-05G0:0031072MFmisfolded protein binding3.89E-05G0:0051400MFpetidase regulator activity5.26E-05G0:001134MFpetidase regulator activity5.46E-05G0:002020MFprotease binding5.26E-05G0:002020MFprotease binding7.18E-05G0:002020MFprotease binding7.18E-05G0:002020MFsterol binding7.18E-05G0:002020MFsterol binding7.18E-05G0:002020M	GO:0030127	CC	COPII vesicle coat	2.95E-06
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G0:0043028MFcysteine-type endopeptidase regulator activity involved in apoptotic process8.04E-07G0:0016620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06G0:0051087MFchaperone binding1.43E-05G0:0042277MFpeptide binding1.89E-05G0:0015903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05G0:0015485MFamide binding2.13E-05G0:0015485MFcholesterol binding3.75E-05G0:0042277MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:0015485MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:00172MFheat shock protein binding3.50E-05G0:0051787MFmisfolded protein binding5.23E-05G0:0051400MFpeptidase regulator activity5.26E-05G0:0015209MFantioxidant activity5.26E-05G0:002020MFprotease binding7.18E-05G0:0021020MFunfolded protein binding7.18E-05G0:0032934MFsterol binding7.18E-05G0:0015248MFsterol binding9.19E-05G0:0046906MFtetrapyrrole binding0.00013578G0:0044387MFenzyme inbilitor activity0.00014015	GO:0019867	CC	outer membrane	1.33E-05
GO:0016620MFoxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor6.96E-06GO:0051087MFchaperone binding1.43E-05GO:0042277MFpeptide binding1.89E-05GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO:003218MFamide binding2.13E-05GO:0015485MFcholesterol binding3.75E-05GO:0043027MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05GO:0051787MFheat shock protein binding3.89E-05GO:0051787MFmisfolded protein binding5.23E-05GO:0051400MFBH domain binding5.23E-05GO:001134MFpeptidase regulator activity5.46E-05GO:001134MFpeptidase regulator activity5.46E-05GO:002020MFprotease binding6.20E-05GO:003234MFunfolded protein binding7.18E-05GO:003234MFsterol binding7.18E-05GO:0015248MFsterol binding7.19E-05GO:0015248MFsterol binding9.19E-05GO:004890MFtetrapyrrole binding0.00013578GO:0044389MFenverse inbihoir activity0.00014405GO:0044389MFubiquitin-like protein ligase binding0.00014405	GO:0043028	MF	cysteine-type endopeptidase regulator activity involved in apoptotic process	8.04E-07
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GO:0042277MFpeptide binding1.89E-05GO:0016903MFoxidoreductase activity, acting on the aldehyde or oxo group of donors1.96E-05GO:0033218MFamide binding2.13E-05GO:0015485MFcholesterol binding3.75E-05GO:0043027MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05GO:0043027MFheat shock protein binding3.89E-05GO:0051787MFmisfolded protein binding4.50E-05GO:0051400MFBH domain binding5.23E-05GO:0061134MFpeptidase regulator activity5.26E-05GO:0016209MFantioxidant activity5.26E-05GO:002020MFprotease binding6.20E-05GO:0032934MFunfolded protein binding7.18E-05GO:0015248MFsterol binding7.19E-05GO:0046906MFterapyrrole binding0.00013578GO:0044389MFubiquitin-like protein ligase binding0.000146315	GO:0051087	MF	chaperone binding	1.43E-05
