WITHDRAWN:
HSPA8 is a New Biomarker of Triple Negative Breast Cancer Associated with Prognosis and Immune Infiltration

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Research Article

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The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Objective

Triple negative breast cancer (TNBC) is a malignant tumor that endangers the health and lives of women all over the world in the 21st century. Heat shock protein member 8 (HSPA8/HSC70) is the chaperone gene of the heat shock protein family. It is involved in many cellular functions.For example, it promotes the circulation between ATP and ADP, participates in protein folding, and can change the vitality of the cell and inhibit its growth. HSPA8 overexpression in several human cancers reportedly leads to its malignant transformation. Nonetheless, the HSPA8 mRNA abnormal expression in TNBC and its diagnostic and prognostic significance remains to be elucidated.

Methods

TCGA, GEO, ONCOMINE,TIMER2.0, UALCAN, HPA, STRING and Kaplan-Meier plotter were conducted for bioinformatics analysis. HSPA8 protein expression was evaluated by Immunohistochemical method in TNBC tissues. Western blotting experiments were carried out to verify the results. Then the clinicopathological characteristics of patients with TNBC were analyzed by R software and Cox regression analysis. On this basis, a nomogram is constructed to estimate the 5-year overall survival. The prognostic Nomogram performance was calibrated and evaluated by calibration curve and receiver operating characteristic (ROC) curve .

Results

In this study, we discovered remarkably upregulated transcription of HSPA8 in breast cancer (BC) samples and TNBC samples relative to normal breast samples through bioinformatic analysis, which was further verified in clinical tissue samples and in experiments. Moreover, the transcriptional level of HSPA8 in BC samples and TNBC samples was positively associated with clinical parameters such as clinical tumor stage. Cox regression analysis revealed that the expression of HSPA8 in TNBC had significant clinical prognostic value. The nomogram and ROC analysis results demonstrated the strong predictive ability of HSPA8 in TNBC. KEGG and GO researches indicated that HSPA8 was mainly involved in partner-mediated autophagy, mRNA catabolism, neutrophil activation, immune response, protein targeting, RNA splicing, RNA catabolism and other biological processes.Immune infiltration analysis indicated that HSPA8 was significantly associated with immune cell subsets.

Conclusions

Our study findings demonstrate the potential diagnostic and prognostic significance of HSPA8 expression in TNBC, and elucidate the potential molecular mechanism of promoting the occurrence and

development of TNBC. These results may provide new opportunities and research approaches for targeted therapies in TNBC.

Introduction

Breast cancer(BC) is the most universal malignant tumors that endanger human health and life in the 21st century [1]. Lack of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor-2 (HER2) in breast cancer subtypes are TNBC [2],accounting for 15–20% of all diagnosed BC, and has the characteristics of high invasiveness, rapid distant metastasis, high mortality and short survival time [3].

Heat shock 70 kDa protein (HSP70) is reported to be involved in tumor cell proliferation and metastasis [4]. HSPA8 is a member of HSP70 belonging to the heat shock protein family. It is a constitutively expressed molecular chaperone, which plays an integral role in cellular stress response [5].HSPA8 has been found overexpressed in various cancer cells, which was indispensable to the growth of cancer cells [4]. Furthermore, depletion of HSPA8 could suppress cell growth, induce apoptosis, and cell cycle arrest in solid human tumors [6]. Previous many scholars believe that HSPA8 is involved in tumor molecular chaperone autophagy [7], and participate in the process of breast cancer through molecular chaperone autophagy [8].Nonetheless, the HSPA8 mRNA abnormal expression in TNBC and its diagnostic and prognostic significance remains to be elucidated.

Here, we aimed to comprehensively and systematically explore the expression of HSPA8 in TNBC through bioinformatics analysis, clinical tissue samples and in experiments using immunohistochemistry and Western blotting. The study findings offer insight into the clinical significance, potential functions, interactive network, and association with immune infiltration of HSPA8 in TNBC, providing a novel prognostic biomarker for accurate survival prediction and precise targeted treatment of TNBC. The following is the flow chart of this article(Fig. 1).

Materials And Methods

Data Resource

Retrieve TNBC patient data from the Breast invasive carcinoma dataset in TCGA database, comprised of 101 TNBC samples and 10 paracancerous tissues (Workflow Type: HTSeq-FPKM). HTSeq-FPKM values were then converted to TPM values (transcript per million) to compare differential expression among samples. The TCGA database also provides the TNBC patients corresponding clinical data.For pan-cancer analysis, the TIME2.0 database was used to obtain the data of HSPA8 differences between various cancers and adjoin normal specimens.

In addition, 112 cases of triple negative breast cancer archived in the affiliated Tumor Hospital of Xinjiang Medical University from January to December 2015 were selected. A total of 112 paraffin specimens of TNBC were collected. All of them were female, and their average age is (54.69 ± 18.12) years.All of them

were operated for breast cancer for the first time and were confirmed to be TNBC by pathology after operation. This experiment was approved by the Medical Ethics Committee of the Tumor Hospital to Xinjiang Medical University.112 people signed the corresponding informed consent form.

Comprehensive evaluation

ONCOMINE is a translational bioinformatics service that provides powerful, genome-wide expression analysis [9]. In our research, HSPA8 expression in BC samples and neighbor normal specimen were compared.The screening criteria are as follows: the P value is set to 0.01, the multiple change is 1.5, and the top 10% genes are ranked.

CCLE is a comprehensive portal that analyzes and visualizes genomic data from more than 1,000 tumor cell lines [10].The corresponding CCLE data were selected and R software (version 4.1.0) was used to evaluate the HSPA8 expression in multiple tumor cell lines.

HPA is a publicly available source that provides immunohistochemical images for analyzing protein expression patterns in approximately 20 common tumors and normal tissues [11]. Immunohistochemical images of clinical BC specimens and normal breast tissue samples were obtained from this database to compare HSPA8 protein expression between the two groups.

UALCAN is a comprehensive network information resource that provides evaluation based on TCGA and MET500 queue data [12].In the current research, we used the UALCAN platform to evaluate the relationship between the HSPA8 mRNA expression and clinicopathological features. $P < 0.05$ was significant.

Kaplan-Meier Plotter is a comprehensive portal for analyzing the survival of cancer patients [13][14], which to evaluate the prognostic value of HSPA8 mRNA in BC and TNBC by analyzing the relationship between HSPA8 level and survival outcomes.Survival results included OS and RFS. The optimal cutoff value is determined by the KM plotter algorithm.

Western Blotting

Cells were lysed and the protein concentration was determined by bicinchoninic acid assay. Proteins were subsequently separated by SDS-polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane. The membrane was blocked with 5% bovine serum albumin diluted with Tris-buffered saline with 0.1% Tween 20 (TBST) at room temperature for 2 h, and then incubated overnight at 4℃ with the primary antibodies anti-β-Tubulin and anti-HSPA8. The membrane was washed with TBST and incubated with secondary antibodies for 1.5 h. The Enhanced Chemiluminescent detection kit was employed to visualize the protein bands.

Immunohistochemistry and result judgement

Immunohistochemical staining was performed by SP method, and the operation process is completed in strict accordance with the product instructions.HSPA8 rabbit anti-human monoclonal antibody was

purchased from British Ab-cam company, and SP detection kit was purchased from Solebo Biological Company (SPO041).The positive expression of HSPA8 was located in the cytoplasm of the cells. The immunohistochemical results were interpreted by two pathologists who read the slices double-blindly .And ten visual fields were randomly collected in each case.The percentage of positive cells and staining intensity were observed. (1) staining intensity: no positive staining or cell chromogenic indistinguishability from the surrounding stroma was 0; light yellow was 1; yellow or brownish yellow was 2; brown was 3; (2) percentage of positive cells: the number of positive cells < 5% was 0; 5% 25% was 1; 26% 75% was 2; > 75% was 3. The above two scores were multiplied as the final score of HSPA8 protein expression: 0 as negative, ≥ 1 as positive, 1–3 as low expression, 4–6 as medium expression, ≥ 7 as high expression.

Differentially expressed genes

233 cases of TNBC and 114 cases of normal breast specimens from GSE86945, GSE106977 and GSE102088 were analyzed.DEGs between TNBC samples and normal breast specimens were identified using the DESeq2 package [15] in R with thresholds of |logFC| > 3 and p < 0.01. Volcano plots and correlation heatmaps of DEGs were constructed using the ggplot2 .

Functional Enrichment Analysis

The pathways of HSPA8 and EDGs were identified by GO annotation and KEGG. Functional enrichment Analysis using clusterProfiler package [16].

