

High Expression of Mediator Complex Subunit 8 Acts as a Prognostic Biomarker in Hepatocellular Carcinoma

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Research

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1 **High expression of Mediator complex subunit 8 acts as a prognostic**
2 **biomarker in hepatocellular carcinoma**

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Abstract

Background: Mediator complex subunit 8 (*MED8*) encodes a subunit of the mediator complex (*MED*), which is critical for transcription. *MED8* is highly expressed in some tumours and is associated with a poor prognosis. However, correlations between *MED8* and clinical features of hepatocellular carcinoma (HCC) have not been reported. **Results:** A univariate analysis showed that high *MED8* expression predicts poor overall survival (HR: 2.495; 95% confidence interval (CI) 1.740, 3.578; $P < 0.001$). A multivariate regression analysis showed that high *MED8* (HR: 3.032 (1.817, 5.060); $P < 0.001$) expression and M stage (HR=4.075 (1.179-14.091) for M1 vs. M0, $P=0.026$) are independent prognostic indicators of poor overall survival in patients with HCC. The areas under the curve (AUC) for receiver operating characteristic (ROC) curves were used to describe the prognostic value of *MED8* (AUC: 0.905 (0.849, 0.941)). Gene Set Enrichment Analysis (GSEA) and Immune infiltration Analysis were applied to reveal significant enrichment differences among TCGA data. A functional analysis showed that the cell cycle checkpoints, mitotic G2-G2-M phases, transcriptional regulation by TP53, and regulation of TP53 activity were significantly enriched in DEGs associated with high *MED8* expression. Th2 cells were positively correlated with *MED8* expression. **Conclusions:** *MED8* predicts poor prognosis in HCC, potentially via the regulation of the cell cycle regulation and Th2 cells.

Key words: Mediator complex subunit 8, Mediator, hepatocellular carcinoma, prognosis, diagnostic biomarker

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer worldwide and the fourth leading cause of cancer-related deaths[1]. The molecular mechanisms underlying HCC are not completely understood[2]. Owing to the lack of effective early diagnosis and targeted therapies,

the 5-year survival rate is only 11%[3]. Mainstream detection methods include computed tomography (CT), magnetic resonance imaging (MRI), and glycoprotein biomarkers (e.g., AFP). However, these methods have various limitations; CT and MRI are expensive and difficult to implement at a broadscale, while glycoprotein biomarkers are limited by a lack of specificity to tumour areas[2; 4]. In view of the importance of early tumour detection for improving the survival rate of patients with HCC, it is very important to explore new markers[5].

Mediator is an evolutionarily conserved polyprotein complex composed of 33 subunits in humans and is an indispensable regulator of transcription[6; 7]. It is divided into four distinct submodules: head, middle, tail, and kinase. Conformational changes by strong interactions with RNA polymerase II (POL II) affect transcription initiation and other important steps in protein expression[8]. Several studies have shown that the expression levels of certain subunits are altered in various human diseases, especially cancer[6; 7; 9; 10; 11]. Mediator complex subunit 8 (*MED8*) is mutated in colorectal cancer cell lines. Furthermore, the expression of *MED8* in renal clear cell carcinoma is associated with a short survival time and high TNM stage, and the expression of *MED8* in metastatic tumours is higher than that in primary tumours[12]. However, the association between *MED8* and HCC has not been evaluated.

In this study, the expression and prognostic value of *MED8* in HCC were analysed based on TCGA data. Furthermore, a multi-dimensional analysis was used to evaluate the *MED8* and functional networks associated with *MED8* in HCC and to explore its role in tumour immunity. We confirmed that *MED8* expression is elevated in liver HCC samples and is associated with worse overall survival. We further identified M stage (M1 vs. M0) and *MED8* as independent prognostic factors for overall survival. Our study provides a new diagnostic and prognostic marker for HCC.

Results

2.1. Demographic characteristics

Basic patient information is shown in Table 1. A total of 371 cases of HCC with clinical information were obtained from TCGA, among which 186 cases had lowMED8 expression and 185 cases had highMED8 expression using the median value as the threshold. The patients included 250 males and 121 females, with a mean age of 61 years. Chi-squared tests or Fisher's exact tests showed that MED8 levels were significantly correlated with the T stage ($P = 0.006$), pathologic stage ($P < 0.001$), race ($P = 0.017$), histologic grade ($P = 0.001$), and TP53 status ($P < 0.001$). The *t*-test or Wilcoxon rank sum tests showed that MED8 levels are significantly correlated with weight ($P = 0.002$), AFP (ng/ml) ($P < 0.001$), and BMI ($P = 0.012$).