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G0:0015485MFcholesterol binding3.75E-05G0:0043027MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:0031072MFheat shock protein binding3.89E-05G0:0051787MFmisfolded protein binding4.50E-05G0:0051780MFBH domain binding5.23E-05G0:00151740MFpeptidase regulator activity5.26E-05G0:0016209MFantioxidant activity5.46E-05G0:002020MFprotease binding6.20E-05G0:0031082MFunfolded protein binding7.18E-05G0:0015248MFsterol binding7.19E-05G0:0046906MFtetrapyrrole binding0.00013578G0:0044389MFubiquitin-like protein ligase binding0.000146315	GO:0033218	MF	amide binding	2.13E-05
G0:0043027MFcysteine-type endopeptidase inhibitor activity involved in apoptotic process3.83E-05G0:0031072MFheat shock protein binding3.89E-05G0:0051787MFmisfolded protein binding4.50E-05G0:0051700MFBH domain binding5.23E-05G0:0061134MFpeptidase regulator activity5.26E-05G0:0016209MFantioxidant activity5.46E-05G0:0016209MFprotease binding6.20E-05G0:0015020MFprotease binding6.20E-05G0:0051082MFunfolded protein binding7.18E-05G0:0015248MFsterol binding7.19E-05G0:0046906MFterdapyorter activity9.19E-05G0:0044389MFubiquitin-like protein ligase binding0.000146315	GO:0015485	MF	cholesterol binding	3.75E-05
G0:0031072MFheat shock protein binding3.89E-05G0:0051787MFmisfolded protein binding4.50E-05G0:0051400MFBH domain binding5.23E-05G0:0061134MFpeptidase regulator activity5.26E-05G0:0016209MFantioxidant activity5.46E-05G0:001200MFprotease binding6.20E-05G0:001502MFunfolded protein binding7.18E-05G0:001502MFsterol binding7.18E-05G0:0015248MFsterol binding7.19E-05G0:0045906MFtertapyrrole binding0.00013578G0:0044389MFubiquitin-like protein ligase binding0.00014405G0:0044387MFenzyme inbibutor activity0.000146315	GO:0043027	MF	cysteine-type endopeptidase inhibitor activity involved in apoptotic process	3.83E-05
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GC:0051400MFBH domain binding5.23E-05GC:0061134MFpeptidase regulator activity5.26E-05GC:0016209MFantioxidant activity5.46E-05GC:002020MFprotease binding6.20E-05GC:0051082MFunfolded protein binding7.18E-05GC:0015248MFsterol binding7.19E-05GC:0046906MFsterol transporter activity9.19E-05GC:0044389MFubiquitin-like protein ligase binding0.00013578GO:0044387MFenzyme inbibitor activity0.000146315	GO:0051787	MF	misfolded protein binding	4.50E-05
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GC:0016209MFantioxidant activity5.46E-05GC:0002020MFprotease binding6.20E-05GC:0051082MFunfolded protein binding7.18E-05GC:0015248MFsterol binding7.19E-05GC:0046906MFterapyrrole binding0.00013578GO:0044389MFubiquitin-like protein ligase binding0.00014405GO:004857MFenzyme inbibitor activity0.000146315	GO:0061134	MF	peptidase regulator activity	5.26E-05
GC:0002020MFprotease binding6.20E-05GO:0051082MFunfolded protein binding7.18E-05GO:0032934MFsterol binding7.19E-05GO:0015248MFsterol transporter activity9.19E-05GO:0046906MFtetrapyrrole binding0.00013578GO:0044389MFubiquitin-like protein ligase binding0.00014405GO:004857MEenzyme inbibitor activity0.000146315	GO:0016209	MF	annoxidant activity	5.46E-05
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GC:00522534Mrsterol binding7.19E-05GO:0015248MFsterol transporter activity9.19E-05GO:0046906MFtetrapyrrole binding0.00013578GO:0044389MFubiquitin-like protein ligase binding0.00014405GO:004887MFenzyme inbibitor activity0.000146315	GO:0051082	ME	unroided protein binding	7.18E-05
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GO-0004857 Mr upiquini-inc protein igase princing 0.00014405 GO-0004857 MF enzyme inhibitor activity 0.00016315	CO-0044390	ME	icitapynoie onding ubiquitin like protein ligase binding	0.00013378
	GO:0044369 GO:0004857	ME	enzyme inhibitor activity	0.00014403