Interaction Analysis

STRING database (https://string-db.org/) is a database that searches online for known protein interactions, and Integrate the corresponding protein-protein interaction data.[17]. STRING was used to evaluate the PPI network of HSPA8 and DEGs of TNBC and further processed using the visualization tool Cytoscape. The hub genes were screened by CytoHubba's Degree algorithm (Degree 50 was used as the screening standard).

Evaluation of immune infiltration

TIMER used 10,897 cancer samples from TCGA to assess the abundance of immune infiltration [18] [19]. TIMER gene module was used to study the expression of HSPA8 in different tumors and the relationship between the expression of HSPA8 and the abundance of immune infiltration [20][21].

Statistical Analysis

Classified measurements are described by counts and percentages, and continuous measurements are represented by mean and range. Chi-square test was used for classified measurement comparison.Kaplan-Meier analysis was employed for evaluating patient survival. Cox regression analysis was used to evaluate the relationship between the expression of HSPA8 and other clinical parameters in

TNBC patients.The receiver operating characteristic was established by using PROC package to analysis the HSPA8 expression significance in diagnosis [22], and the area under the ROC curve (AUC) indicated the magnitude of diagnostic efficiency. AUC > 0.7 and 0.5–0.7 indicated good accuracy and weak accuracy, respectively. Based on the expression values of HSPA8 and other clinical parameters, we established a nomogram model to analyze the TNBC patients overall survival. The statistical methods of this study are carried out by R software (version 4.1.0). $P < 0.05$ is statistically significant. Continuous variables are expressed as mean ± standard deviatio

Results

Pan-Cancer Analysis of HSPA8 mRNA Expression Level

HSPA8 mRNA expression in multiple cancer using independent datasets from different sources. First, the transcriptional of HSPA8 in various human tumors and their counterpart normal breast specimen were investigated in the TCGA datasets. HSPA8 mRNA expression in tumor tissues specimen was significantly higher than in normal tissues specimen for multiple cancers, including BRCA and Head and Neck squamous cell carcinoma (Figs. 2A, B). Then the HSPA8 expression levelin pan-cancer was evaluated by ONCOMINE, which revealed the same expression trend as above. (Fig. 2B). Further, evaluationof HSPA8 expression in multiple common tumor cells from the CCLE database indicated that breast cancer cells had relatively higher HSPA8 expression than other tumor cells (Fig. 2C).And the HSPA8 expression level in TNBC cell lines (such as HCC1569, SUM159PT, HCC2157, etc.) was higher than that in other breast cancer molecular subtypes (Fig. 2D).

HSPA8 mRNA Expression in Breast carcinoma and Triple Negative Breast Cancer

Although accumulating evidence suggests that HSPA8 is a novel tumor biomarker, transcriptional analysis of HSPA8 in human TNBC has not been well documented. Therefore, Using the TCGA data to compare transcriptional levels of HSPA8 between TNBC samples and normal breast samples. The HSPA8 mRNA expression in TNBC tissues was significantly higher than that in normal breast tissues ($p < 0.001$) (Fig. 3B).This conclusion was also verified in Breast carcinoma and normal tissues ($p < 0.001$) (Fig. 3A). The same conclusion was further confirmed in the ONCOMINE data ($p < 0.05$). Specifically, the Curtis and Sorlie datasets demonstrated that HSPA8 was up-regulated in BC specimen relative to normal specimen, with FCs of 1.579–2.092(Figs. 3C, 3D). Furthermore, high HSPA8 protein expression was observed in BC tissues based on the HPA dataset (Fig. 3E). Besides, our IHC staining results on TNBC tissue demonstrated that HSPA8 expression in TNBC specimen was significantly increaser than that in adjacent non-tumor specimen (Figs. 3F,3G). The HSPA8 quantitative evaluation in TNBC and paracancerous tissues by Western blot was shown in Fig. 3H ($P < 0.05$).Finally, the difference of HSPA8 expression between normal breast cell lines and four TNBC cell lines was verified by Western blot quantitative analysis and Western blotting analysis (Figs. 3I).

Relationship of HSPA8 and Clinicopathological parameters of BC and TNBC

In this study, we used UALCAN to assess the relationship between HSPA8 and the clinicopathologic parameters of BC, including molecular subtype ,clinical tumor stage, pathological tumor grade and TP53-mutation status (Fig. 4A-4D). There were significant differences of HSPA8 was found between TNBC group and normal group ($p < 0.001$) (Fig. 4A). And there were significant differences among different TNBC subtypes ($p < 0.001$) (Fig. 4C).The highest mRNA levels of HSPA8 were predominantly found in patients in stages II and III. Pathological tumor grading has important prognostic significance. According to pathological tumor grading criteria, patients with high-grade tumors tended to exhibit higher HSPA8 mRNA levels ($p < 0.05$) (Fig. 4B). The p53 variation reportedly is closely related to the development of tumors (30). As expected, differences in HSPA8 expression were identified between the TP53-mutation group and the normal and TP53-nonmutation groups ($p < 0.001$) (Fig. 4D).Moreover, HSPA8 expression in TNBC patients was significantly associated with lymph node metastasis, T stage, N stage in TCGA database($p < 0.05$) (Fig. 4H). In addition, the clinicopathological data of 112 TNBC patients in the affiliated Tumor Hospital to Xinjiang Medical University were analyzed.Evaluated by Logistic statistical method,the HSPA8 expression was closely related to a variety of clinical characteristics of poor prognos, such as AJCC stage (OR = 5.846, 95%CI = 2.322–15.641, $p < 0.001$),Lymph node metastasis (OR = 6.361, 95%CI = 2.021–28.238, $p = 0.004$), CK5/6 expression (OR = 7.666, 95% CI = (2.439–34.021, $p = 0.002$), and HSPA8 expression(OR = 3.991, 95% CI = 1.601–10.733, $p < 0.05$) (Table 1). Furthermore, various survival parameters were also evaluated for their relationship with HSPA8 mRNA levels in TNBC patients. Survival analysis demonstrated that the OS (defined by period from suffering to death), RFS (referring to time from primary treatment to recurrence) rates of BC and TNBC patients with high-HSPA8 expression were significantly lower than those in low-HSPA8 patients ($p < 0.001$) (Figs. 4E,4F). The clinical data of this experiment were used for further survival analysis.The same conclusion was reached (Figs. 4G).

In addition, using the clinical information of experimental data, Cox proportional hazard regression analysis was used to evaluate the independent prognostic value of HSPA8.This study demonstrated that the high HSPA8 transcriptional was independently correlated with significantly shorter OS ($p = 0.0035$) for TNBC (Table 2). The HSPA8 transcriptional level was confirmed to be an independent prognostic factor for the overall survival of patients with TNBC.

Table 1
Logistic analysis of the relationship between HSPA8 expression and clinical parameters in TNBC.

Characteristics	Odds Ratio (OR)	P-value
Age(< = 60vs. >60)	0.991(0.209–3.568)	0.144
Menstruation(N0 vs Menopause)	0.444(0.137–1.219)	0.137
AJCC stage (Stage IV& Stage III vs.Stage II & Stage I)	5.846(2.322–15.641)	< 0.001
Lymph node metastasis(No vs Yes)	6.361(2.021–28.238)	0.004
Histologic grade (III vs. I&II)	2.287(0.874–6.775)	0.108
CK5/6	7.666(2.439–34.021)	0.002
Distant metastasis (No vs Yes)	–	0.992
HSPA8 expression (low vs high)	3.991(1.601–10.733)	0.004

Table 2
Cox regression analysis of variables for OS in TNBC patients

Characteristics	Univariate analysis		Multivariate analysis	
	Hazard ratio(95%CI)	P-value	Hazard ratio(95%CI)	P-value
Age(< = 60vs. >60)	0.89(0.27–2.97)	0.853	–	–
Menstruation(N0 vs Menopause)	0.46(0.17–1.22)	0.119	0.47(0.18–1.25)	0.129
AJCC stage (Stage IV& Stage III vs.Stage II & Stage I)	4.35(1.94–9.76)	< 0.001	4.48(1.53–13.18)	0.006
Lymph node metastasis(No vs Yes)	5.3(1.59–17.67)	0.007	4.90(1.36–47.65)	0.015
Histologic grade (III vs. I&II)	2.13(0.86–5.32)	0.104	0.55(0.18–1.73)	0.308
CK5/6 expression (No vs Yes)	6.59(1.98–21.98)	0.002	9.00(2.02–40.22)	0.004
Distant metastasis (No vs Yes)	–	0.997	–	–
HSPA8 expression (low vs high)	3.23(1.4–7.44)	0.006	2.92(1.26–6.77)	0.013

Diagnostic Significance of HSPA8 Expression in TNBC

Next, the HSPA8 diagnostic **significance** in different clinical features of TNBC patients was evaluated.Using the results of multi-factor Cox analysis ,we established a nomogram combining HSPA8 expression and key clinical factors to estimate the 1-year, 3-year and 5-year survival of TNBC patients (Figs. 5G). A higher nomogram score for OS indicated a worse prognosis .And the C-index is 0.801.The calibration curve of the nomogram is very close to the 45-degree line, indicating that the nomogram is

well calibrated.More specifically(Figs. 5D-5F).ROC analysis reveals that the survival line chart can accurately predict the 1 year, 3 year and 5 year survival rates of patients with TNBC.Their AUC values are 0.9, 0.824, 0.823 respectively.(Fig. 5A-5C) .These results implied that the transcriptional level of HSPA8 was relatively sensitive and specific for the diagnosis of TNBC.

