HSPA4 Expression is Correlated with Melanoma Cell Proliferation, Prognosis, and Immune Regulation

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Purpose: Heat shock protein A4 (*HSPA4*) is associated with a variety of human diseases. However, its function in cutaneous malignant melanoma (CMM) remains uncertain.

Patients and Methods: The gene and protein expression level of *HSPA4* in CMM was investigated with public databases. Cell Counting Kit-8 (CCK8) assay was performed to assess the effect of *HSPA4* on the proliferation of melanoma cells. Then, the diagnostic and prognostic value of *HSPA4* in CMM were analyzed. Gene variations and methylation levels, and the correlation between *HSPA4* expression and immune cell infiltration were evaluated, followed by the construction of *HSPA4* related protein-protein interaction networks and functional enrichment analysis.

Results: The mRNA and protein expression level of *HSPA4* was significantly higher in CMM. Knocking down *HSPA4* in A-375 cell line could inhibit tumor cell growth. The receiver operating characteristic (ROC) curve analysis confirmed the diagnostic value of *HSPA4*. Survival analysis showed that high expression of *HSPA4* was associated with poor prognosis. *HSPA4* gene alterations were observed in 3% of CMM patients. Five CpG sites are associated with the prognosis of CMM. *HSPA4* is negatively correlated with most immune cells in CMM. The protein interaction network shows that *HSPA4* is closely related to proteins such as DnaJ heat shock protein family (Hsp40) member B1 (*DNAJB1*) and DnaJ heat shock protein family (Hsp40) member B6 (*DNAJB6*), and the expression of *DNAJB1* is positively correlated with *HSPA4*. Functional enrichment analysis indicated that *HSPA4* may be associated with immune suppression and immune escape within the tumor microenvironment of CMM.

Conclusion: *HSPA4* may participate in the regulation of tumor development and microenvironment, which may be a potential diagnostic and prognostic marker of CMM.

Keywords: heat shock protein, melanoma, *HSPA4*, TCGA, bioinformatics

Introduction

Cutaneous malignant melanoma (CMM) is the leading cause of mortality among human skin-related tumors, accounting for approximately 80%, making it the most dangerous skin tumor.[1] Depending on the clinical stage at the time of diagnosis, the 5-year survival rate for patients varies from 15% to 60%.[2,3] Survival rates are closely associated with tumor characteristics such as Clark staging, ulceration, and lymph node involvement.[4–6] Current treatment methods include surgical resection, interferon therapy, targeted therapy, and immunotherapy.[7] In recent years, the prognosis for advanced CMM patients has improved to some extent with the use of targeted drugs such as nivolumab and vemurafenib.[7] However, the high cost of these medications imposes a heavy burden on patients. Therefore, the application of more effective biomarkers for early diagnosis of CMM remains important.

Recently, more and more genes and proteins have been identified to play important roles in the occurrence and development of CMM.[8] Mutations in the B-Raf proto-oncogene (*BRAF*) gene are common in CMM, which lead to an aberrant activation of the MAPK signaling pathway, a central step in the development of CMM.[9,10] Similarly, alterations in the

NRAS gene can also activate the MAPK pathway.[11] Targeted therapies, such as vemurafenib, which inhibits *BRAF* V600E mutation, have been observed with a significantly reduced risk of death in advanced CMM patients.[12] Immune checkpoint inhibitors are also the commonly used anti-melanoma medications. Pembrolizumab is a *PD-1* inhibitory IgG4 monoclonal antibody, and nivolumab is the second *PD-1* inhibitory monoclonal antibody used in advanced melanomas.[13,14] Understanding these genetic and proteomic factors not only helps in elucidating the pathogenesis of CMM but also provides potential biomarkers for early diagnosis and novel therapeutic targets.

Heat shock proteins (HSPs) are a group of relatively conserved proteins that are typically expressed at very low levels under normal conditions.[15] They were initially discovered to be produced by cells in response to heat, chemical, or physical stress as chaperones to protect proteins from damage.[16,17] HSPs also contribute to the repair of misfolded proteins.[16] It has been shown that HSPs can interact with protein kinases and regulate their activity.[18] The HSP family is divided into five major classes based on molecular weight: small HSPs, HSP40, HSP70, HSP90, and HSP110.[19] A significant number of genes encode for these HSPs, with each class comprising multiple gene members. Glucose-regulated protein 78 (*GRP78*) is a member of the HSP70 family, and its overexpression is implicated in multidrug resistance of cancer cells.[20] Increased *Hsp10* is independently implicated in immune modulation.[21] Heat Shock Protein A4 (*HSPA4*), with a molecular weight of around 70kDa, belongs to the HSP70 family.[15]

Increasing evidence suggests that *HSPA4* plays an important role in the development and progression of various types of cancers. For instance, a study conducted by Wang et al showed that *HSPA4* may play an important role in the metastasis of nasopharyngeal carcinoma.[22] Jo et al reported frameshift mutations in the *HSPA4* gene in gastric and colorectal cancer.[23] Ma et al found a strong correlation between *HSPA4* and the prognosis of hepatocellular carcinoma patients.[24] Gu et al discovered that tumor-induced B cell selectively promotes lymph node metastasis in breast cancer through targeted IgG against *HSPA4*.[25] Although there is substantial evidence implicating the involvement of *HSPA4* in various types of cancer, its role in the pathogenesis of CMM remains unknown.

With bioinformatics analysis of data from public databases, this study aim to explore the role of *HSPA4* in the development, diagnosis, and prognosis of CMM, investigate the relationship between *HSPA4* and the pathogenesis as well as clinical characteristics of CMM, and identify potential biomarkers and therapeutic targets that may be beneficial for patients with CMM.

Materials and Methods
HSPA4 Expression Level in CMM
CMM data was extracted from the TCGA (https://portal.gdc.cancer.gov/) and GTEx (https://gtexportal.org/home/) databases, including mRNA expression profiles and clinical information from 469 cases of melanoma tissues and 1 case of adjacent normal tissues in TCGA-SKCM, as well as 812 cases of normal skin tissues in the GTEx database. Additionally, mRNA expression data from the GSE3189 dataset were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo/) to compare the expression differences of *HSPA4* between CMM and nevus cells. Then, a comprehensive evaluation of antibody staining cell proportion and staining intensity in the HPA database (https://www.proteinatlas.org/) was performed to analyze the protein expression differences of *HSPA4* between CMM and normal skin melanocytes. To investigate the diagnostic and prognostic value of *HSPA4* in CMM, a survival analysis was conducted. The Kaplan-Meier method and Log rank test were performed using the survival and survminer packages. The diagnostic ability and prognostic significance of *HSPA4* in CMM were analyzed using the pROC and survival packages. The GSE65094 dataset was utilized for validating the prognostic analysis. Proportional hazards hypothesis testing and Cox regression analysis were performed using the R package "survival", and a nomogram model was subsequently constructed and visualized using the "rms" package. A statistical significance level of *P*<0.05 was considered significant.