2.2. Identification of differentially expressed Genes in HCC

To investigate whether MED8 plays an important role in the development of HCC, we used DESeq2 to analyse expression differences based on HTseq-counts between the groups with high and lowMED8 expression. Under the threshold values of $|\log_{2}FC| > 2$ and $P_{adj} < 0.05$, 582 DEGs were obtained, among which 46 Genes were down-regulated and 536 Genes were up-regulated. The volcano and heat maps are shown in Figure 1A and Figure 1B, respectively.

2.3. Functional enrichment analyses of MED8

Using the clusterProfiler package, DEGs associated with MED8 were evaluated by a MED8 Ontology (GO) analysis, identifying enrichment for 47 terms, including 14 terms in the biological process (BP) category, 16 in the cellular components (CC) category, and 17 in the molecular function (MF) category. The results are presented in Figure 2A–C.

2.4. MED8-related signalling pathways based on GSEA

A GSEA was performed to identify meaningful signalling pathways in the MED8 data sets for the comparison between the groups with high and low MED8 expression. In the MSigDB (c2.cp.v62.symbols) dataset, many significant differences (FDR < 0.05, normalized P < 0.05) were observed. The most significantly enriched pathways were obtained according to their NES values, including cell cycle checkpoints, mitotic G2-G2-M phases, transcriptional regulation by TP53, and regulation of TP53 activity, as shown in Figure 2D-G.

2.5. Correlations between MED8 expression and immune cell infiltration

Spearman correlation coefficients were used to analyse the relationships between MED8 expression and 24 kinds of infiltrating immune cells in the tumour microenvironment. MED8 expression was positively correlated with various cells, including Th2 cells, aDCs, T helper cells, and TFH, and negatively correlated with TH17 cells, pDCS, eosinophils, and neutrophils (Figure 3A, P < 0.05). What's remarkable is that MED8 showed a very strong positive correlation with the abundance of Th2 cells (Figure 3B, P < 0.001).

2.6. Role of MED8 in patient survival

The Wilcoxon signed rank test was used to compare MED8 data from TCGA between 50 HCC samples and paired adjacent samples as well as between 50 normal samples and 371 HCC samples. Levels of MED8 were significantly higher in HCC samples than in control samples (P < 0.001) (Figure 4A, Figure 4B). Analysis of MED8 protein patterns using IHC revealed higher expression in HCC compared to normal tissues (Figure 4C). As determined by the Kruskal–Wallis rank sum test, high levels of MED8 were significantly correlated with the pathologic stage, T stage, and histologic grade in liver HCC based on TCGA data (P < 0.05), as shown in Figure 5A–C. Kaplan–Meier plots were drawn using the survminer package to assess the prognostic value of MED8 for overall survival. A correlation between high MED8 expression and

a worse overall survival was detected (HR = 2.49 (1.74–3.58), $P < 0.001$), as shown in Figure 5D. Correlations between clinicopathological features and the classification of MED8 expression into high and low groups was analysed by logistic regression. Levels of MED8 were significantly correlated with the T stage (OR=1.98 (1.31-3.00), $P = 0.001$), pathologic stage (OR=2.04 (1.33-3.13), $P = 0.001$), histologic grade (OR=2.26 (1.46-3.15), $P < 0.001$), AFP (ng/ml) (OR=2.15 (1.22-3.83), $P = 0.009$), and TP53 status (OR=6.83 (4.01-12.11), $P < 0.001$), as summarized in Table 2. High expression of MED8 in the pathologic stage I vs. stage II and stage III and stage IV subgroup was associated with a worse overall survival (HR = 2.47 (1.69–3.62), $P < 0.001$) (Figure 5E). A forest plot was used to demonstrate the prognostic value of MED8 for overall survival in various subgroups of liver HCC based on TCGA data. MED8 was significantly related to the T stage subgroups T1&T2 (HR = 2.593 (1.608–4.180), $p < 0.001$) and T3 (HR = 2.159 (1.151–4.050), $p = 0.016$). The pathologic stage subgroups stage I (HR = 1.958 (1.065–3.601), $p = 0.031$), stage II (HR = 3.670 (1.367–9.852), $p = 0.010$), and stage III (HR = 2.587 (1.346–4.971), $p = 0.004$) were significant, as shown in Figure 5F.

2.7. Development of a prognostic model based on MED8 and clinicopathological factors

Univariate and multivariate Cox regression analyses were used to evaluate whether MED8 is an independent prognostic indicator for HCC. Variables with $P < 0.1$ in single-factor Cox regression were included in a univariate Cox regression, including T stage ($P < 0.001$), M stage ($P = 0.018$), pathologic stage ($P < 0.001$), and MED8 ($P < 0.001$). Further, multivariate Cox regression showed that M stage ($P = 0.026$) and MED8 ($P < 0.001$) were independent prognostic factors for overall survival ($P < 0.05$), as shown in Table 3. A nomogram was MED8 rated to evaluate the prognostic model, as shown in Figure 6A, including M stage and MED8 (c-index: 0.650 (0.624–0.677)). A calibration curve was used to verify the performance of models including M stage and

MED8, as shown in Figure 6B. We analysed the diagnostic efficacy of MED8 in HCC by an ROC analysis, and the area under the curve (AUC) was 0.905, suggesting that MED8 is a potential diagnostic marker, as shown in Figure 6C.