Identification of Differentially Expressed Genes

To explore the abnormal changes in downstream pathways caused by TNBC differential genes, three GEO data sets (GSE86945, GSE106977 and GSE102088, containing 233 TNBC samples and 114 normal breast tissue samples) were selected.4691 DEGs were detected in TNBC and normal breast specimens, of which 2337 DEGs expression was up-regulated and 2354 DEGS expression was down-regulated.These results generate volcanic charts and bar charts, which visually show the distribution of DEGs.(Figs. 6A, 6C), and heatmaps depicted the top 100 significantly up-regulated and down-regulated DEGs (Figs. 6B).The top 1000 down-regulated DEGs and the top 1000 up-regulated DEGs were input into STRING database for analysis.The hub genes were screened by Degree algorithm in CytoHubba in Cytoscape software(Degree genes ≧ 50 as the screening standard) (Figs. 6D).Among them, HSPA8 is one of the top five hub genes (Figs. 6D).

Enrichment Analysis of HSPA8 and TNBC differential genes

To further clarify the potential mechanisms of in the progress of TNBC, we used GO and KEGG techniques to predict the functions and pathways of the 50 hub genes. The biological processes for these genes were predominantly enriched in translation initiation, mRNA catabolism of nuclear transcription, SRP-dependent cotranslation protein targeting membrane, rRNA processing and so on (Fig. 7A).Enrichment analysis demondtrated that HSPA8 was involved in partner-mediated autophagy, mRNA catabolism, neutrophil activation, immune response, protein targeting, RNA splicing, RNA catabolism and other biological processes (Fig. 7B).The results of KEGG enrichment revealed several main pathways: ribosome, RNA transport, estrogen signal pathway, PI3K-Akt signal pathway, antigen processing and presentation, proteoglycan in cancer (Fig. 7C).The genes corresponding to the pathway are listed, such as HSP90AA1 and HSPA8 are involved in the estrogen signal pathway (Fig. 7D).All the above-enriched pathways were markedly related to the occurrence and evelopment of malignant tumors.

HSPA8 Expression and Various Immune Infiltrates in TNBC Patients

Immune cells in the tumor microenvironment largely influence the biological behavior of the tumor [23] [24]. Investigating infiltration of different immune cells, we demonstrated in the TNBC micro-environment that HSPA8 mRNA was positively related to the abundance of immunocytes such as CD4+, CD8 + Tcells, neutrophils, monocytes and macrophage, but negatively related to the abundance of innate immune cells such as B cell NK cell(Figs. 8). Moreover, we used TIMER software to perform Cox proportional hazard regression analysis to evaluate the independent prognostic value of immune cell infiltration and HSPA8mRNA expression.The results indicated that except for CD8 + T cells, B cells ,NK cell and

neutrophils, the infiltration degree of the other 3 kinds of immune cells and the HSPA8 expression were closely related to the clinical characteristics of TNBC(Table 3).

Table 3
The cox proportional hazard model for HSPA8 mRNA expression in TNBC and six tumor-infiltrating immune cells (TIMER).

Characteristics	Coef	HR	95%CI_l	95%CI_u	P-value
CD4 + T cell	−34.450	0.0001	0.0001	0.001	0.012*
CD8 + T cell	−0.705	0.494	0.113	2.154	0.348
B cell	−0.434	0.648	0.017	24.094	0.814
Neutrophil	−2.174	0.114	0.004	3.105	0.198
Monocyte	14.379	$1.757x10^{6}$	42.334	$7.290x10^{10}$	0.008**
Macrophage	14.103	$1.333x10^{6}$	6.679	$6.453x10^{11}$	0.035*
NK cell	0.133	1.142	0.256	1.885	0.603

Discussion

Breast cancer is the most universal malignant tumor in the world. there are about 19.3 million new cancer patients worldwide in 2020, of which female breast cancer accounts for 11.7%, surpassing lung cancer (11.4%) for the first time to become the cancer with the largest number of newly diagnosed cancers in the world. Among them, TNBC has the clinical manifestations of rapid invasion, strong heterogeneity and short prognosis [25].Due to the lack of effective therapeutic targets, TNBC is ineffective to endocrine therapy and is currently the most difficult malignant tumor to treat.The standard therapy for TNBC is limited to chemotherapy and radiotherapy, but there is a high risk of recurrence. Therefore, effective biomarkers and novel therapeutic targets for TNBC are urgently needed.

HSPA8 is a fascinating chaperone protein and plays an essential role in many biological processes [26]. Decreased expression of HSPA8 is beneficial for suppressing the proliferation of cancer cells, inducing cell proliferation arrest, and acting as a modulator of viability and autophagy for cancer cells [26]. Besides, HSPA8 high expression has been identified in various cancer cells, including hepatocellular carcinoma and endometrial carcinoma, and is involved in cancer cell growth [27, 28] and regulating the autophagy in tumor cells [29]. However, the exact role of HSPA8 gene in TNBC remains unknown. In our study, we systematically characterized HSPA8 in TNBC, revealing its expression profile, predictive and prognostic significance, potential functions, interactive network and association with infiltration levels of immune subsets.

Firstly, the HSPA8 transcriptional levels in different kinds of cancers were examined using independent data sets from different platforms (TIMER, ONCOMINE).HSPA8 was highly expressed in a variety of

tumors, including BRCA, Thyroid carcinoma, Cholangiocarcinoma,Liver hepatocellular carcinoma and Colon adenocarcinoma. Similarly, high HSPA8 expression was also identified in various tumors cells and TNBC cells in the CCLE database. Taken together, these analysis illuminate that HSPA8 may have a potential promoting role in tumor development.Subsequently, it was revealed significantly higher HSPA8 transcriptional levels in BC and TNBC specimens than in normal samples. HSPA8 was highly expressed in various tumors including Hepatocellular carcinoma and Prostate cancer [30][31].Nian et al. identified that HSPA8 is closely related to endometrial carcinoma by relative and absolute quantitation (iTRAQ)-based proteomic analysis. In studies have shown that HSPA8 knockout can significantly inhibit the proliferation of endometrial tumor cells and promote their apoptosis [32].Yang et al. Identified that in HBV-related early-stage hepatocellular carcinoma, HSPA8 is thought to be up-regulated in tumor tissue and correlated with barren prognosis of patients [33].In the current study, higher HSPA8 transcription were identified in BC and TNBC samples compared to normal breast samples in various databases. HSPA8 protein expression in breast cancer and TNBC specimens was also significantly higher than that in normal breast tissues in HPA dataset and TNBC clinical tissue samples. To further verify our conclusion, we detected the relative expression levels of HSPA8 in TNBC tissue, various TNBC and a normal breast cell line by Western blotting, obtaining results that were consistent with our bioinformatics analysis.

The relationship between HSPA8 mRNA expression and the clinical characteristics of TNBC was further investigated, revealing that HSPA8 expression was correlated with tumor stage, molecular subtype, TP53 mutation status and various TNBC molecular subtypes in breast cancer. HSPA8 expression was closely related to lymph node metastasis, T stages and N stages in TNBC patients through logistic regression .The Kaplan-Meier test indicated that high expression of HSPA8 was suggestive of undesirable OS and RFS prognoses of TNBC patients. We confirmed that high HSPA8 expression was an independent adverse prognostic factor for OS in TNBC through univariate and multivariate regression analysis.At present, a prediction profile of TNBC based on HSPA8 expression has not been reported, so we conducted a multi-Cox regression analysis by integrating various clinical parameters from the experimental data, which showed that TNBC tumor stage, lymph node metastasis, CK5/6 expression and HSPA8 expression were independent prognostic factors, and further established a Nomogram to predict the death risk of individual patients and help optimize treatment decisions. The C-index index was 0.801. Then our ROC curve also conveied that HSPA8 expression had significant value in the diagnosis of TNBC.