Cell Culture and Treatment
A-375 melanoma cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM medium (PromoCell) supplemented with 10% fetal bovine serum (FBS, Sijiqing) at 37°C in a humidified incubator with 5% CO_2. Cell transfection was performed using the Lipo8000™ transfection reagent

(Beyotime) according to the manufacturer's protocol. Briefly, cells were seeded in six-well plates (Corning) and grown to a cell density of 30% and then transfected and cultured at 37°C for a further 48h, followed by harvesting for quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and other experiment.

RNA Extraction and qRT-PCR

Total RNA from approximately 1×10^6 cells were isolated using TRIzol reagent (Pufer, Shanghai, China) according to the manufacturer's protocol. The primer sequences used for qRT-PCR were as follows: *HSPA4* forward-5'GCAGACACCA GCAGAAAATAAGG-3', Reverse: 5'-TCGATTGGCAGGTCCACAGT-3'; for *ACTB*: Forward: 5'-CATGTACGTTG CTATCCAGGC-3', Reverse: 5'-CTCCTTAATGTCACGCACGAT-3′ (Biomed, China). QRT-PCR parameters were: 95°C 3 min; (95°C 10s, 55°C 30s, and 72°C 32s) × 40 amplification cycles. Relative expression levels were normalized to internal controls and calculated according to the $2^{-\Delta\Delta CT}$ method.

CCK8 Assay

In each experiment, the logarithmic growth phase of cells was trypsinized and resuspended overnight in complete culture medium. At the scheduled time, cell proliferation was measured using the Cell Counting Kit-8 (CCK-8) reagent (APEXBIO, USA), following the manufacturer's protocol. The optical density was read at 450 nm using a microplate reader (Molecular Devices).

Genetic Alteration of HSPA4 in Patients with CMM

To investigate the genetic variations of *HSPA4* in CMM, an analysis of *HSPA4* gene alterations was performed in 622 patients with CMM using the cBioPortal database (https://www.cbioportal.org/).[26] These patients were derived from three datasets, including Melanoma (MSKCC, NEJM 2014), Metastatic melanoma (DFCI, Science 2015), and Skin cutaneous melanoma (TCGA, PanCancer Atlas).

DNA Methylation of HSPA4 in CMM

The MethSurv database (https://biit.cs.ut.ee/methsurv/) was utilized to analyze the DNA methylation levels of the *HSPA4* gene in CMM in TCGA database.[27] Additionally, the prognostic value of *HSPA4* CpG methylation was assessed.

Correlation Analysis of HSPA4 with Immune Cell Infiltration

We used the GSVA package to analyze and visualize the relationship between *HSPA4* expression and immune infiltration in melanoma using a lollipop plot. Patients with CMM were divided into two groups according to the median expression level of *HSPA4: HSPA4* low- and high-expression groups. The stromal score, immune score, and ESTIMATE score between the two groups were evaluated using estimate algorithm. Stromal score is an indicator that assesses the content and composition of the extracellular matrix in tumor tissue.[28] A high stromal score may indicate that immune cells have difficulty entering the tumor, resulting in a potentially lower response to immunotherapy. Immune score is an indicator that evaluates the level of immune cell infiltration in tumor tissue.[29] A higher immune score represents a greater number of immune cells entering the tumor tissue, suggesting a better immune response and prognosis. ESTIMATE score takes into account both stromal score and immune score, providing a comprehensive indicator for evaluating immune infiltration.[30] The estimate of the stromal score, immune score, and estimate score between the two groups can indicate the relationship between *HSPA4* gene expression and immune infiltration in melanoma and further explore its potential role in tumor treatment. Statistical significance was considered with a *P*-value <0.05.

Functional Enrichment Analysis

We retrieved the protein-protein interaction (PPI) network related to *HSPA4* using the STRING database, considering a protein interaction score greater than 0.9 as statistically significant.[31] Functional enrichment analysis of *HSPA4* and its associated proteins was then performed using the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Gene Set Enrichment Analysis (GSEA) was utilized to predict the relevant pathways involving *HSPA4* in CMM. The annotated gene set (c2.cp.v7.2.symbols.gmt) was selected as the reference dataset, and each

analysis was run with 1000 permutations of the genome. A significance threshold of $P < 0.05$ was considered for enrichment analysis.

Statistical Analyses

The statistical analysis was performed using the R package (v.3.6.3, R Foundation for Statistical Computing, Vienna, Austria). The differences between two groups were compared using the non-parametric Wilcoxon test. Correlation analysis was conducted using either the Pearson or Spearman correlation test. Survival analysis was represented using Kaplan-Meier curves, and the differences in survival rates between groups were assessed using the Log rank test. A significance level of $\alpha = 0.05$ was used for all analyses, and $P < 0.05$ was considered statistically significant.

Results
Higher HSPA4 Expression Levels in CMM Than in Normal Skin

To explore the possible role of *HSPA4* in tumors, the mRNA expression level of *HSPA4* in CMM tissues and normal skin samples were analyzed using TCGA and GTEx data. The results shown that *HSPA4* expression was significantly up-regulated in CMM tissues compared to normal skin samples ($P < 0.001$) (Figure 1A). To further validate the differential expression of *HSPA4* in CMM, we retrieved the GSE3189 dataset from the GEO database. The results in GSE3189 also confirmed higher expression of *HSPA4* mRNA in CMM tissues compared to normal nevi tissues (Figure 1B). Furthermore, our search in the HPA database revealed that in the HPA028675 antibody panel, 3 out of 12 CMM samples showed strong positive staining (Figure 1C), 2 showed moderate positive staining, 5 showed weak positive staining, and 2 CMM samples were negative, while both melanocytes in normal skin tissue were negative (Figure 1D).
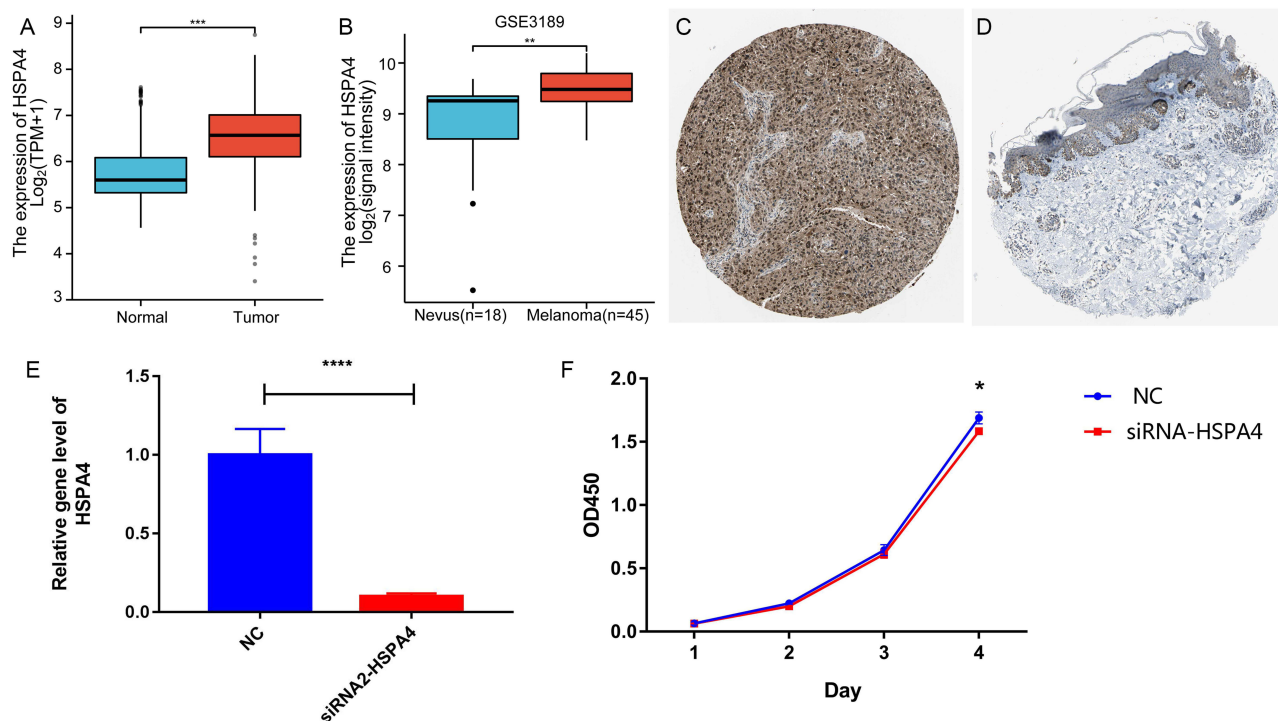