Discussion

In eukaryotes, mRNA transcription is dependent on RNA polymerase II. Although many factors are involved in the regulation of POLII activity, the majority of POLII transcripts require the expression of MED. The head and intermediate modules of MED[6] can directly interact with POLII and act as bridges between transcription factors and the mechanism underlying the binding of upstream regulatory elements[8]. The MED head module is mainly composed of proteins encoded by the SRB. MED8, MED18, and MED20 represent submodules of the mediation head domain. MED8 is considered a multi-domain protein, consisting of an n-terminal helical domain, a flexible ligand, and a c-terminal helix interacting with Med18 (25)[12; 13]. MED8 plays an important role in the transcription of all eukaryotic, and changes in its function and/or composition may have important functional consequences, contributing to various diseases, including cancer[8; 9; 14]. Few studies have evaluated the association between MED8 and cancer. High levels of MED8 in renal clear cell carcinoma have been detected by immunohistochemistry; however, the proliferation and motor capacity of renal clear cell carcinoma cells are significantly reduced after MED8 is silenced with siRNA[12]. Other studies have shown that MED8 is mutated in colon cancer. However, the association between MED8 expression and HCC development and prognosis has not been reported.

To characterize the role of MED8 in HCC, we analysed data for 371 patients with HCC with complete clinical information from TCGA. The expression level of MED8 was higher in HCC tissue samples than in normal tissue samples. Moreover, high MED8 expression was closely

correlated with pathological parameters, such as the T stage, pathologic stage, histologic grade, and TP53 status, suggesting that the high expression of MED8 is involved in the invasion and metastasis of HCC. Similarly, patients with HCC in the group with highly expressed MED8 had a worse overall survival rate than that of patients in the group with lowMED8 expression, suggesting that MED8 may serve as a new diagnostic and prognostic marker for HCC.

To further explore the mechanism by which MED8 contributes to the development of HCC, we used data from TCGA for a GSEA. Based on this analysis, genes related to highMED8 expression are enriched for cell cycle checkpoints, mitotic G2-G2-M phases, transcriptional regulation by TP53, and regulation of TP53 activity. As a tumour suppressor gene, p53 encoded by TP53 plays an important role in cell cycle regulation via three pathways: 1) P53 can bind to p21 and activate its transcription, thereby inhibiting CDK activity, preventing cells from entering the S phase from G1 phase, and making cells stop at G1 phase; 2) P53 can induce the synthesis of GADD45, thus inhibiting entry to the S phase. 3) Bax is induced and co-regulates apoptosis with bcl-2[15; 16; 17]. Our results indicated that genes correlated with highMED8 expression are significantly enriched for cell cycle regulation and TP53 pathways. Therefore, we hypothesized that MED8 (1) promotes the proliferation of HCC cells by regulating the transcription and expression of TP53 and (2) enables cells to pass through the S/M phase rapidly via cell cycle regulation, thus facilitating cell proliferation. The two effects explain the positive role of highMED8 expression in the development of HCC.

Another important finding of our study is the observed relationship between MED8 expression and immune cell infiltration. The expression of MED8 was positively correlated with Th2 cells, aDCs, T helper cells, TFH, etc. It should be noted that MED8 showed a very strong positive correlation with the abundance of Th2 cells. The tumour microenvironment in the state of

chronic inflammation makes infiltrated immune cells differentiate along the direction of tumour growth, invasion, and metastasis, accelerating tumour development and the process of immune escape[18]. Th2 cells mainly secrete IL-4, IL-5, IL-10, IL-13, and other cytokines to participate in humoral immunity. It is MED8 rally believed that Th2 cytokines can inhibit the differentiation of CD4+T cells into T1 cells, weaken the anti-tumour immune response, and thus promote tumour development. Some studies have confirmed that the levels of T1 cytokines (IL-2, IL-12, tumour necrosis factor- β , and interferon- γ) in HCC show a decreasing trend, while the levels of Th2 cytokines show an increasing trend, consistent with our analysis results[19]. Th2 cytokines are associated with more aggressive and metastatic HCC phenotypes, and our analysis showed that highMED8 expression is significantly correlated with the T stage, pathologic stage, and histologic grade of patients with HCC. This suggests the following corollary: MED8 may adjust Th2 cytokine levels, thereby promoting the metastasis and invasion of HCC. Other studies have shown that adjuvant T/Th2 cells, immature dendritic cells, and macrophages are negatively correlated with overall survival in patients with cancer[20], which may effectively explain the poorer prognosis in patients with highMED8 expression.