To explore the expression of HSPA8 in TNBC and the downstream pathway of DEGs in TNBC,We identified the DEGs between TNBC and normal breast tissue.The enrichment results of GO and KEGG expressed that the above DEGs were mainly involved in ribosome, RNA transport, estrogen signal pathway, PI3K-Akt signal pathway,proteoglycan in cancer, etc. All the above-enriched pathways were markedly correlated with the occurrence and progression of malignant tumors.

Some studies have shown that the correlation between the high expression of HSPA8 and several oncogenic activities and signaling pathways, including PI3K-Akt and calcium signaling pathways. For example, targeting the PI3k-Akt signaling pathway results in an anti-leukemic effect by activating oncogenes upstream (FLT3-ITD, KIT, NRAS, etc.) [34][35]; and dysregulated Ca2 + homeostasis plays a

crucial role in the pathogenesis of various cancers [36].HCC patients with moderate/severe or mild/no depression indicates a correlation between high HSPA8 expression and activation of the VEGF/VEGFR2–PI3K–AKT pathway. It is tempting to speculate that this activation contributes to poor DFS by inducing endothelial cell proliferation and migration, which promotes angiogenesis and tumor growth, as well as by inhibiting expression of BAD and caspase 9, which reduces tumor cell apoptosis [37][38].Alteration of the phosphoinositide-3 kinase (PI3K) signaling pathway has been related with angiogenesis, tumor proliferation and inhibition of apoptosis. Activating mutations of oncogenes (PIK3CA, AKT, mTOR) or inactivating mutations of tumor suppressor genes (INPP4B, PTEN) are associated with tumor growth and treatment resistance [39]. PI3K's activation is associated with tumor evolution and chemotherapy resistance, and is a common genomic abnormality detected in TNBCs [40][41]. Therefore, we speculate that there may be a correlation between the high expression of HSPA8 and the activation of PI3K signal pathway in patients with TNBC.

Some scholars have found that high activation of CMA is observed in tumors and is required for tumor growth and survival [42][43].The elevated CMA has been proven to be necessary to sustain enhanced glycolysis to meet the bioenergetic demand of rapid proliferation [44].HSPA8 is a crucial molecular regulator of chaperone-mediated autophagy (CMA), as a detector of substrates that will be processed by this specialized autophagy pathway [45].

Under adverse conditions such as hypoxia, prolonged starvation [46], oxidative stress [47], or DNA damage, the chaperone Hsc70 binds different co-chaperones. Thus induce autophagy and protect tumor cells against cellular death.Furthermore, it was also reported that CMA is upregulated and required for the survival of breast cancer cells [48]. For examples ,some studies revealed that both the expression of HSPA8 (key proteins of CMA pathway) in breast cancer tissue samples were higher than the corresponding normal breast adjacent tissues from the same patient, indirectly suggesting higher CMA activity in tumor tissues [49].TNBC tumors are characterized by a more aggressive behavior and early relapse [50]. Higher levels of basal autophagy were found in the metastatic cell lines when compared to the non-metastatic, suggesting that autophagy could promote invasiveness and possibly increase tolerance to the cellular stress occurring during the metastatic process [51]. TNBC tumors are more hypoxic than non-TNBC and it has been suggested that they are less sensitive to hypoxic conditions because of perpetually higher levels of autophagy [52]. Therefore, we speculate that the overexpression of HSPA8 promotes the process of autophagy, which leads to the malignant transformation of TNBC.

An increasing body of evidence supports the hypothesis that immune cell infiltration influences the occurrence and progression of cancer, which adversely affects clinical prognosis and immunotherapy effectiveness [53]. According to related studies, it has been found that HSPA8 is related to neurodegenerative diseases, cardiac diseases, stroke, metabolic diseases, cancer, asthma, aging and others [54][55][56][57][58].And HSPA8 expression has been found to change in many immune diseases.For example, flow cytometry research have clarified increased expression of HSPA8 on B cells, T cells, specifically activated T cells and CD11b + Gr-1 + granulocytes / macrophages in the spleen of MRL/LPR lupus susceptible mice [59][60].Another significant finding of this study was the relationship

between HSPA8 mRNA and the degree of immune cell infiltration in TNBC.HSPA8 mRNA expression was significantly associated with the abundance of CD8 + T cells,B cells,neutrophils, monocyte, macrophage,and especially CD4 + T cells.Previous researches have illuminated that HSPA8 plays a central role in different key steps of polypeptide antigen presentation by CD4 + T cells, which may regulate the activation of T and B cells and the final secretion of antibodies by plasma cells [61][62][63][64][65][66][67][68]. Consistent with the above information, we revealed that HSPA8 expression was increasely associated with the infiltration of CD4 + T cells in TNBC.Udono and Srivastava have shown that HSPA8 in tumor cells binds to tumor-specific antigenic polypeptides to facilitate their recognition by the host immune system [69]. HSPA8 can also induce maturation of antigen-presenting cells, promoting the transformation of Th cells into Th1 cells, directly activating TCRγδT cells and natural killer cells [70].

Natural killer (NK) cells, a type of cytotoxic lymphocytes, are crucial constituents of the innate immune system whose function in enhancing the anti-tumor immunity in TNBC has been studied extensively [71].Some scholars have found that baseline circulating tumor cells (CTCs) status is positively associated with peripheral NK cells among those receiving first-line treatment in 75 patients with TNBC. Baseline CTCs combined with peripheral NK enumeration (CTC-NK) can predict PFS of TNBC patients more precisely [72]. NK cells are the major effectors of antibody (Ab)-dependent cell-mediated cytotoxicity (ADCC) and thus play an important role in Ab-based therapies. In vivo and in vitro studies revealed that tissue factor (TF)-targeting antibody-like immunoconjugate (called L-ICON)-CAR-NK cells have direct killing effects against TNBC cells and also mediate L-ICON ADCC to acquire a stronger effect [73]. Avelumab, a human IgG anti-PD-L1 mAb, triggers ADCC against a panel of TNBC cells and enhances NK-cell mediated cytotoxicity [74].Therefore, HSPA8 may directly kill TNBC cells by directly activating natural killer cells.Notably, macrophage subsets in TNBC tumors tended to co-express typical M1 and M2 signals, which is consistent with recent findings in human breast and lung cancer [75][76].Furthermore, the Cox proportional hazard model revealed that CD4 + T cells, monocyte and macrophages were explicitly associated with undesirable clinical outcomes of TNBC patients.

Although this study revealed the potential significance and possible mechanism of HSPA8 in the occurrence and development of TNBC, there were some limitations. Firstly, the functional assessment of HSPA8 was based on database and some experiments, which had not been confirmed in vivo, and needs to be further explored in future studies. Secondly, although the expression of HSPA8 and its prognostic significance are verified in experimental clinical samples, and the results are similar to those of public data sets, it will lead to some errors due to the slight difference in pathological data and the small number of TNBC patients.Finally, although this study demonstrated that HSPA8 plays a part in regulating the cell cycle and influencing immune infiltration, the underlying molecular mechanisms and signaling pathways have not been explored. We will conduct future studies to elucidate the mechanism of HSPA8 in TNBC.

Conclusion

In conclusion, we comprehensively and systematically evaluated the expression patterns, prognostic and diagnostic value, and potential mechanisms of HSPA8 in the occurrence and development of TNBC. Our results provide novel insight to help identify new prognostic biomarkers and therapeutic targets, which may assist clinicians to more accurately predict the survival of TNBC patients and inform their treatment decisions.

Abbreviations

HSPA8,Heat shock protein member 8;

CK5/6,Cytokeratin 5/6;

BC, Breast cancer;

TNBC,Triple negative breast cancer;

BRCA Breast invasive carcinoma;

UALCAN (http://ualcan.path.uab.edu/analysis.html);

ONCOMINE (www.oncomine.org) ;

CCLE, The Cancer Cell Line Encyclopedia;

HPA, Human Protein Atlas (https://www.proteinatlas.org) :

STRING (https://string-db.org/)

PPI, protein-protein interaction;

GO, gene ontology;

KEGG, Kyoto Encyclopedia of Genes and Genomes;

Kaplan-Meier Plotter (https://kmplot.com/analysis/)

TIMER,Tumor Immune Estimation Resource (https://cistrome.shinyapps.io/timer/);

TCGA, the cancer genome atlas(https://cancergenome.nih.gov/);

GEO,Gene Expression Omnibus(https://www.ncbi.nlm.nih.gov/geo/);

GSEA, Gene set enrichment analysis;

GTEx, Genotype-Tissue Expression;

ROC, Receiver operating characteristic;

AUC, area under the ROC curve;

95%CI, 95% confidence interval;

DEGs, Differentially expressed genes;

FC, fold change;

OS, overall survival;

RFS recurrence-free survival;

*P < 0.05, **P < 0.01, ***P < 0.001.