Figure 1 The expression of *HSPA4* in tumors and normal tissues. (**A**) The mRNA expression level of *HSPA4* in the TCGA and GTEx databases. (**B**) The mRNA expression level of *HSPA4* in the GSE3189 dataset. (**C**) According to the HPA database, HSPA4 exhibits elevated expression in CMM. (**D**) According to the HPA database, HSPA4 is not expressed in normal skin melanocytes. (**E**) QRT-PCR results showed that *HSPA4* gene expression was knocked down by siRNA in A-375 cells. (**F**) Knockdown of *HSPA4* significantly inhibited cell proliferation in A-375 cells. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

HSPA4 Knockdown Inhibited the Malignant Behaviors

The results of qRT-PCR revealed that the specific siRNA obviously decreased *HSPA4* expression level (Figure 1E). After siRNA interference of *HSPA4* expression in A-375 cells, the cell proliferation was evaluated at 1, 2, 3, and 4 days. It was observed that the *HSPA4* knockdown group had slower tumor cell proliferation compared to the control group. Specifically, on the 4th day, there was a significant decrease in cell proliferation with statistical significance ($P < 0.05$) (Figure 1F).

The Diagnosis and Prognostic Value of HSPA4 Expression in CMM Patients

According to the Kaplan-Meier survival curves, the high-expression group of *HSPA4* in CMM showed worse overall survival (OS, $P = 0.006$, Figure 2A) and disease-specific survival (DSS, $P = 0.035$, Figure 2B) compared to the low-expression group, indicating a significant association between high expression of *HSPA4* and poor prognosis in CMM. These findings were also confirmed in the validation dataset GSE65094, where CMM cases with high expression of *HSPA4* exhibited worse OS ($P = 0.004$, Figure 2C) and DSS ($P = 0.007$, Figure 2D) compared to the low-expression group, with significant differences (grouping based on minimizing p-values). The diagnostic value of *HSPA4* in CMM was assess with receiver operating characteristic (ROC) curves and shows that *HSPA4* has good accuracy and sensitivity in distinguishing tumor tissue from normal tissue in CMM (AUC = 0.812, Figure 2E). We also utilized a forest plot (Figure 2F) to demonstrate how *HSPA4* levels predict the 1-, 3-, and 5-year survival rates in CMM patients.

Genetic Alteration of HSPA4 in Patients with CMM

To investigate the genetic alterations of *HSPA4* in melanoma, we analyzed *HSPA4* gene alterations in 622 CMM patients. The results showed that *HSPA4* gene alterations were observed in 3% of the patients with CMM (Figure 3A). The
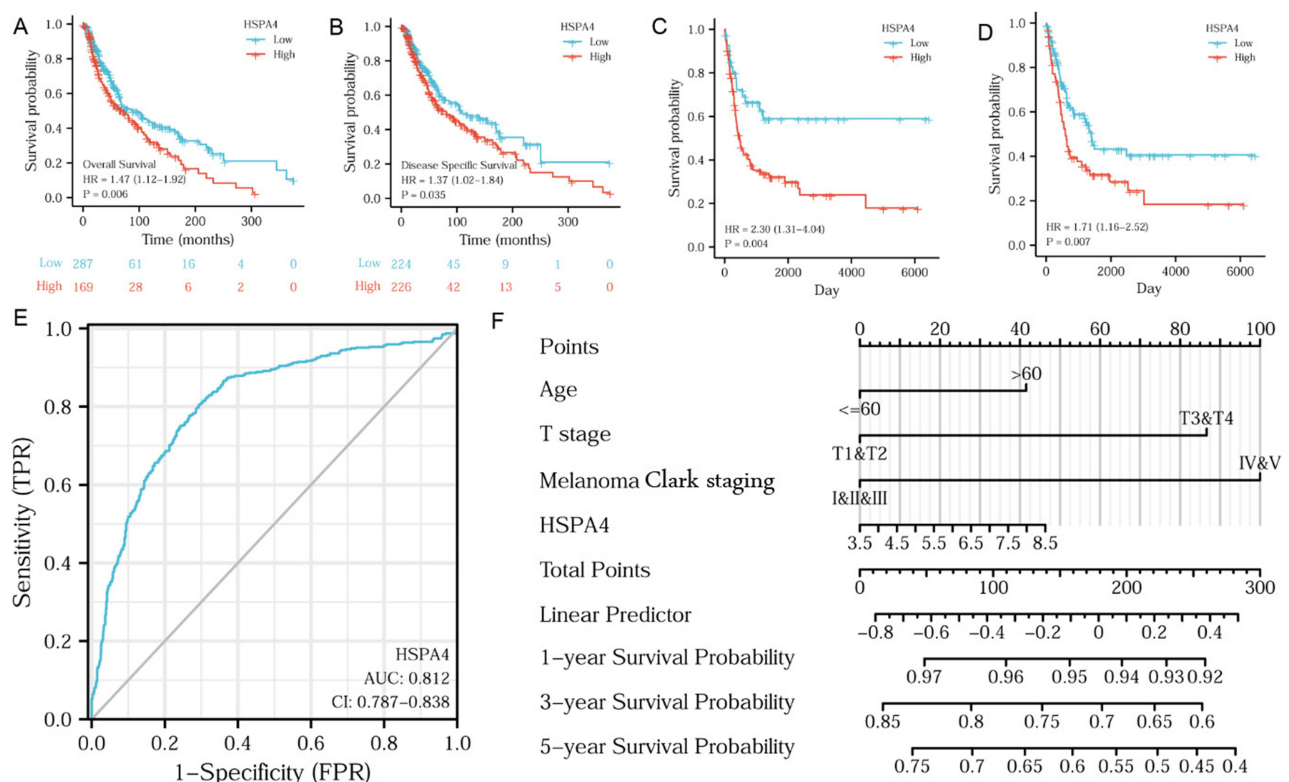