\*Only top 20 BP,CC, MF were displayed.

The STRING (version 11.0, http://www.string-db.org/) database [37] was used to perform protein-protein interactions (PPI) on critical module genes from the WGCNA results and the ER stress-related differentially expressed genes. We selected Required Confidence (combined score) > 0.4 as the threshold value of the PPI relationship. Cytoscape software [38] was used to construct the PPI network of interaction genes, and the top ten hub genes were extracted using the cytohbhs plug-in Ref. [39].

## Table 2

KEGG enrichment.

ID	Description	pvalue	gene
hsa04141	Protein processing in	8.78E-	PRKCSH/TRAM1/RPN2/AMFR/GANAB/CANX/RBX1/UBQLN2/SEC24D/HSPA6/ATF4/DNAJC1/
	endoplasmic reticulum	14	EIF2S1/DNAJC10/EIF2AK2/SEC23B/HSPA1A/SAR1A/SEC23A
hsa04612	Antigen processing and	9.66E-	HLA-DRA/CD74/TNF/HSPA4/IFNG/CANX/HSPA6/TAP1/CTSB/TAP2/HLA-B/HSPA1A
	presentation	11	
hsa05417	Lipid and atherosclerosis	4.84E-	TNF/CYBB/BAD/TLR4/HSPA4/BCL2L1/IL1B/HSPA6/ATF4/PIK3CA/SOD2/APAF1/EIF2S1/
	*	10	CYBA/HSPA1A/OLR1/ABCA1
hsa04210	Apoptosis	6.40E-	TNF/BAD/MCL1/BCL2L1/ACTG1/ATF4/PIK3CA/APAF1/EIF2S1/ACTB/BIRC2/CTSB
	r · r · · · ·	08	
hsa05164	Influenza A	1.03E-	HLA-DRA/VDAC1/TNF/TLR4/IFNG/IL1B/KPNA2/ACTG1/PIK3CA/APAF1/EIF2S1/ACTB/
		07	EIF2AK2
hsa04145	Phagosome	2 19F-	HI A-DRA/CYBB/TI B4/ACTG1/CANX/ACTB/CYBA/PIK3C3/TAP1/TAP2/HI A-B/OI B1
1000 11 10	1 magooome	07	
hsa05020	Prion disease	7 09F-	VDAC1/TNE/CVRR/CRER311/SDHC/RAD/II1R/HSDA6/ATE4/DIK3CA/ADAE1/EIE2S1/CVRA/
1138103020	i non discase	07	HCDA1A/ATE?
hsa04621	NOD-like recentor signaling	1 72F-	VDAC1/TNE/DNM11/CVRR/TI R4/RCI 211/II 1R/TYNID/RIRC2/CVRA/CTSR/CARARAD
11500 1021	nathway	06	
hsa05166	Human T-cell leukemia virus 1	2 07F-	HI & DR & /VD & C1 /TNF /CR FR3I 1 /RCI 2I 1 /CANY /MVC /ATE4 /DIK 3CA /II 1 R1 /DTEN /HI & R /
115000100	infection	06	ΔΤΕΊ
bca04217	Necroptosis	2 70F	VDAC1/TNE/DNM11/CVRR/CLUD1/TLP4/JENC/JL1R/HMCR1/EJE2AK2/RIDC2
1138104217	Necroptosis	2.7 0L-	VD/RGI/ INI/ DAMITE/ GIDD/ GEODI/ TERA/ II NG/ IEID/ IIMGDI/ EII Z/RZZ/ DIRGZ
bca05134	Legionellosis	4 56F	TNE /TI D/ /II 1B /LISDA6 /ADAE1 /LISDA1A /SAD1A
113803134	Legionenosis	4.50L-	
bca05162	Maaslas	5 576	
115805102	WEASIES	0.57E=	DAD/ 1ER4/ DGE2E1/ 1E1D/ 115F R0/ FIRSGA/ AFAF1/ EIF251/ EIF2AR2/ 115F ATA
hee04140	Automboon onimol	6.225	DAD /DCI 911 /JIMCD1 /DIV9CA /DTEN /EIE901 /DIV9C9 /CTCD /ATC7 /CADADAD
115204140	Autophagy - anniai	0.33E-	DAD/ DCL2L1/ HWGD1/ PIK3CA/ PIEW/ EIF231/ PIK3C3/ CI3D/ AIG/ / GADAKAP
hee05121	Chicollogia	6745	
115205151	Singenosis	0./4E-	VDAG1/INF/ILR4/DGL2L1/ILID/UDG/AG1G1/RDA1/PAN/PIK5GA/ILIR1/AG1D/PIK5G5
bco04669	THE signaling pathway	6 925	THE (DRM11 /CDED211/II 1D /ATE4 /DIV2CA /DIDC2 /CEDDD /ATE2
115404008	The signaling pathway	0.63E-	INF/DINIIL/CRED5LI/ILID/AIF4/PIK5CA/DING2/CEDPD/AIF2
hee05145	Townloamonia	6.025	
118403143	Toxopiasillosis	0.65E-	HLA-DRA/ INF/ BAD/ ILR4/ BGL2L1/ IFNG/ H3PA0/ BIRG2/ H3PAIA
105160	TT	1.075	
nsa05163	Human cytomegalovirus	1.3/E-	INF/CREB3L1/IL1B/MYC/A1F4/PAN/PIK3CA/IL1R1/IAP1/IAP2/HLA-B/A1F2
105140	Infection	05	
nsa05140	Leisnmaniasis	3.41E-	HLA-DKA/INF/CYBB/ILK4/IFNG/ILIB/CYBA
1 0 - 0 - 0		05	
nsa05010	Aizneimer disease	4.50E-	VDAG1/INF/GYBB/SDHC/BAD/ILIB/APOE/ATF4/PIK3GA/APAF1/EIF2S1/EIF2AK2/SNCA/
1		05	PIK3C3/GAPDH
hsa05152	Tuberculosis	5.33E-	HLA-DRA/CD/4/INF/BAD/TLR4/IFNG/IL1B/APAF1/PIK3C3/CEBPB
		05	