Declarations

Author Contributions

BCY ,WTX and YTL designed and performed the experiments. BCY wrote the draft of the manuscript. BCY, WTX,YN,and YTL analyzed and interpreted data.BCY and YN collected the TNBC samples and clinical information. YTL supervised the project.

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Competing interests

The authors declare no competing interests.

Notes for publishers

All propositions expressed in this article represent only those of the author, not necessarily those of their affiliated organizations, nor do they necessarily represent those of publishers, editors and reviewers.

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Figures

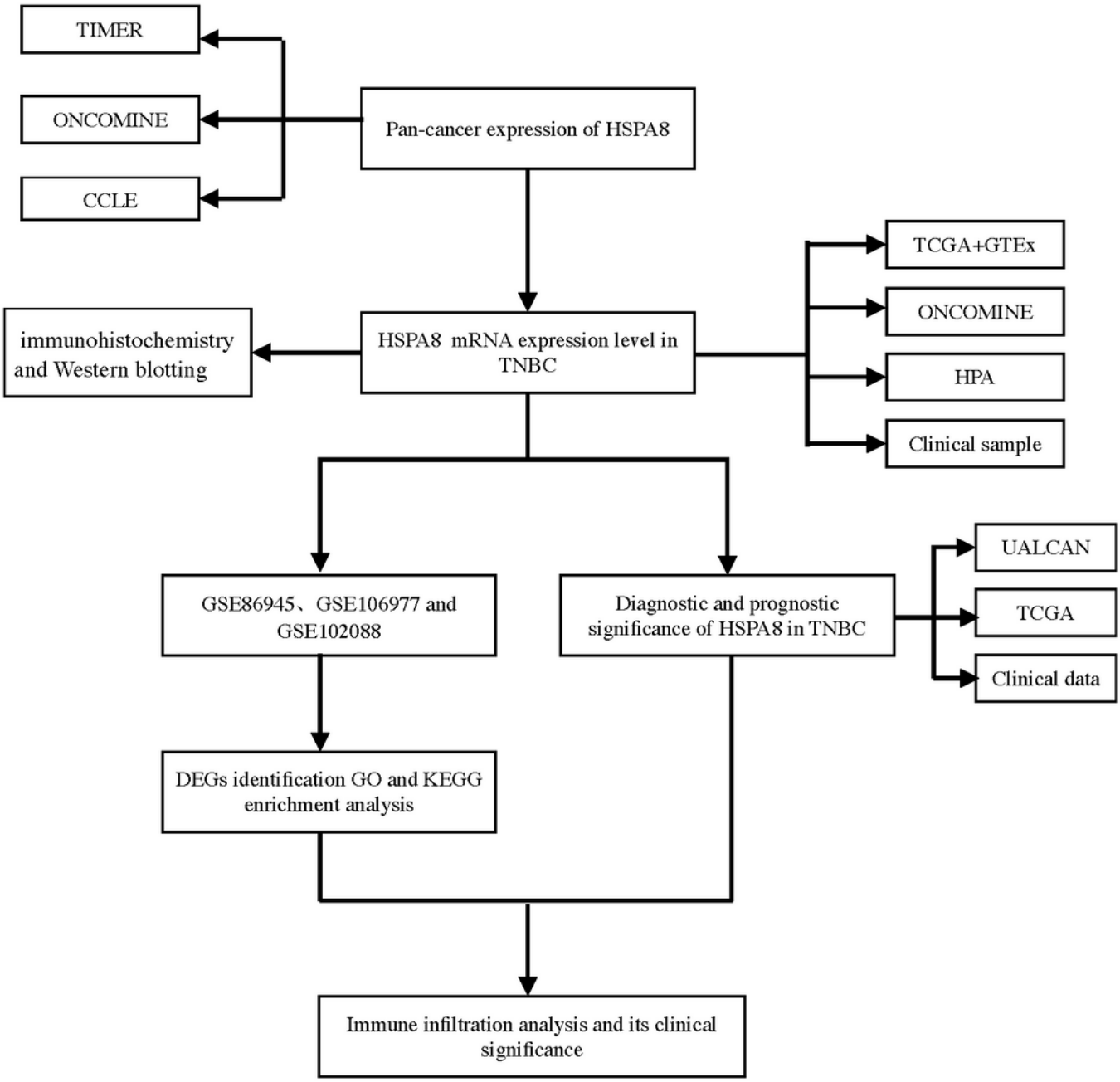

Figure 1

The flow chart of this study

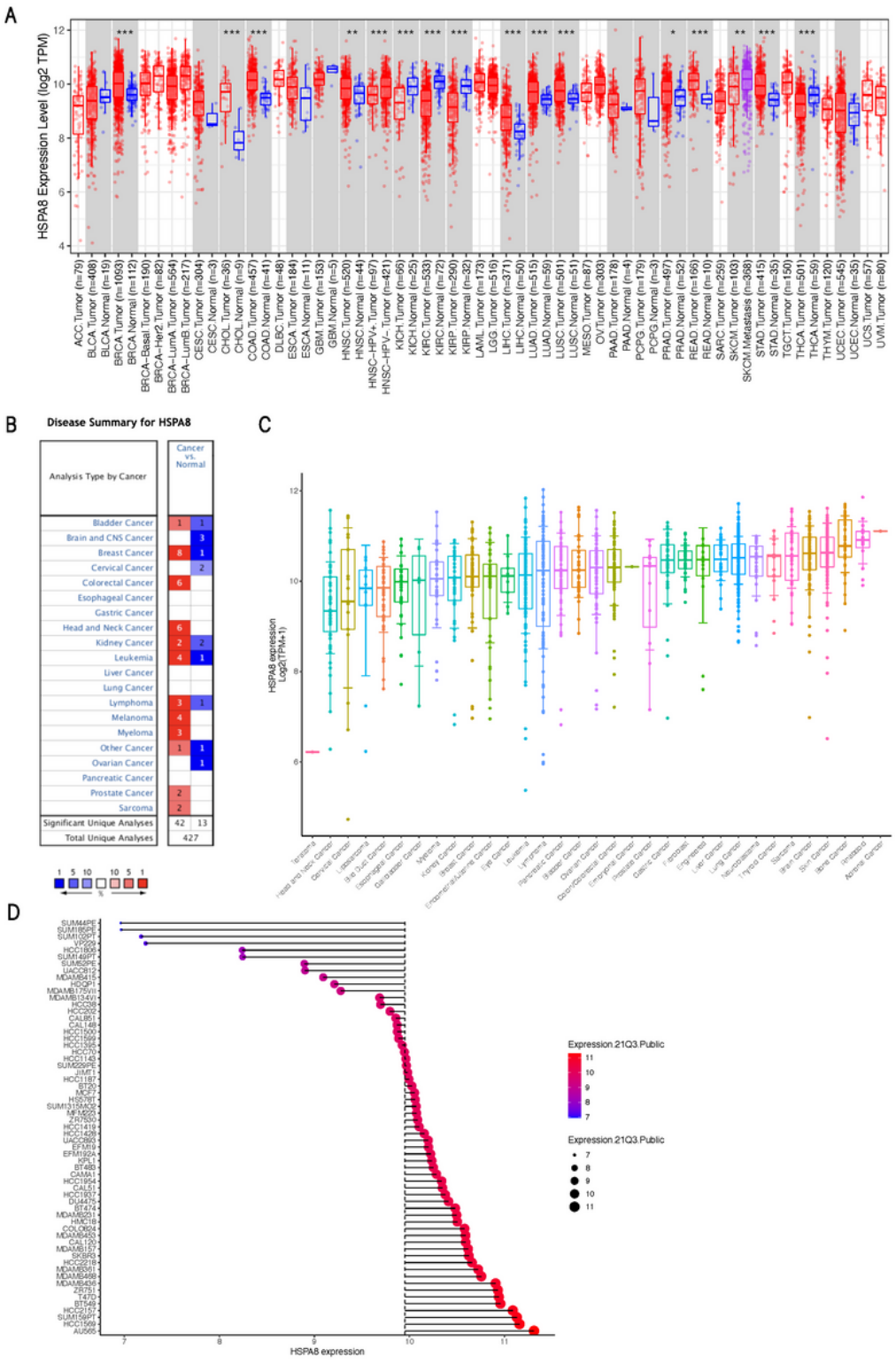

Figure 2

Difference of HSPA8 expression between tumor and paracancerous normal tissue(A) HSPA8 mRNA expression in33 kinds of tumor tissues and normal tissues adjacent to cancerin the TIMER2.0 datasets. Blue indicates the expression of normal tissue. Red indicates the expression of tumor tissue.(B) Differential HSPA8 mRNA levels between tumor and adjacent normal tissues across various tumor types in the Oncomine database (screening criteria P< 0.01, 1.5fold changes, top 10%gene rank). (C) the

HSPA8 expression in various tumor cell lines (CCLE). (D) the HSPA8 expression in different molecular subtypes of breast cancer cell lines (CCLE).