Although our study improves our understanding of the association between MED8 and HCC, there were some limitations. First, our results were not confirmed by cytological experiments. Second, owing to the limitations of the database, the sample size included was not sufficiently large. Finally, our study did not comprehensively account for all clinical factors associated with HCC. In follow-up experiments, we will verify the functional mechanism by which MED8 promotes HCC by cellular and zoological experiments and perform more detailed stratification and subgroup analyses.

Conclusions

In conclusion, MED8 predicts poor survival rates and is associated with clinicopathological parameters in HCC. Furthermore, MED8 may play an important role in cell cycle regulation and the Th2-mediated tumour immune microenvironment. Our results indicate that MED8 may be an effective biomarker for diagnosis and prognosis in HCC.

Materials & Methods

5.1 Patients and samples

RNA-seq data (level 3 HTSeq-FPKM format) for 424 patients diagnosed with HCC and corresponding clinical information were downloaded from TCGA database [21; 22]. RNA-seq data that did not contain clinical information and cases with survival <30 days were excluded. Data in level 3 HTSeq-FPKM format were converted into TPM (transcript per million reads) format, and RNA-seq data for 371 cases containing clinical information were finally obtained for subsequent analyses. Unavailable or unknown clinical information was treated as missing values and these data are summarized in Table 1. Setting the median MED8 expression level as the cut-off value, HCC tumour samples were divided into two groups: high expression and low expression.

5.2 Identification of DEGs

The DESeq2 package was used to analyse differentially expressed genes (DEGs) based on HTSeq-count files between the groups with high and low expression in HCC samples. Log fold change ($\log_{2}FC$) > 2 and adjusted $P < 0.01$ were set as the thresholds for DEGs. Volcano [22] and heat maps were used to visualize the results.

5.3 Metascape analysis

The Metascape database was used to evaluate enrichment for MED8 Ontology (GO) terms in the three broad categories, biological processes (BPs), molecular functions (MFs), and cellular

components (CCs), among DEGs between the groups with high and low MED8 expression.

Parameter settings were as follows: adjusted $P < 0.05$, minimum count > 3 , enrichment factor > 1.5 .

5.4 MED8 Set Enrichment Analysis (GSEA)

A GSEA was implemented in the R package clusterProfiler (version 3.6.0)[21] to analyse and visualize signalling pathways that might be associated with DEGs between the groups with high and low expression. Adjusted $P < 0.05$ and FDR $q < 0.2$ indicated statistical significance.

5.5 Immune infiltration analysis by ssGSEA

To analyse the infiltration of 24 immune cell types in tumour tissues, the ssGSEA method in the GSVA package was applied. Spearman correlation coefficients were used to describe the correlations between MED8 and the relative abundances of these 24 cells. The rank-sum test was used to analyse the relationship between high MED8 expression and the infiltration of immune cells.

5.6 Statistical analyses and protein expression analysis by immunohistochemistry(IHC)

Statistical analyses were performed using R (v.6.2). The Wilcoxon rank sum test was used to compare the expression of MED8 between HCC and control tissues. Spearman correlation coefficients and Fisher's exact test were used to analyse relationships between the level of MED8 expression and clinicopathological factors. The correlation between survival and MED8 was evaluated by a multivariate Cox regression analysis. All hypothesis tests were two-tailed and statistically significant if $P < 0.05$. Immunohistochemical images of Human protein analysis (THPA) were served to determine the distribution and subcellular localization of MED8, as well as protein expression between different tumor samples and matched normal tissues.

5.7 Construction and evaluation of a prognostic model

To screen out independent prognostic factors related to survival, univariate and multivariate COX regression analyses[23] were conducted by combining MED8 expression data with clinicopathologic factors. The Rms package was used to construct a nomogram and MED8 rated calibration plot. Risk scores (RS) were calculated from the multi-factor Cox model. A risk factor association graph showing MED8 expression, patient survival time, survival status, and the distribution of risk scores was MED8 rated. Taking the median MED8 expression level as the cut-off value, the TCGA HCC cohort was divided into a high-risk group and low-risk group, and a survival curve was drawn by Kaplan-Meier programming with survminer Package. The prognostic model was evaluated by the c-index in the ROC analysis. The results were statistically significant at $P < 0.05$.

Declarations

Ethical Approval and Consent to participate: Not applicable

Consent for publication: This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal.

Availability of data and materials: The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: There are no conflicts of interest to declare.