\*Only top 20 items were displayed.

# 6 Analysis of Immune Cell Infiltration

CIBERSORT is based on the principle of linear support vector regression to deconvolve the transcriptome expression matrix and estimate the composition and abundance of immune cells in mixed cell populations [40]. We used the gene expression matrix data to analyze the infiltration of 22 immune genes in the samples using the CIBERSORT algorithm. The samples with P < 0.05 were filtered to obtain the immune cell infiltration matrix. The corplot package [41] was used to draw correlation heatmaps to visualize the correlations of the 22 immune cell infiltrations. The gepubr package (https://CRAN.R-pnojiect.org/package-ggpuby) was used to draw violin plots to visualize the differences in the infiltration of the 22 immune cells. We plotted the correlations between the hub genes and immune cells.

## 7 Statistical analysis

All statistical analyses were performed using means  $\pm$  standard deviation. The data were analyzed using R software (version 3.6.3). A *P*-value <0.05 was considered statistically significant, and all statistical tests were two-sided. The overall analysis protocol used in this study is shown in Fig. 1.

# 3. Results

## 1 GEO data variance analysis

The downloaded standardized data was preprocessed to obtain a boxplot of the data before and after preprocessing (Fig. 2A and B). The results of the PCA analysis revealed specific differences between the metastatic and non-metastatic groups (Fig. 2C). The

#### Table 3

GSEA Enrichment results.

Description	ES	NES	p-val
huntingtons disease	0.46630952	1.58440333	0.00172712
cell cycle	0.50538946	1.64173798	0.00177936
spliceosome	0.51580512	1.62686146	0.00177936
parkinsons disease	0.51169624	1.61820978	0.00178891
ribosome	0.63942248	1.9899259	0.00179211
asthma	-0.8866284	-2.3835054	0.00204499
primary immunodeficiency	-0.6713973	-1.8398342	0.00205761
allograft rejection	-0.845997	-2.3734866	0.00208768
type i diabetes mellitus	-0.7803001	-2.2486644	0.0021097
nod like receptor signaling pathway	-0.7042556	-2.0714756	0.00212766
graft versus host disease	-0.8501564	-2.4277465	0.0021322
cytosolic dna sensing pathway	-0.6094253	-1.7984544	0.00214592
systemic lupus erythematosus	-0.8388346	-2.488651	0.00214592
autoimmune thyroid disease	-0.8391577	-2.4826173	0.00215054
intestinal immune network for iga production	-0.822676	-2.3955756	0.00215054
viral myocarditis	-0.7628128	-2.379273	0.00215983
b cell receptor signaling pathway	-0.5974895	-1.8628274	0.00218818
toll like receptor signaling pathway	-0.6431166	-2.1193684	0.00220264
complement and coagulation cascades	-0.7051765	-2.1764987	0.00220751
leishmania infection	-0.789533	-2.4331695	0.00221239
cell adhesion molecules cams	-0.6483611	-2.2007646	0.00223214
natural killer cell mediated cytotoxicity	-0.6285108	-2.1406363	0.00224719
lysosome	-0.5284886	-1.7686821	0.00228833
hematopoietic cell lineage	-0.6706642	-2.1650725	0.00230415
antigen processing and presentation	-0.7504096	-2.4231959	0.00231481
chemokine signaling pathway	-0.5715468	-2.0123182	0.00236967
cytokine cytokine receptor interaction	-0.5242236	-1.9269993	0.00239234
jak stat signaling pathway	-0.4284559	-1.4716202	0.00473934
ubiquitin mediated proteolysis	0.45041173	1.49145857	0.00714286

\*Only top 20 items were displayed.