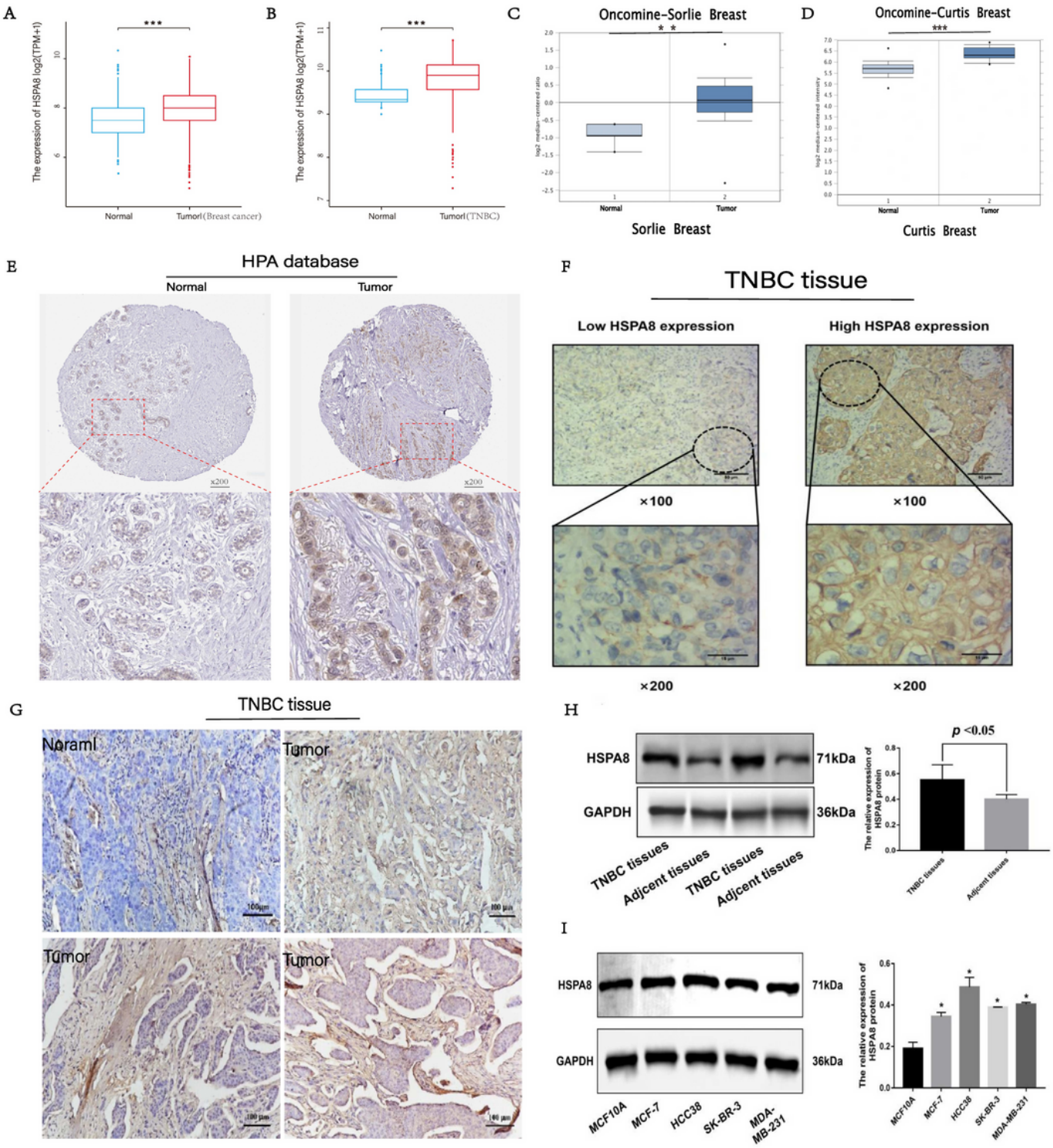

Figure 3

The relative expression of HSPA8 in BC tissue and TNBC at the cell and tissue levels. (A,B) Expression of HSPA8 in tumor specimens and adjacent tissues (TCGA and GTEx). (C,D) HSPA8 expression in normal

and tumor specimens (ONCOMINE). (E) HSPA8 protein expression level in BC and normal breast tissues (HPA). (F,G) The IHC staining results of HSPA8 level in TNBC and adjacent non-tumor tissue (clinical tissue of TNBC) . Statistical significance was determined by t-test.(H) Western blotting results of HSPA8 expression in TNBC and paracancerous tissues. (I) Western blot results of HSPA8 in normal breastand TNBC cell lines.

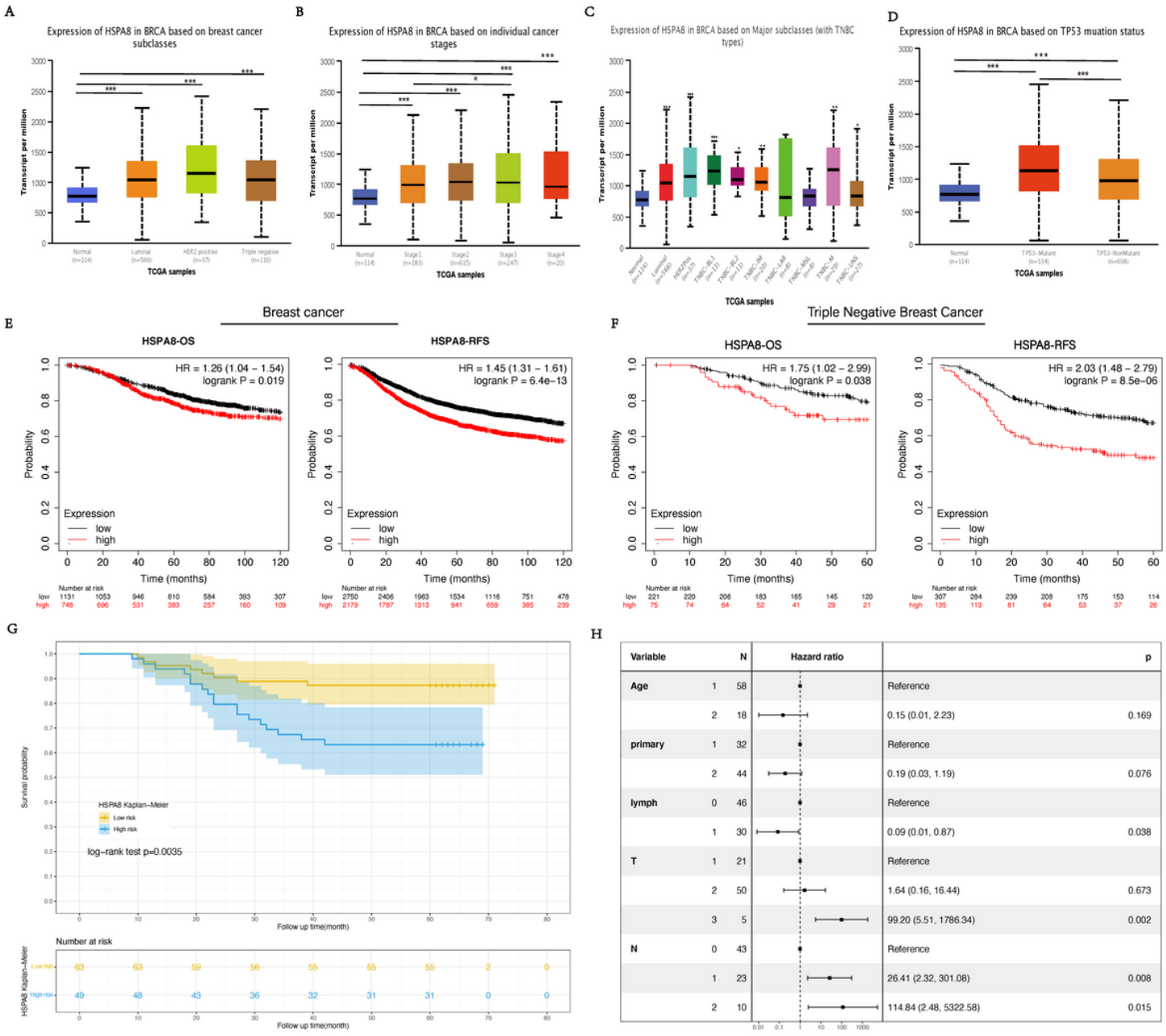

Figure 4

Correlation between HSPA8 expression and the clinical parameters of BC and TNBC patients and its prognostic significance. (A–D) Relationship of HSPA8 mRNA levels with individual molecular subtype, cancer stages, tumor grade, and TP53 mutation status of BC patients. (E–F) Relationship of HSPA8 expression with OS and RFS in TCGA database. (G) Relationship between HSPA8 expression and OS in

experimental data.(H) Forest plot showing the impact of HSPA8 on OS at different T stage, N stage ,Age , primary and Lymph node metastasis.