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Authors' contributions: Taoyuan Zhang contributed to the conception of the study; Yuan Cao, Senyuan Luo performed the experiment; Sancheng Cao contributed significantly to analysis and

manuscript preparation; Shuang Wu performed the data analyses and wrote the manuscript; Qiao Li helped perform the analysis with constructive discussions.

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Table Legends

Table 1. TCGA hepatocellular carcinoma patients characteristics

Table 2. Association between clinicopathological features and the classification of MED8 expression into high and low groups was analysed by logistic regression

Table 3. Associations with overall survival and clinicopathologic characteristics in TCGA patients using Cox regression

Figure Legends

Figure 1. Identification of differentially expressed genes in TCGA dataset. (A) Volcano plot of the differentially expressed genes. (B) Heat map of the top 50 DEGs between the groups with high and low MED8 expression.

Figure 2. The enrichment analysis of MED8 and neighboring genes. (A) Biological process enrichment related to genes associated with MED8. (B) Cellular component enrichment related to genes associated with MED8. (C) Molecule function enrichment related to genes associated with MED8. (D) GSEA results of cell cycle checkpoints. (E) GSEA results of mitotic G2-G2-M phases. (F) GSEA results of regulation of TP53 activity. (G) GSEA results of transcription regulation by TP53. ES, enrichment score; NES, normalized ES.

Figure 3. Correlations of MED8 expression with immune infiltration level in HCC. (A) Correlation between the relative abundances of 24 immune cells and MED8 expression level. (B) Correlation between the relative enrichment score of Th2 cells and the expression level of MED8.

Figure 4. Analysis of correlation between expression level of MED8 and clinical parameters in HCC. (A) Expression levels of MED8 in paired tumor and adjacent samples and (B) non-paired samples were analyzed by Wilcoxon signed rank tests. Association between MED8 expression and clinicopathologic characteristics, including (C) Histologic grade, (D) Pathologic stage, (E) T stage, impact of MED8 expression on OS (F) and MED8 expression in the pathologic stage on OS (G) in patients with HCC in TCGA datasets. (H) A forest plot demonstrates the prognostic value of MED8 for overall survival in various subgroups.

Figure 5. Relationship between the MED8 and other clinical factors. (A) Nomogram for predicting the probability of 1-, 3-, and 5- year OS for HCC patients. (B) Calibration curve of the nomogram in the TCGA datasets. (C) ROC created by plotting the true positive rate against the false positive rate at various threshold settings with corresponding AUC labeled around the curve