Figure 2 The prognostic and diagnostic value of *HSPA4* in CMM. (**A** and **B**) The TCGA database exhibits Kaplan-Meier plots for overall survival (**A**) and disease-specific survival (**B**) comparing high-expression and low-expression groups of *HSPA4*. (**C** and **D**) The GEO database exhibits Kaplan-Meier plots for overall survival (**C**) and disease-specific survival (**D**) comparing high-expression and low-expression groups of *HSPA4*. (**E**) The ROC curve demonstrates the ability of *HSPA4* to differentiate CMM tumor tissues from normal tissues. (**F**) Nomogram shows the relationship between *HSPA4* levels and the predictive survival rates of CMM patients at 1-, 3-, and 5-years with relevant clinical factors.
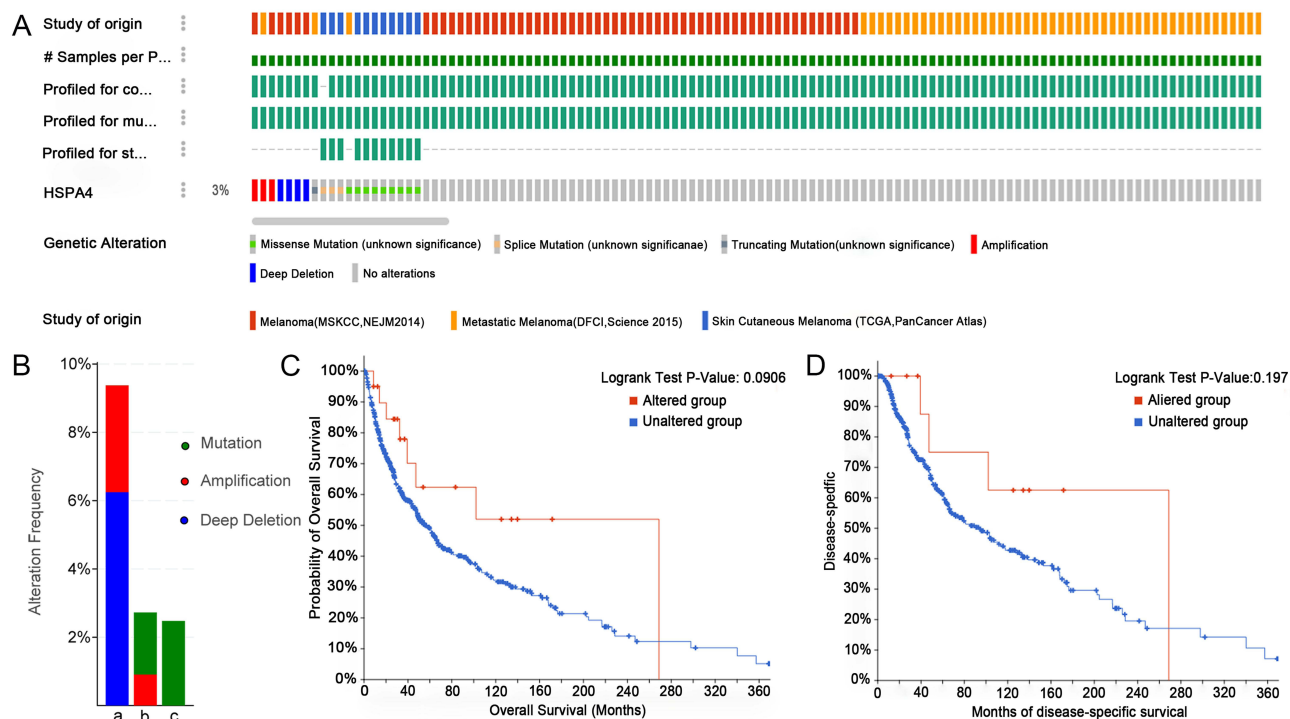
Figure 3 Gene mutations of *HSPA4* in CMM. (**A**) The overall condition of *HSPA4* gene mutations. (**B**) The mutation status of *HSPA4* in CMM in the TCGA database. a. Melanoma (MSKCC, NEJM 2014), 9.38%; b. Metastatic Melanoma (DFCI, Science 2015), 2.73%; c. Skin Cutaneous Melanoma (TCGA, PanCancer Altas) 2.48%. (**C** and **D**) Comparison of overall mortality (**C**) and disease-specific survival (**D**) between the group with *HSPA4* gene alterations and the group without alterations.

alteration frequencies ranged from 9.38% (6/64) to 2.48% (11/444) (Figure 3B). Kaplan-Meier analysis and Log rank test indicated no significant differences in OS ($P = 0.0906$, Figure 3C) and DSS ($P = 0.197$, Figure 3D) between the group with *HSPA4* gene alterations and the group without alterations.

HSPA4 Methylation Analysis in Patients with CMM

To investigate the changes in methylation levels of the *HSPA4* gene in CMM patients, we utilized the MethSurv database to explore the association between DNA methylation levels at individual CpG sites of the *HSPA4* gene and their prognostic value. The MethSurv database results identified 13 methylated CpG sites of *HSPA4* in CMM patients, among which cg05996250, cg07474441, cg10645426, and cg11250576 displayed higher methylation levels (Figure 4). Further analysis showed that 5 methylated sites (cg02067788, cg12202022, cg23946014, cg13778073, and cg10645426) were associated with overall mortality rates ($P < 0.05$) (Table 1). Kaplan-Meier analysis for 5 methylated sites suggested that higher methylation levels at the cg02067788, cg12202022, cg23946014, and cg10645426 CpG sites, as well as lower methylation levels at the cg13778073 site of the *HSPA4* gene, in CMM patients are associated with better OS compared to the group with lower methylation levels (Figure 5).