GSE21257 data set screened the expression data for 24,994 genes with  $|\log FC| > 0.263$ , p.adj<0.05 as the threshold. Finally, 1960 differentially expressed genes were identified, of which 1145 were up-regulated and 815 were down-regulated (Fig. 2D and E). The ER stress-related genes were downloaded from the GeneCard database. Then, 138 potential ER stress-related differentially expressed genes from the GSE21257 data set (Fig. 2F).

We used the consensus clustering method to cluster the samples. In the CDF curve of the consensus matrix, when K = 4, the CDF curve presented a relatively flat middle segment (Fig. 3A and B). Furthermore, when K = 4 was selected for consensus clustering analysis, the interference between subgroups was reduced considerably (Fig. 3C). Therefore, we identified four subgroups, including cluster 1 (n = 10), cluster 2 (n = 5), cluster 3 (n = 14), and cluster 4 (n = 4). The expression profiles between the four clusters were compared (Fig. 3D).

As described in the methods, the 138 intersection genes were subjected to enrichment analysis, including 1337 significant items for biological processes (BP), 116 significant items for cellular components (CC), 86 significant items for molecular function (MF), and 112 significant pathways. The significant items were displayed using bubble charts (Fig. 4A–D). As shown in Fig. 4 below, the BP, CC, and MF items revealed the top 20 items based on the *P*-value (Fig. 4E–Table 1). It was noted that the differentially expressed genes related to ER stress-affected pathways such as Protein processing in the ER, Apoptosis, and Antigen processing and presentation (Fig. 4F–Table 2). Based on the GSEA analysis, the GSE21257 data set had 29 significant gene set pathways (Table 3), and the TOP5 pathways included Huntington's disease, cell cycle, the spliceosome, Parkinson's disease, and ribosomes (Fig. 4G).

We performed a weighted gene co-expression network analysis (WGCNA) on the gene expression data using the R package WGCNA algorithm to identify the co-expression patterns of the genes during osteosarcoma metastasis. First, we performed sample clustering analysis to detect any variation across the 35 samples and outliers. Next, we set a soft threshold of 3 to approximate the network to a scale-free network (Fig. 5A). Then, the Pearson correlation coefficient of paired genes was calculated to obtain a similarity matrix. The similarity matrix was transformed into an adjacent matrix using the threshold and power values listed above. Linkage hierarchical clustering was then averaged to identify the modules where genes were closely linked, and genes not assigned to a specific module were shown in grey (Fig. 5B). We found one module of significantly co-expressed genes (MEblue, P = 0.01) (Fig. 5C). The identified genes were closely associated with osteosarcoma metastasis, suggesting that this was a critical gene set to investigate the risk of osteosarcoma metastasis further (Fig. 5D).

The PPI interaction network analysis was performed on the ER stress-related differentially expressed genes (Fig. 6A) in the 81 blue modules. As shown in Fig. 6B and C, there were 218 interaction relationships among 68 key genes, and there were 38 interaction relationships among the top ten critical genes, as seen in Fig. 6D.

The CIBERSORT algorithm was used to assess changes in immune cell infiltration levels before and after osteosarcoma metastasis as described in the Methods (Fig. 7A and B). The results of the correlation analysis revealed a positive correlation between osteosarcoma



Fig. 5. WGCNA analysis: (A) Soft Threshold. (B) Module and Trait Data Heatmap. Orange means positive correlation, blue means negative correlation, the darker the color, the stronger the correlation. (C)Module Clustering Plot. (D)Blue Significant Difference Module Scatter Plot. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

# 5 PPI Interaction Network

metastasis and the infiltration levels of various immune cells, such as naive CD4 T cells and neutrophils (Fig. 7C). A negative correlation was observed for other immune cells such as CD8 T cells and M0 macrophages. The differential analysis demonstrated that the infiltration levels of various immune cell subsets were significantly different between the metastatic and non-metastatic groups (Fig. 7D), including M0 macrophages, CD8 T cells, and others. The correlation analysis showed that the expression of hub genes was primarily related to memory B cells, CD4 T cells, memory resting, and others (Fig. 7E-N).

To elucidate the immune microenvironmental status of these four subgroups, we explored the expression of ten Hub genes across the four clusters. All ten genes were significantly differentially expressed in clusters 1 and 4 (Fig. 8A–J).