Figures

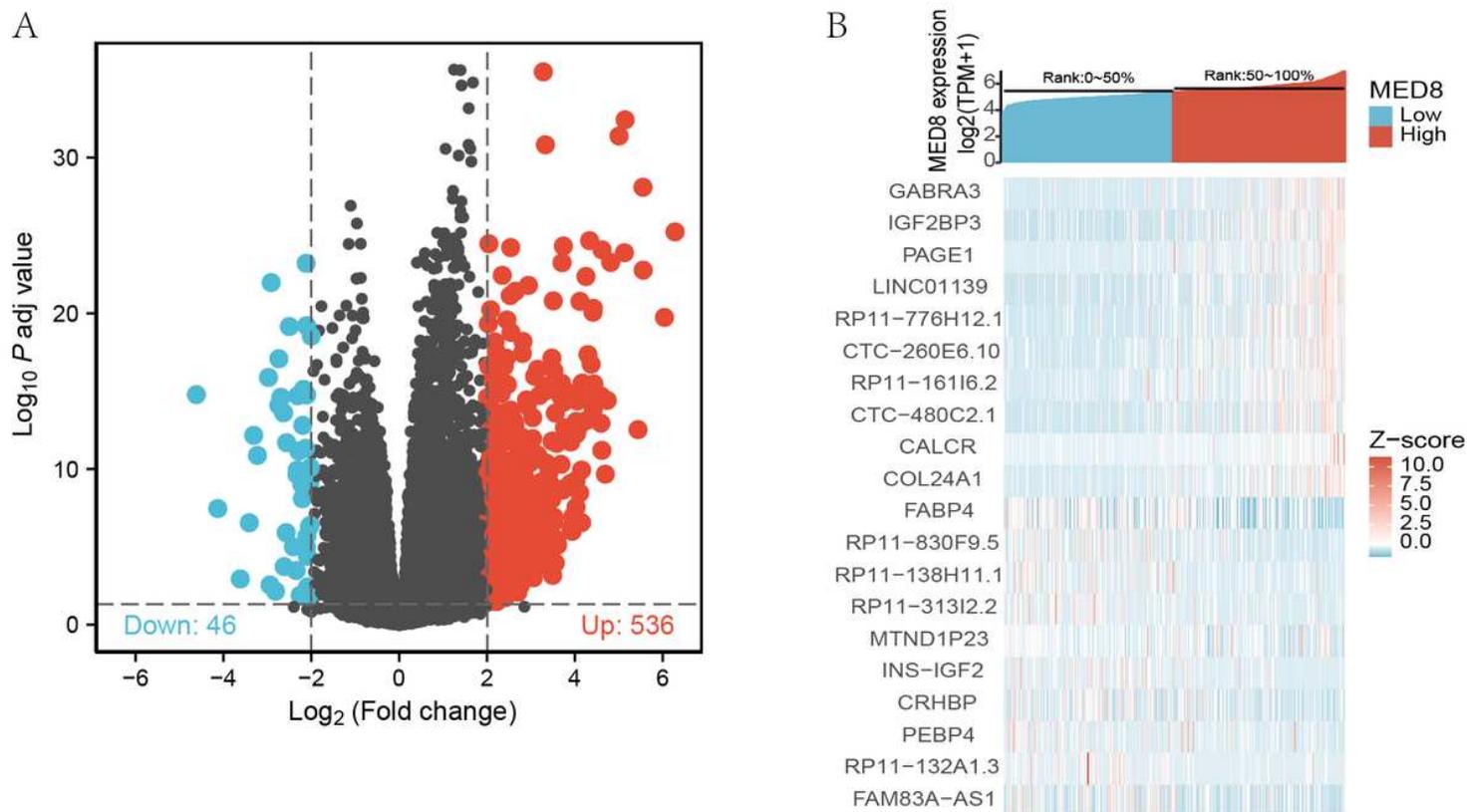


Figure 1

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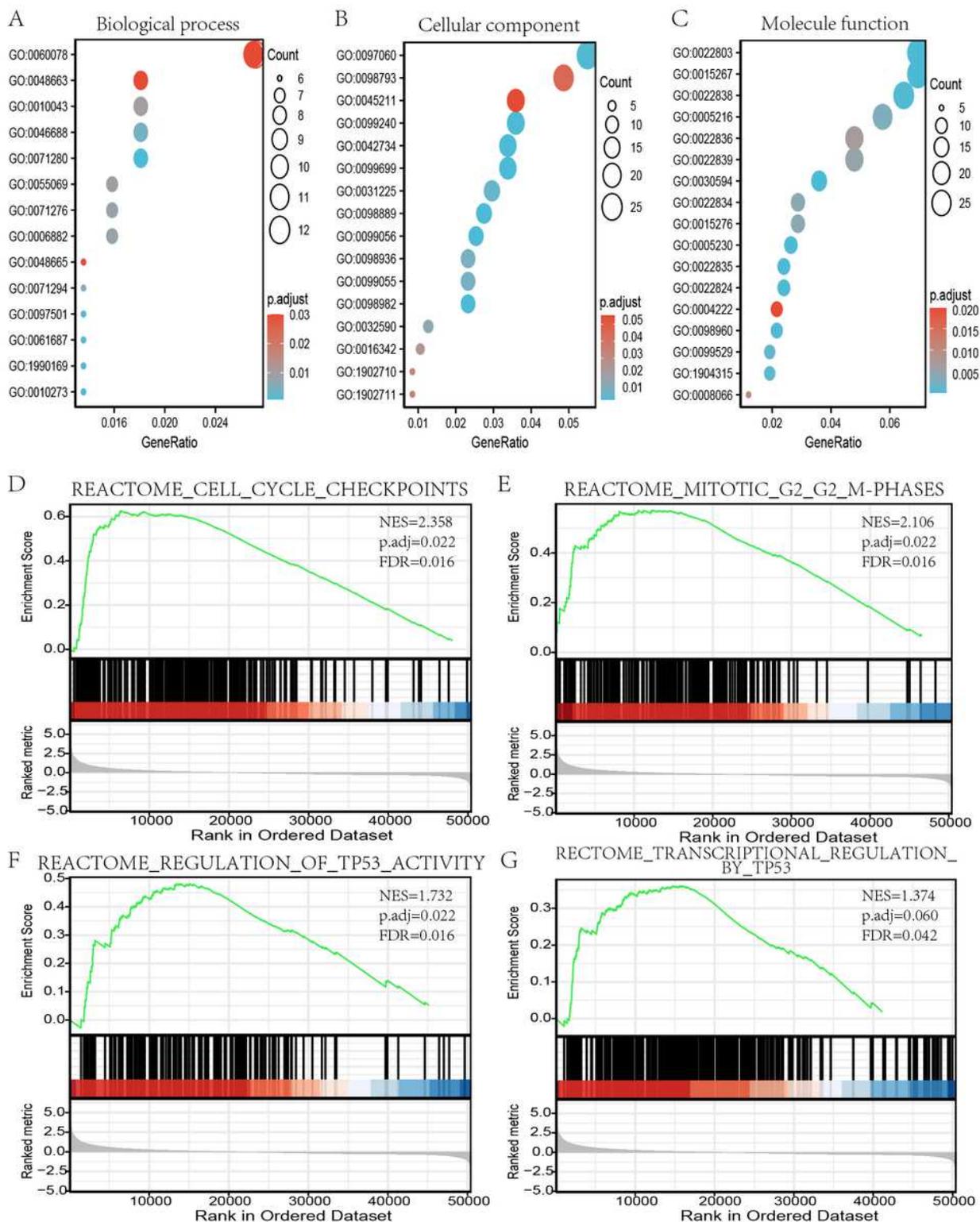
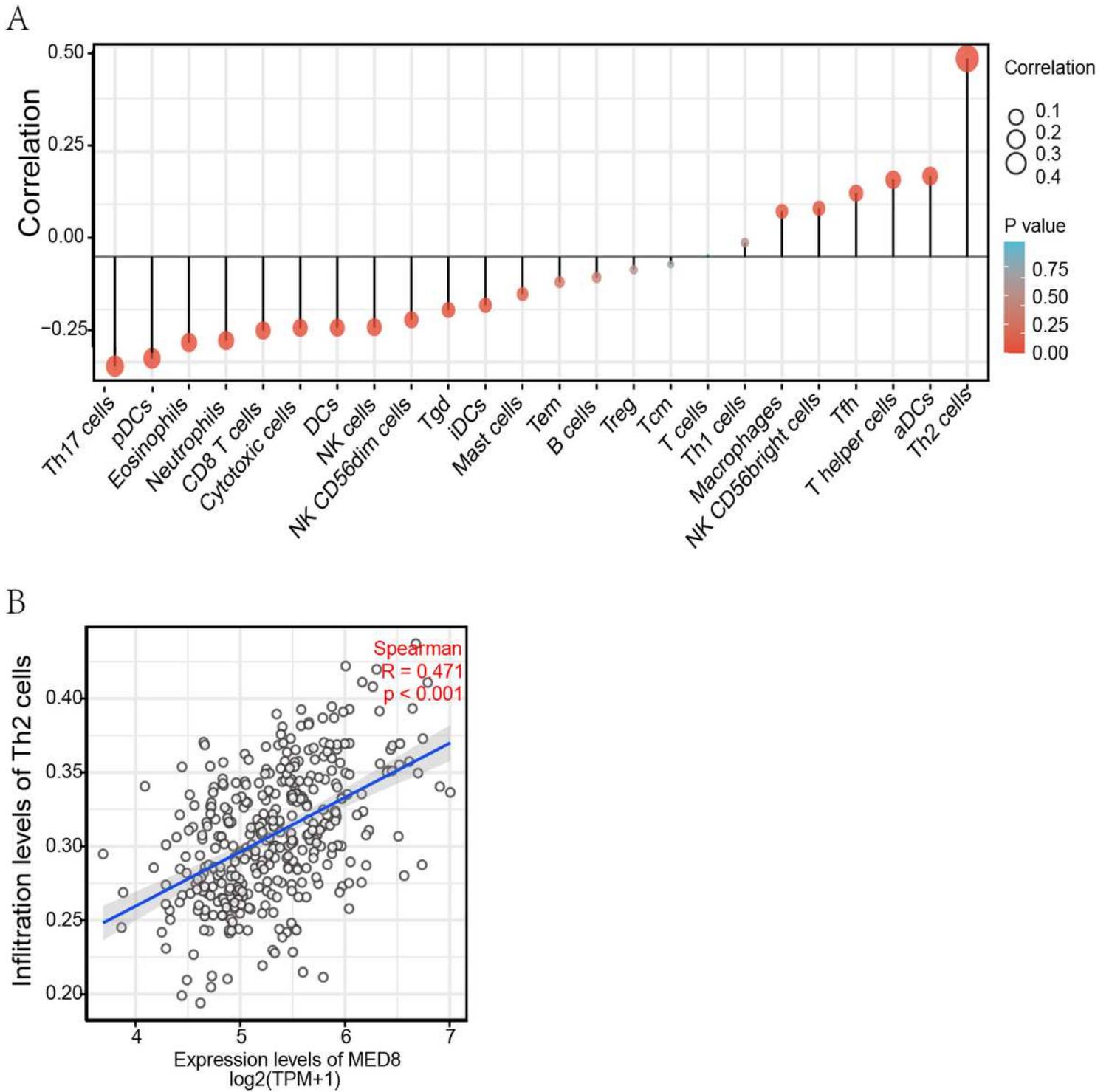


Figure 2

The enrichment analysis of MED8 and neighboring genes. (A) Biological process enrichment related to genes associated with MED8. (B) Cellular component enrichment related to genes associated with MED8. (C) Molecule function enrichment related to genes associated with MED8. (D) GSEA results of cell cycle checkpoints. (E) GSEA results of mitotic G2-G2-M phases. (F) GSEA results of regulation of TP53 activity. (G) GSEA results of transcription regulation by TP53. ES, enrichment score; NES, normalized ES.