Relationship Between HSPA4 Expression and Immune Cell Infiltration

To determine the correlation between *HSPA4* and immune cell infiltration in CMM, we used a lollipop plot (Figure 6A) to illustrate the association between *HSPA4* expression and various immune infiltrates. The results showed that in CMM tissues, *HSPA4* expression was positively correlated with T helper cells, Tcm (central memory T cells), and Th2 cells, and negatively correlated with CD56bright natural killer cells, pDCs (plasmacytoid dendritic cells), TReg cells (regulatory T cells), CD56dim natural killer cells, DCs (dendritic cells), cytotoxic cells, Eosinophils, Th17 cells, iDCs (mature dendritic cells), mast cells, NK cells, TFH cells (T follicular helper cells), aDCs (activated dendritic cells), T cells, B cells, Th1 cells, Macrophages, and Neutrophils ($P < 0.05$). The results showed that compared to the *HSPA4* low-
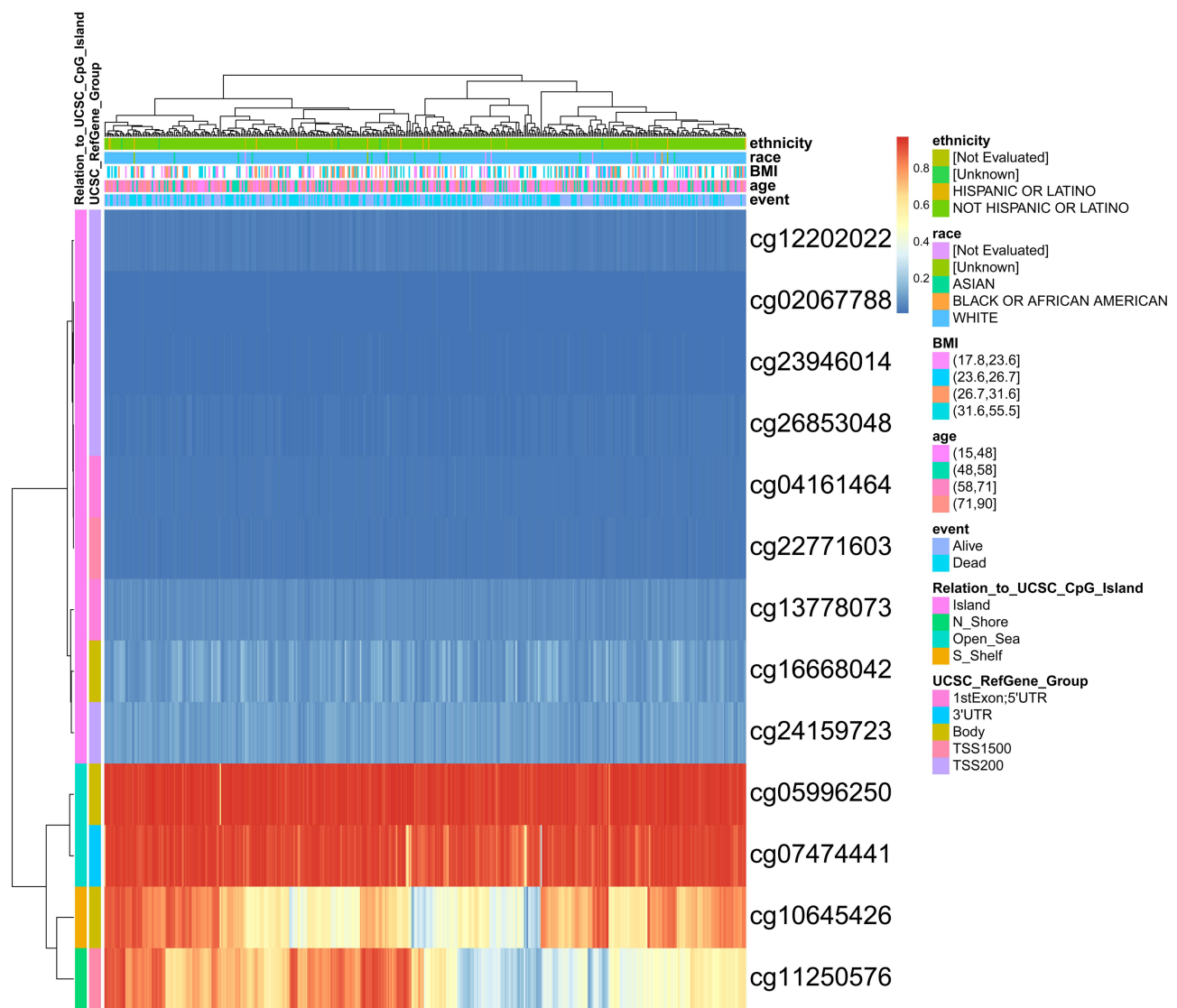
Figure 4 The methylation level of *HSPA4* in CMM.

expression group, the *HSPA4* high-expression group exhibited significantly lower stromal score, immune score, and estimate score (*P* <0.001, Figure 6B).

GO and KEGG Pathway Analyses

By searching the STRING database, we constructed a PPI network of HSPA4 and its related proteins. We selected the top 10 proteins that are most associated with HSPA4, including STUB1, DNAJB1, DNAJB6, HSP90AA1, SNCA, STIP1, BAG1, BAG2, BAG3, and ST13 (Figure 7A). The table in Figure 7B shows the annotation information of the interacted proteins with HSPA4. GO enrichment analysis consisted of three main parts: biological processes, cellular components, and molecular functions (Table 2). The biological processes include regulating cellular response to heat stimulus, response to unfolded protein, negative regulation of DNA metabolism and cell cycle, regulating apoptosis signaling pathways, regulating innate immune response, antigen processing and presentation, regulating IL-1, and interferon-alpha production. The cellular components include forming chaperone complexes, DNA repair complexes, and mitochondria complexes. The molecular functions include heat shock protein binding, unfolded protein binding, and involvement in the Hedgehog signaling pathway. KEGG pathway analysis revealed that *HSPA4* and its associated molecules are enriched in heat stress-related pathways (Figure 7C). GSEA enrichment analysis revealed that *HSPA4* is involved in the negative

Table I The Association Between Methylated CpG Sites of HSPA4 and the Prognosis in CMM

CpG	HR	P-Value
TSS200-Island-cg02067788	**0.639**	**0.0011**
TSS200-Island-cg12202022	**0.734**	**0.036**
TSS200-Island-cg23946014	**0.718**	**0.014**
TSS200-Island-cg24159723	1.229	0.17
TSS200-Island-cg26853048	0.836	0.19
1stExon;5′URT-Island-cg04161464	1.243	0.11
1stExon;5′URT-Island-cg13778073	**1.358**	**0.024**
Body-Island-cg16668042	1.138	0.37
TSS1500-Island-cg22771603	1.242	0.16
Body-Open_Sea-cg05996250	1.307	0.072
3′URT-Open_Sea-cg07474441	1.207	0.21
Body-S_shelf-cg10645426	**0.682**	**0.0049**
TSS1500-N_Shore-cg11250576	1.133	0.36

Note: Methylation CpG sites that were found to be significantly related to prognosis ($P < 0.05$) were indicated in bold font.

regulation of pathways related to IL-2, IL4-2, IL12-2, IL17, IL18, IL23, IL27, CD8-TCR, T cell receptor, Wnt, JAK-STAT, PD-1, and PD-1 blockade-related cancer immunotherapy (Table 3). Additionally, the expression of *HSPA4* was found to be positively correlated with the expression of *DNAJB1* (Figure 7D). We also confirmed that high expression of *DNAJB1* is associated with poor prognosis in CMM ($P = 0.01$) (Figure 7E).