## 4. Discussion

ER stress has been found to be associated with the incidence, progression, spread, and resistance to drugs of tumors [14,16,18]. Once the accumulation of improperly folded proteins in the ER surpasses a crucial threshold, it triggers a signal transduction pathway known as the unfolded protein response (UPR). The heightened activation of UPR signaling is implicated in the epithelial-mesenchymal transition (EMT) of tumor cells preceding metastasis [16]. The UPR is initiated by three ER transmembrane proteins: PERK, IRE1 $\alpha$ , and ATF6 [15]. The up-regulation of LAMP3 by PERK has been shown to facilitate the migration and invasion of cancer cells [42]. Tumor metastasis is closely associated with the epithelial-mesenchymal transformation (EMT) process in tumor cells [4,43]. Inhibition of the EMT process through small-molecule PERK inhibitors has been found to effectively suppress the migration of tumor cells [44]. Additionally, IRE1 $\alpha$  and ATF6 play significant roles in regulating various physiological and pathological processes in different cell types [45–47].

In this study, the author searched for a set of differentially expressed genes associated with ER stress and osteosarcoma metastasis. Through bioinformatics analysis of gene data of 14 metastatic osteosarcoma patients and 19 non-metastatic patients, enrichment



Fig. 6. PPI interaction analysis of key genes: (A) Venn diagram of the intersection of ER stress-related differential genes and key genes in the blue module. (B) PPI interaction network diagramThe blue dots are key genes, and the connections interactions. (C) PPI interaction NetworkAnalyzer visualization. The smaller the significance, the larger the dots are.The thickness of edge is the combine score, and the color is down-regulated to up-regulated from blue to red. (D) Hub gene interaction diagram. The color from red to light is the MCC score. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

6 Immune infiltration correlation analysis

analysis, immune infiltration analysis, PPI protein interaction network analysis of differentially expressed genes associated with ER stress, construction and verification of a molecular subtype model related to metastatic osteosarcoma, 10 key genes were finally determined, namely ACTB, ACTG1, ANXA5, APAF1, HSPA1A, HSPA4, MYC, NPM1, SIRT1 and VDAC1, providing possibility for inhibiting the occurrence of osteosarcoma metastasis. ACTB, also known as  $\beta$ -actin (ACTB), plays a crucial role in cancer metastasis, particularly in liver cancer [43]. The processes of polymerization, localization, cytoskeleton formation, and overexpression of ACTB are closely associated with cell growth and migration [43,48]. In the context of prostate cancer (PCa), ACTG1 may impact tumor metastasis through the MAPK/ERK signaling pathway [49]. Additionally, Annexin ANXA5 serves as a link between the innate and adaptive immune systems and contributes to immune stimulation within the tumor microenvironment (TME). ANXA5-carbon nanotube conjugates have been utilized for the physical ablation of tumors through photothermal therapy [50]. Apoptosis protease activating factor-1 (APAF-1), a protein weighing 130 kDa, plays a crucial role in regulating programmed cell death. In metastatic colorectal cancer, the frequent absence of APAF-1 expression is strongly linked to unfavorable prognosis. Furthermore, the loss of APAF-1 expression is more prevalent among patients experiencing early recurrence, emphasizing its significance in tumor metastasis [51]. HSPA1A plays a crucial role in facilitating the proper folding of recently synthesized proteins and inhibiting the aggregation of pre-existing proteins within the cytoplasm and organelles. In the context of squamous cell carcinoma, HSPA1A exhibits a direct binding affinity and interaction with LASP1, thereby effectively stimulating the proliferation, metastasis, and invasion of malignant cells [52]. In hepatocellular carcinoma (HCC), the downregulation of HSPA1A, HSPA4, and VDAC1 has been found to impede the invasion, migration, and proliferation of HCC cells [53,54]. Additionally, HSPA1A, HSPA4, and VDAC1 have been identified as independent prognostic factors. The inhibition of miR-93b expression by MYC through direct binding to the promoter region of miR-193b, along with the indirect inhibition of MYC expression by miR-194b, suggests that miR-193b may exhibit an anti-tumor effect on osteosarcoma by targeting KRAS and STMN1. The reciprocal negative regulatory loop involving MYC and miR-93b potentially leads to a persistent upregulation of MYC and downregulation of miR-193b, subsequently leading to enhanced expression of KRAS and