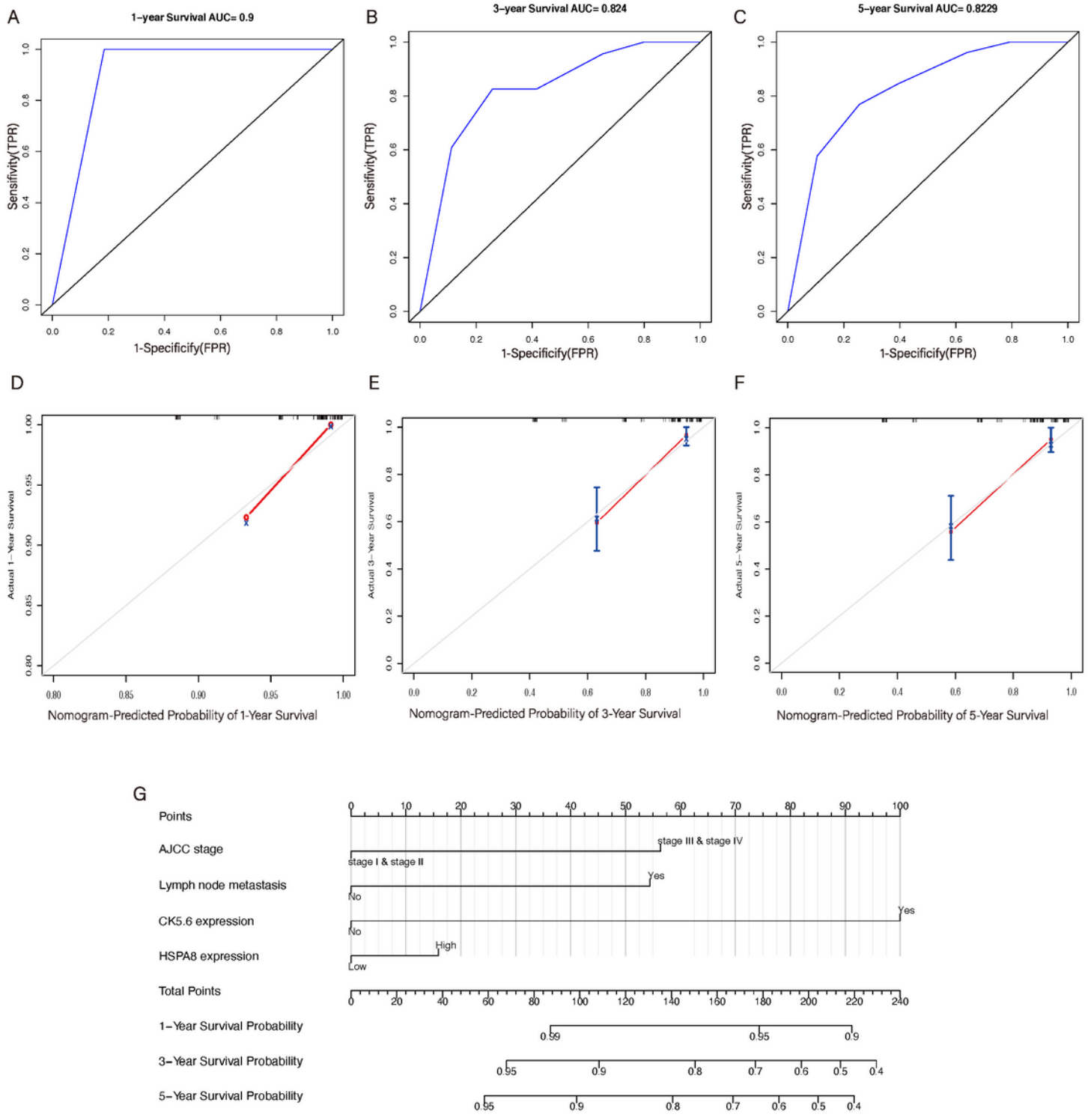

Figure 5

Diagnostic value of HSPA8 mRNA level in TNBC. (A-C) The ROC curve of OS in TNBCpatients at 1 year, 3 years and 5 years.(D-F) The calibration curveof overall survival in TNBC patients. (G) Nomogram predicts

the probability of overall survival in TNBC patients .

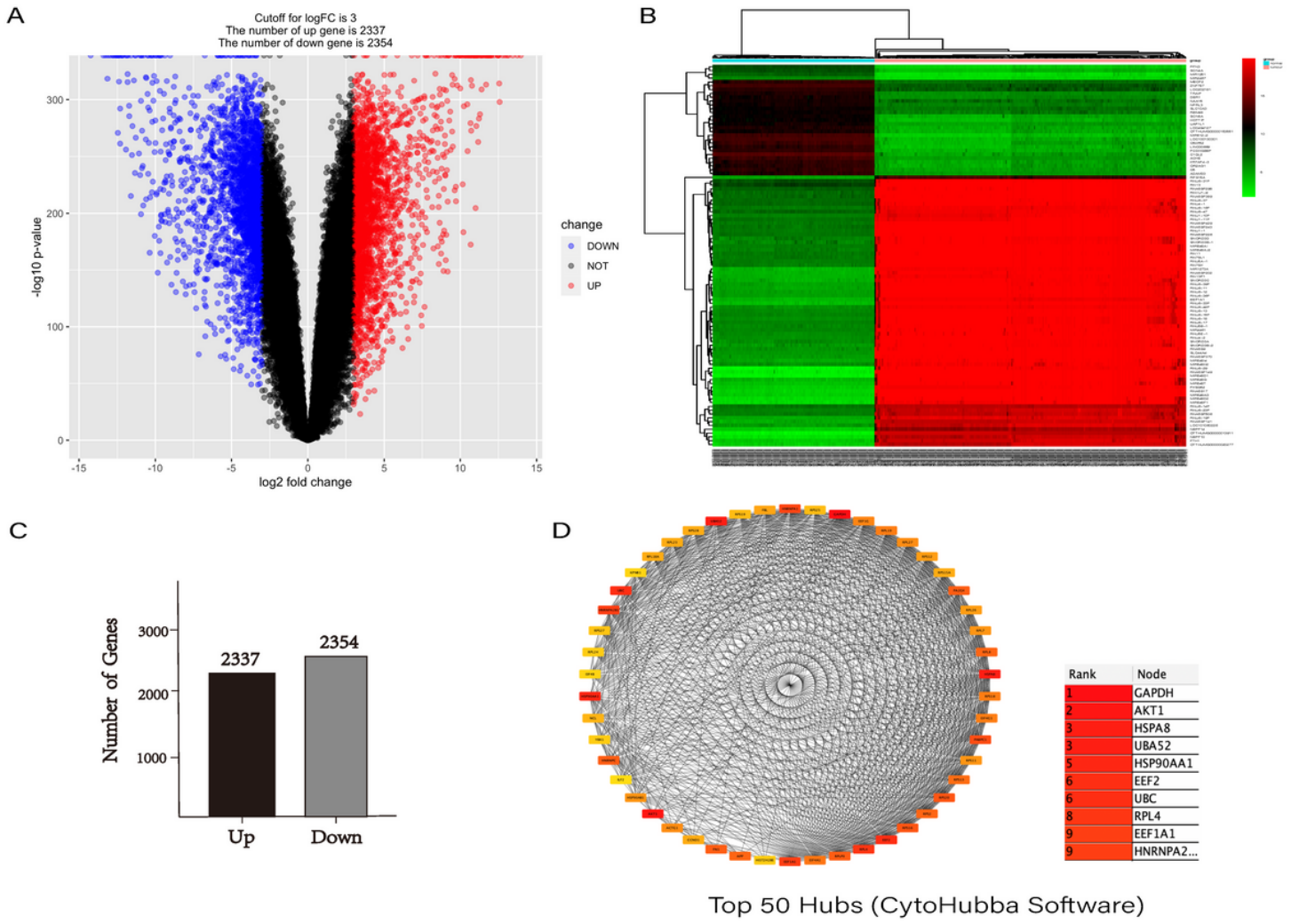

Figure 6

Volcano plots and heatmap plots of DEGs. (A) The volcano plot described 4691 DEGs (|log2fold change| > 3 and p< 0.01).(B) The heatmaps depicted the expression of 100 significant up-regulated and down-regulated genes in TNBC and Normal breast specimen. (C) The histogram showing the number of up-regulated or down-regulated genes. (D) Top 50 hub genes (CytoHubba).