Discussion

Melanoma is a highly malignant skin tumor with a mortality rate approximately 8 times higher than other types of skin tumors.[32] Early detection and aggressive treatment of localized lesions can lead to a 5-year survival rate of over 90%.[32] Therefore, early diagnosis and timely intervention are crucial for the management of this disease. The search for biomarkers that can enable early diagnosis and prognosis assessment holds significant importance for melanoma.

The HSP70 protein family plays a crucial role in protein homeostasis by regulating protein folding reactions within cells. On one hand, they prevent nonspecific protein aggregation and assist in the proper folding of proteins. On the other hand, they possess anti-apoptotic functions and play a role in tumor cell proliferation, infiltration, metastasis, and survival.[33] HSPA4 is a member of the HSP70 protein family and expressed in various tissues, typically in response to stimuli including carcinogens.[34] It has been reported that *HSPA4* also promotes cell proliferation and inhibits apoptosis, and down-regulation of *HSPA4* results in a significant decrease in tumor cell invasion and metastasis.[35] Analysis of data
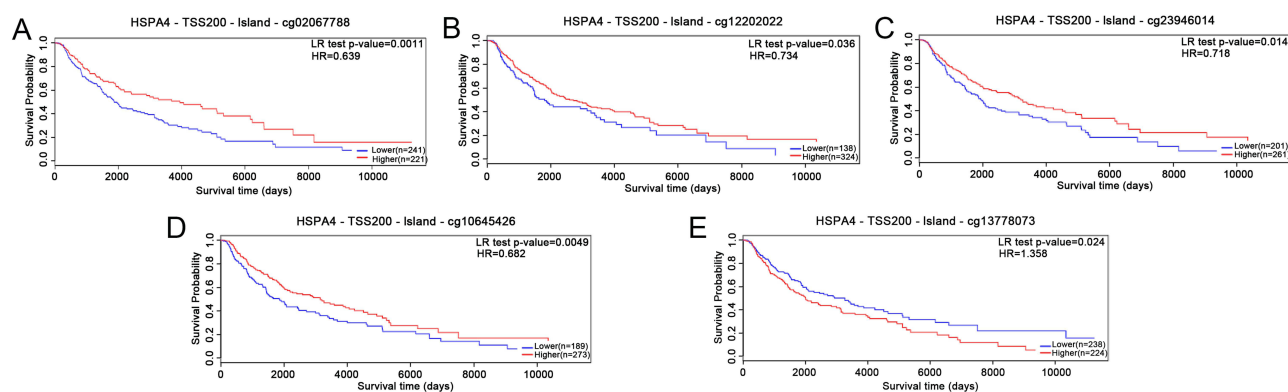

Figure 5 Kaplan-Meier analysis for 5 methylated sites that were associated with overall mortality rates. (**A**) cg02067788, (**B**) cg12202022, (**C**) cg23946014, (**D**) cg10645426, (**E**) cg13778073.
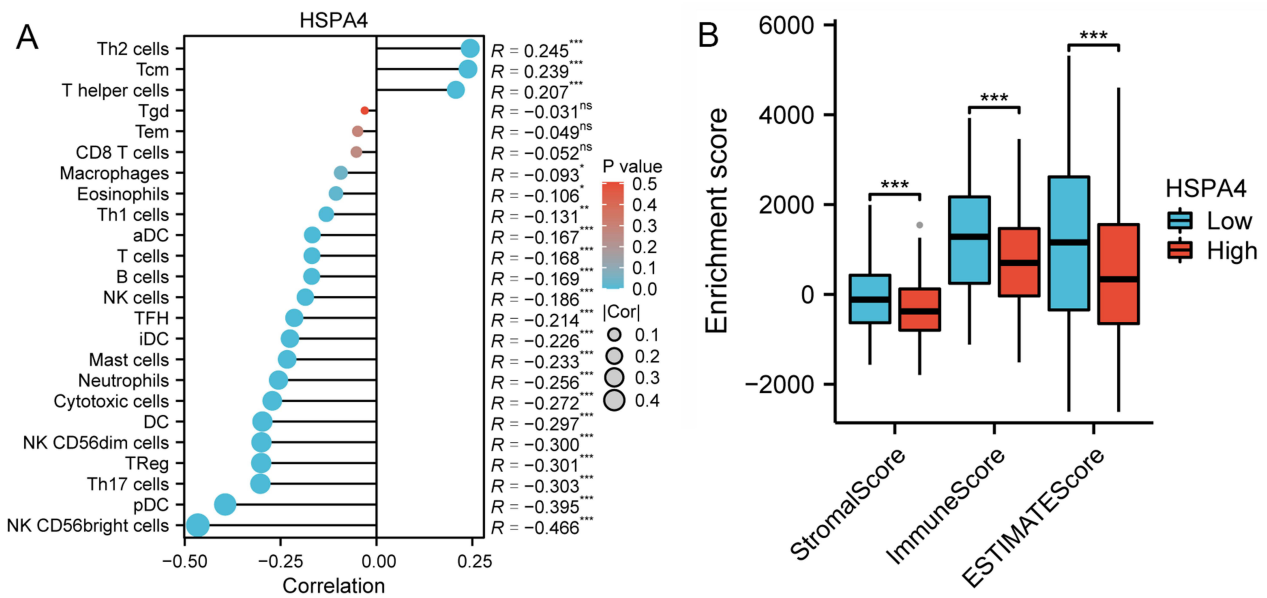
Figure 6 The relationship between *HSPA4* expression and immune cell infiltration in CMM. (**A**) The lollipop plot shows the correlation between the abundance of 24 immune cells and *HSPA4* expression. (**B**) The stromal score, immune score, and ESTIMATE score between the *HSPA4* low- and high-expression groups. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns indicates not significant.
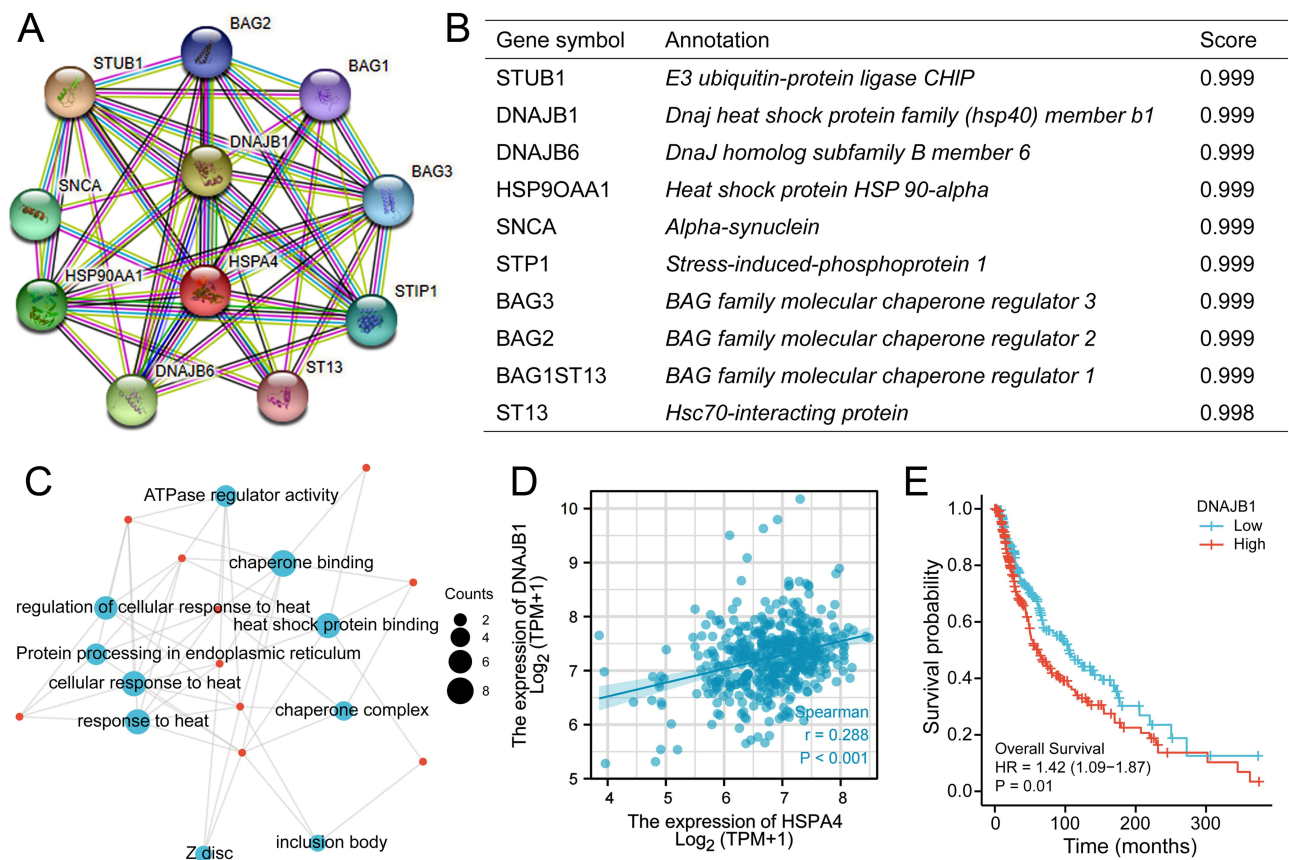