**Fig. 7. Analysis of immune infiltration: (A) Histogram of immune infiltration distribution.** The horizontal axis is the cell type, and the vertical axis is the estimated proportion. **(B) Histogram of the distribution of immune infiltration samples.** The horizontal axis is the sample, the vertical axis is the estimated proportion, and different colors represent different immune cells. **(C) Heat map related with immune infiltration.** Correlation of cellular immune infiltration in each sample, positive correlation in blue, negative correlation in red. **(D) Violin plot of the correlation of cellular immune infiltration between the metastatic group and the non-metastatic group.** The horizontal axis is the type of immune cells, the vertical axis is the cell immune infiltration score, the blue is the metastatic group, the red is the non-metastatic group. **(E–N) Bar graph related with hub genes immune infiltration.** The horizontal axis is the correlation score, and the vertical axis is the immune cell type. The size of the dots is the correlation, the higher the correlation, the larger the dots. The redder the color, the more significant the *P* value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

7 Correlation analysis between Hub genes and different molecular subtypes

STMN1, ultimately culminating in the development and metastasis of osteosarcoma. Suppression of MYC expression has been demonstrated to effectively impede osteosarcoma metastasis [55]. In vitro experiments involving the treatment of osteosarcoma cell lines KHOS and U2OS with the MYC inhibitor 10058-F4 resulted in a significant reduction in migration distance [56]. The NPM1/ERK/NF- $\kappa\beta$  pathway has been found to facilitate the growth and metastasis of osteosarcoma, with NPM1 identified as a crucial molecule in this process [57]. Sirtuin-1 (SIRT1), a class III histone deacetylase, is extensively implicated in gene regulation, genome stability maintenance, apoptosis, autophagy, aging, proliferation, and tumor metastasis [58,59]. SIRT1 plays a pivotal role in governing the proliferation and metastasis of cancer cells under stress by modulating p53-dependent aging and cell reprogramming [60]. Furthermore, it has been determined that the aforementioned 10 crucial genes exhibit a strong correlation with the degree of immune

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![](_page_11_Figure_2.jpeg)

Fig. 8. Correlation diagram between Hub genes and different molecular subtypes (A–J) Violin plots of correlation between different molecular subtypes and hub genes.

cell infiltration and the immune microenvironment. The researcher effectively utilized these genes to validate the molecular subtype model of osteosarcoma. Notably, there was a significant disparity in the expression of these 10 pivotal molecules between cluster 1 and cluster 4, underscoring the potential of the molecular subtype model to facilitate the categorization of osteosarcoma patients and provide valuable guidance for personalized therapeutic interventions [61]. The ten identified genes are anticipated to serve as molecular markers for distinguishing between metastatic and non-metastatic osteosarcoma. Furthermore, these genes hold potential for identifying osteosarcoma micrometastasis and osteosarcoma with a pronounced propensity for extensive metastasis, pending further development and application.

Inhibiting osteosarcoma metastasis is a reliable scheme to improve the cure rate of osteosarcoma. Early prevention of osteosarcoma metastasis is expected to improve the cure rate of osteosarcoma. In this study, 10 key genes associated with ER stress and osteosarcoma metastasis were identified, but the results lack further clinical validation, and the involved cell signaling pathways have not been further validated in vivo and in vitro. The study primarily adopts an observational approach, thus it does not establish causal relationships between ER stress and osteosarcoma metastasis. Additional experimental studies are required to validate the mechanistic connections between these phenomena. Nonetheless, this study successfully constructs a molecular subtype model of osteosarcoma using bioinformatics methods and identifies key molecules, thereby offering significant scientific and potential clinical value.

## 5. Conclusion

The identification of 10 key genes associated with osteosarcoma metastasis and ER stress holds potential clinical significance. Furthermore, the molecular subtype of osteosarcoma has the potential to inform clinical treatment decisions However, further investigations and future clinical validations are necessary to establish the identified key genes and their correlations definitively.

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## Ethics approval and consent to participate

Not applicable.

## **Consent for publication**

Not applicable.

## CRediT authorship contribution statement

Wang-Qiang Wu: Data curation. Cheng-Da Zou: Writing – original draft. Di Wu: Formal analysis. Hou-Xin Fu: Software. Xiao-Dong Wang: Supervision, Funding acquisition. Feng Yao: Writing – review & editing, Writing – original draft, Funding acquisition.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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