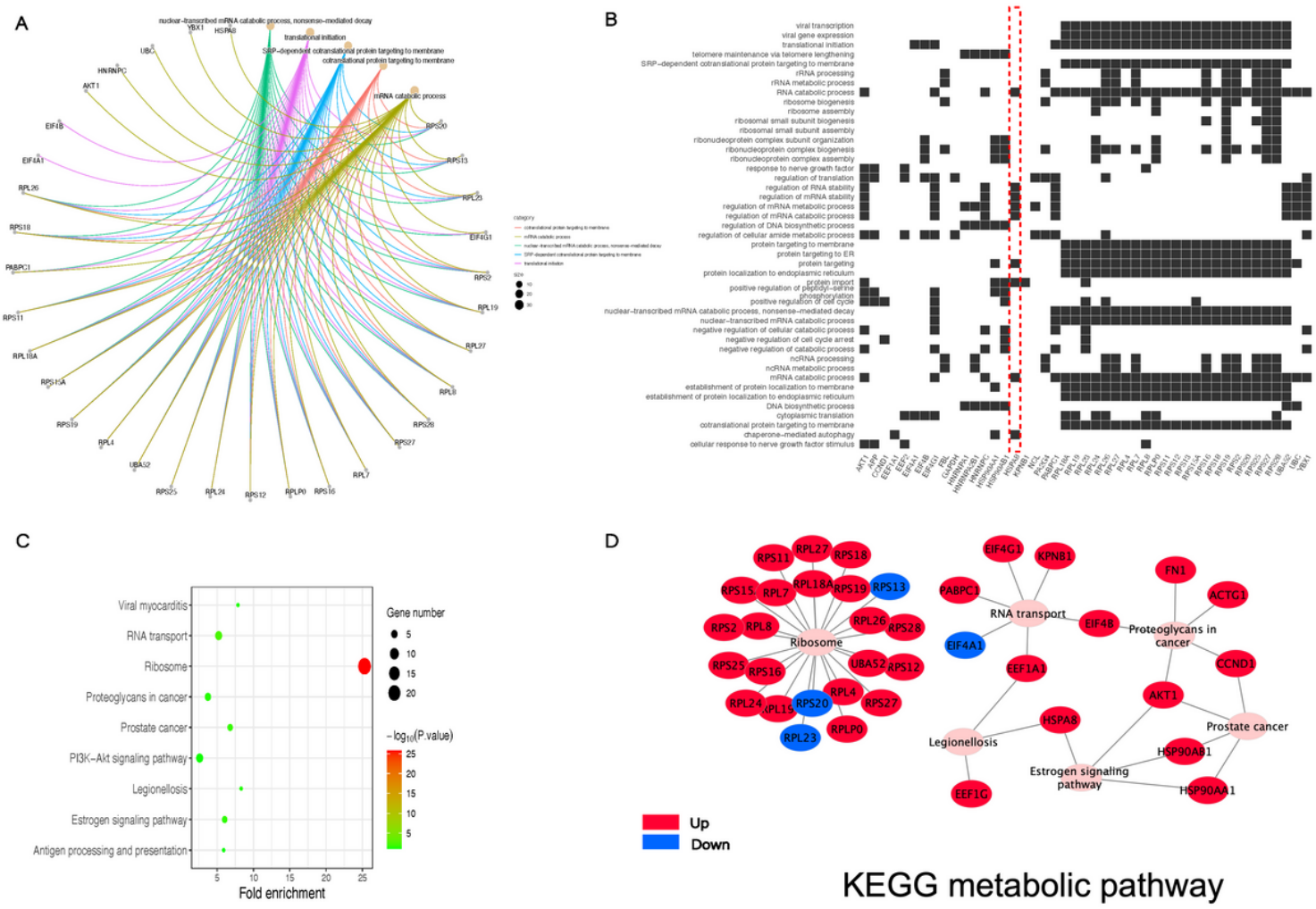

Figure 7

Results of enrichment analysis. (A) the results of GO analysis of 50 hub gene, (B) the results of GO analysis of HSPA8 gene, (C). KEGG analysis results bubble chart ,(D) Metabolic pathway of KEGG.

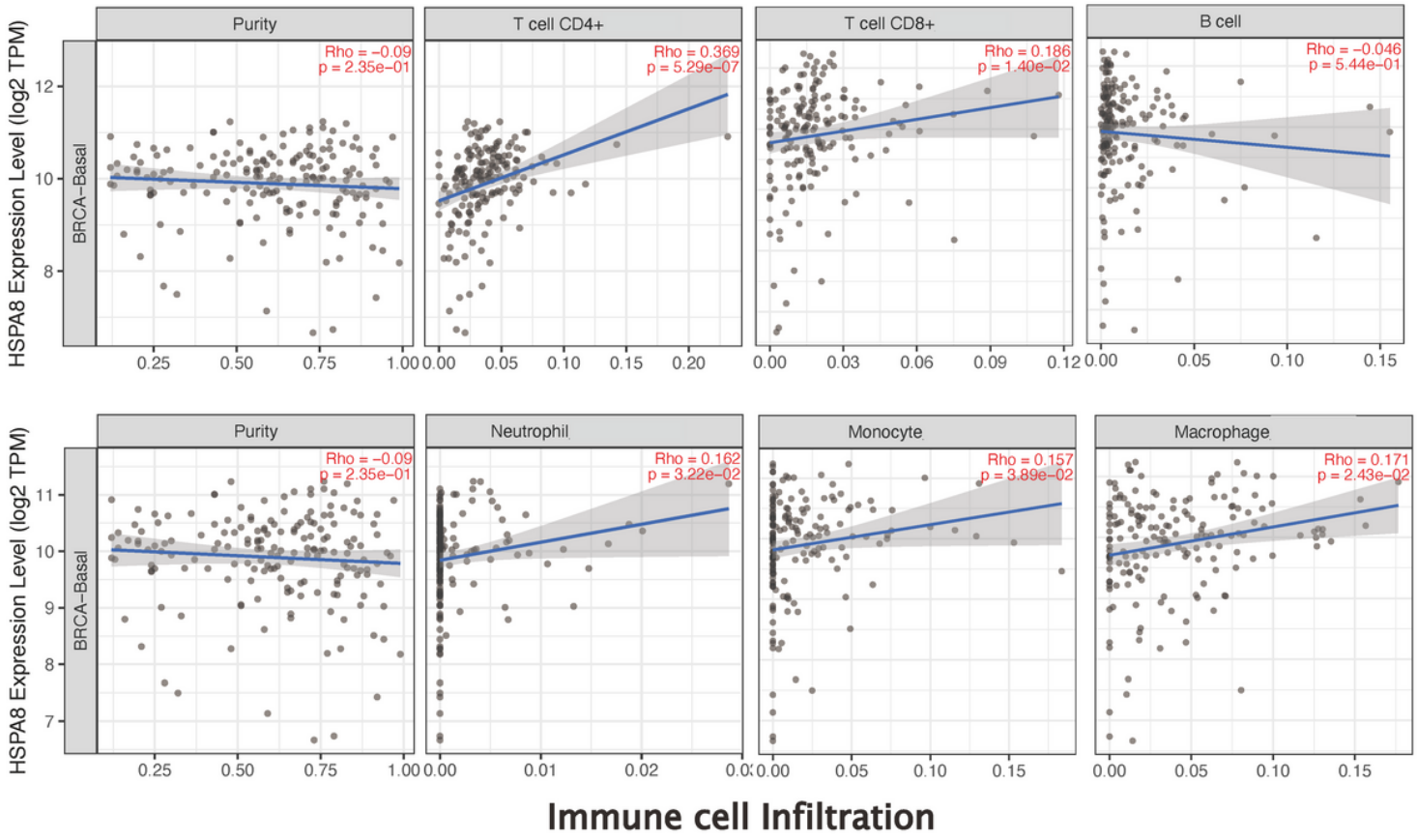

Figure 8

Relationship between HSPA8 mRNA expression and immune cell infiltration (TIMER).