Figure 7 Prediction of molecular functions and signaling pathways for *HSPA4* in melanoma. (**A**) Proteins interacting with *HSPA4* in melanoma. (**B**) Annotation of genes interacting with *HSPA4*. (**C**) Enrichment analysis of GO and KEGG pathways. (**D**) The relationship between *HSPA4* expression and *DNAJB1* expression. (**E**) Survival analysis of different expression groups of *DNAJB1* in CMM.

Table 2 Enrichment Analysis of HSPA4 and Related Genes in Melanoma Using GO and KEGG Database

Categories	ID	Description	P-Value
BP	GO:0006457	Protein folding	1.59818E-08
BP	GO:0009408	Response to heat	3.60221E-06
BP	GO:0032481	Positive regulation of type i interferon production	0.0001848
BP	GO:0097193	Intrinsic apoptotic signaling pathway	0.000581081
BP	GO:0045088	Regulation of innate immune response	0.000692487
BP	GO:0019886	Antigen processing and presentation of exogenous peptide antigen via mhc class ii	0.000808079
BP	GO:0045089	Positive regulation of innate immune response	0.002495396
BP	GO:0019882	Antigen processing and presentation	0.002761461
BP	GO:2001242	Regulation of intrinsic apoptotic signaling pathway	0.004153597
BP	GO:0030111	Regulation of wnt signaling pathway	0.004360815
BP	GO:0032727	Positive regulation of interferon-alpha production	0.004501988
CC	GO:0101031	Chaperone complex	1.54883E-05
CC	GO:1990391	DNA repair complex	3.55528E-05
CC	GO:0000428	DNA-directed RNA polymerase complex	3.56383E-05
CC	GO:0031965	Nuclear membrane	4.29056E-05
CC	GO:0098798	Mitochondrial protein complex	0.000170402
CC	GO:0042470	Melanosome	0.00107805
MF	GO:0051082	Unfolded protein binding	4.8093E-09
MF	GO:0044389	Ubiquitin-like protein ligase binding	2.36345E-07
MF	GO:0003697	Single-stranded DNA binding	3.91785E-07
MF	GO:0003725	Double-stranded RNA binding	4.39375E-07
MF	GO:0031072	Heat shock protein binding	0.0037425
MF	GO:0003684	Damaged DNA binding	0.004481083
KEGG	Hsa04141	Protein processing in endoplasmic reticulum	9.55148E-06
KEGG	Hsa03018	RNA degradation	0.000471396
KEGG	Hsa04340	Hedgehog signaling pathway	0.001812155

from TCGA and GEO databases revealed a significant up-regulation expression of *HSPA4* in CMM tumor tissues compared to normal skin and benign nevus cells. Additionally, using data from the HPA database, we confirmed that HSPA4 protein expression in CMM tumors is significantly higher than in normal melanocytes. Knocking down *HSPA4* expression using siRNA in A-375 melanoma cell lines resulted in a significant reduction in cell proliferation. Furthermore, CMM patients with high *HSPA4* expression showed shorter OS and DSS compared to those with low *HSPA4* expression, demonstrating a significant association between high *HSPA4* expression and adverse prognosis in CMM. We also found that *HSPA4* can effectively distinguish tumor cells from normal melanocytes, demonstrating its potential diagnostic value and predictive ability for 1-, 3-, and 5-year survival. These findings suggest that *HSPA4* has diagnostic and prognostic value in CMM.

It is well-known that genetic variations play an important role in the development and progression of tumors, such as the significant role of *BRAF* gene mutations in CMM.[32] Our study revealed an overall alteration rate of *HSPA4* in approximately 3% of CMM cases. However, no clear relationship was observed between *HSPA4* gene mutations and OS. DNA methylation, as a common epigenetic mechanism in tumors, also plays a significant role in the regulation of gene expression.[36] We investigated the correlation between *HSPA4* and DNA methylation levels, and found that several CpG sites, including cg05996250, cg07474441, cg10645426, and cg11250576, exhibited high levels of methylation. Additionally, methylation at five specific sites was statistically significantly correlated with OS, indicating potential implications.

It is true that the treatment options for advanced CMM are currently limited and often yield unsatisfactory results.[32] Several studies have highlighted the crucial role of the immune system in the occurrence, development, metastasis, and recurrence of CMM within the tumor microenvironment.[37,38] Our research indicates that *HSPA4* is negatively correlated with most immune cells, including B cells, NK cells, and cytotoxic T cells, in CMM. However, it shows a positive correlation with Th2 cells, Tcm, and helper T cells. Our findings suggest that individuals in the *HSPA4* high-expression

Table 3 GSEA Enrichment Analysis of HSPA4-Associated Signaling Pathways

ID	NES	P_Adjust
WP_IL18_SIGNALING_PATHWAY	−1.667629179	0.017857278
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	−2.032839378	0.017857278
REACTOME_IMMUNOREGULATORY_INTERACTIONS_BETWEEN_A_LYMPHOID_AND_A_NON_LYMPHOID_CELL	−2.713911314	0.017857278
REACTOME_SIGNALING_BY_THE_B_CELL_RECEPTOR_BCR_	−2.19331462	0.017857278
REACTOME_INTERLEUKIN_4_AND_INTERLEUKIN_13_SIGNALING	−2.028308782	0.017857278
REACTOME_ANTIGEN_ACTIVATES_B_CELL_RECEPTOR_BCR_LEADING_TO_GENERATION_OF_SECOND_MESSENGERS	−2.661379064	0.017857278
PID_IL12_2PATHWAY	−2.066439476	0.017857278
PID_IL4_2PATHWAY	−1.93058561	0.017857278
REACTOME_INTERLEUKIN_10_SIGNALING	−2.021084832	0.017857278
PID_IL23_PATHWAY	−2.079733409	0.017857278
PID_IL27_PATHWAY	−1.962926447	0.017857278
REACTOME_PD_1_SIGNALING	−2.305484808	0.017857278
REACTOME_WNT_LIGAND_BIOGENESIS_AND_TRAFFICKING	−1.926133023	0.017857278
BIOCARTA_IL17_PATHWAY	−1.965881146	0.017857278
REACTOME_APOPTOTIC_CLEAVAGE_OF_CELL_ADHESION_PROTEINS	−2.03767306	0.017857278
REACTOME_INTERLEUKIN_2_SIGNALING	−1.800181858	0.017857278
KEGG_JAK_STAT_SIGNALING_PATHWAY	−1.610137016	0.027737397
KEGG_T_CELL_RECEPTOR_SIGNALING_PATHWAY	−1.645836877	0.027737397
REACTOME_COSTIMULATION_BY_THE_CD28_FAMILY	−1.837861813	0.027737397
REACTOME_NEGATIVE_REGULATION_OF_TCF_DEPENDENT_SIGNALING_BY_WNT_LIGAND_ANTAGONISTS	−1.78057378	0.028358084
PID_IL12_STAT4_PATHWAY	−1.846835103	0.037816671
PID_WNT_SIGNALING_PATHWAY	−1.798059507	0.037858423
BIOCARTA_IL12_PATHWAY	−1.771289476	0.037916667
REACTOME_INTERLEUKIN_1_FAMILY_SIGNALING	−1.556698568	0.041816896
PID_CD8_TCR_DOWNSTREAM_PATHWAY	−1.68015364	0.042778041

group had significantly lower stromal score, immune score, and estimate score compared to those in the *HSPA4* low-expression group. These results indicate a potential correlation between high expression of *HSPA4* and decreased stromal and immune infiltration in the studied population. Further research is needed to better understand the underlying mechanisms and implications of these findings. GSEA results revealed that *HSPA4* and its related proteins are involved in the negative regulation of signaling pathways associated with IL-2, IL-4, IL-12, IL-17, IL-18, IL-23, IL-27, CD8-TCR, T-cell receptor, Wnt, JAK-STAT, PD-1, and PD-1 blockade-related cancer immunotherapy. These findings suggest that *HSPA4* may be associated with immune suppression and immune escape within the tumor microenvironment of CMM.

The tumor microenvironment, composed of immune cells and immune molecules, plays a crucial role in tumor cell proliferation, survival, and metastasis, making it an indispensable aspect of tumor formation.[39] Cellular immunity, in which natural killer (NK) cells and cytotoxic T cells are key players, plays an important role in tumor immunity.[40] NK cells have been shown to recognize "non-self" alterations in target cells and to identify tumor cells with low or no expression of major histocompatibility complex (MHC), enabling targeted killing of tumor cells in the bloodstream and tumor tissue, as well as eradication of melanoma lung metastasis.[41] Cytokines, such as IL-2, IL-12, and IL-18, regulate the cytotoxic and secretory functions of NK cells, and NK cells secrete various cytokines such as TNF-α, IL-12, and IFN-γ.[42] NK cells can inhibit tumor cell proliferation and differentiation, and promoting tumor cell apoptosis.[43] It has been suggested that NK cells can activate T lymphocytes by secreting IL-12, mediating acquired immunity.[44] IL-12 is primarily secreted by mature antigen-presenting cells such as dendritic cells, macrophages, and monocytes. As a cytokine that coordinates innate and adaptive immune functions, IL-12 can effectively stimulate the production of IFN-γ, promote the maturation and activation of T cells and NK cells, and induce the occurrence of self-immunity.[45]

IL-18 enhances the activity of NK and Cytotoxic T lymphocyte cells, promotes T cell proliferation, induces the production of Th1 and Th2 cytokines, and enhances Fas-mediated cytotoxicity by immune cells.[46] Zhang et al demonstrated in animal models that IL-18 has a strong inhibitory effect on solid tumor growth.[47] Nagai H et al showed that the expression of the IL-18 gene significantly inhibited the growth of B16 melanoma cells.[48] Wang et al found that different concentrations of IL-23 can promote the secretion of VEGF by mouse melanoma cells B16 in a concentration-dependent manner, suggesting that IL-23 may promote angiogenesis in mouse melanoma through the induction of VEGF secretion by B16 cells.[49] Anat et al discovered that IL-23 promotes brain metastasis of mouse melanoma by up-regulating the expression of MMP-2 and MMP-9 in melanoma cells.[50] Langowski et al demonstrated that IL-23 promotes the occurrence and growth of skin cancer by up-regulating MMP-9, promoting angiogenesis and reducing infiltration of CD8+ cells.[51]

There were some limitations in this study. First, this study was based on data from the public databases. Second, the effect of *HSPA4* on immune microenvironment requires further exploration. Third, the role of *HSPA4* in CMM requires further research with larger clinical samples and functional experiments.

Conclusion

This study primarily focused on investigation of the potential value of *HSPA4* in the occurrence, development, diagnosis, and treatment of CMM. These findings suggest that *HSPA4* may not only be involved in the pathogenesis and progression of CMM but also contribute to immune evasion in CMM. Therefore, we propose that *HSPA4* could be a potential biomarker for diagnosis and therapeutic target in CMM. Further in-depth research on the function and mechanisms of *HSPA4* in CMM will help elucidate the relationship between the two, leading to greater clinical significance.

Data Sharing Statement

All data reported in this paper will also be shared by the lead contact upon request.

Ethics Approval and Consent to Participate

The Ethical Committee of Seventh Medical Center of Chinese PLA General Hospital (S2024-066-01) approved this study and waived the requirement for written informed consent for all data are taken from public databases and are exempt from ethical review.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

Dr Xudong Wang reports a patent ZL 2022 1 1058264.3 licensed to Wang Xudong; Xu Jianhong; Wang Jun; Li Zhiyong; Xu Xiaofei. The author(s) declare no other conflict of interest.

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