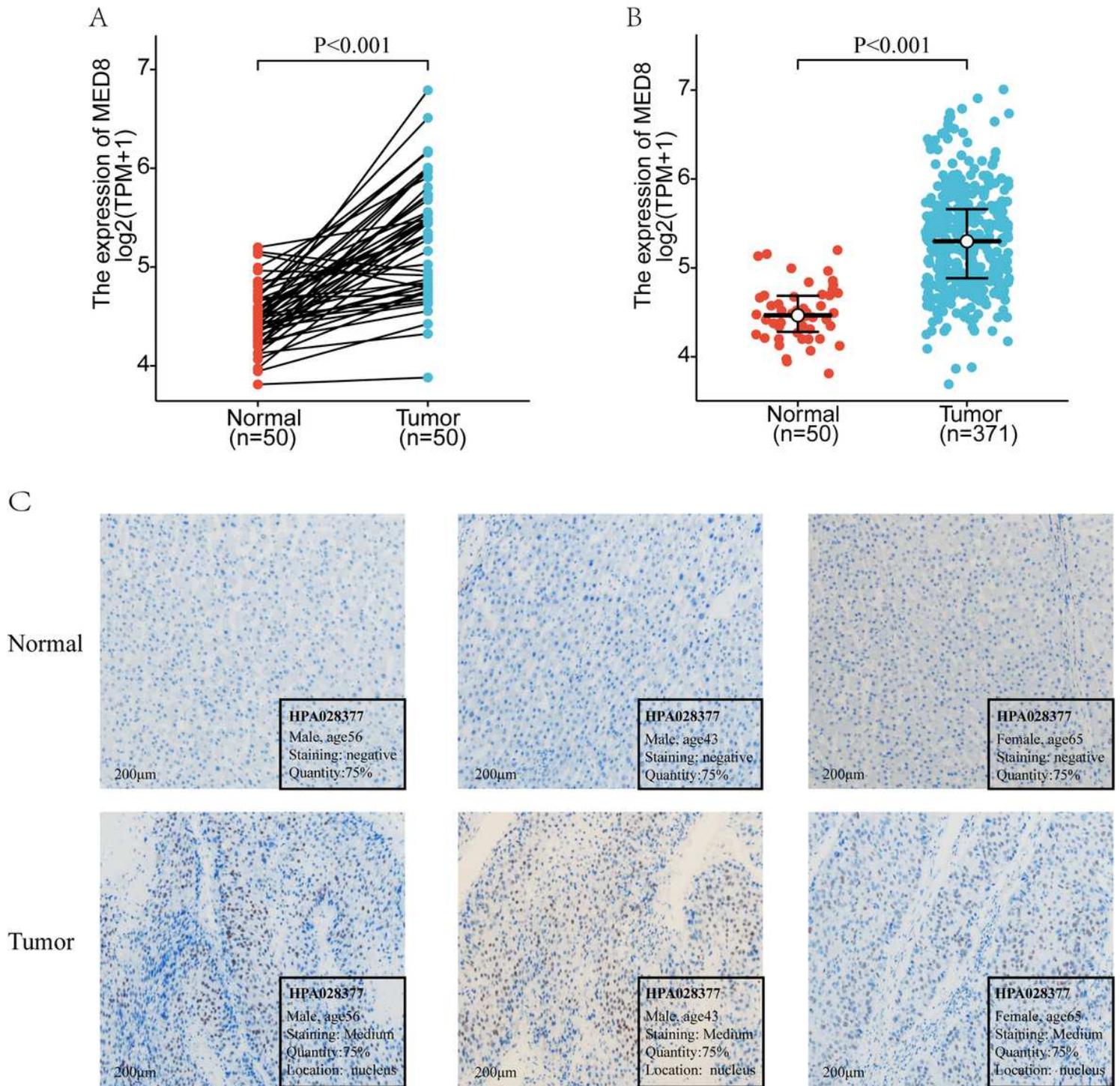


Figure 4

Analysis of correlation between expression level of MED8 and clinical parameters in HCC. (A) Expression levels of MED8 in paired tumor and adjacent samples and (B) non-paired samples were analyzed by Wilcoxon signed rank tests. Association between MED8 expression and clinicopathologic characteristics, including (C) Histologic grade, (D) Pathologic stage, (E) T stage, impact of MED8 expression on OS (F)

and MED8 expression in the pathologic stage on OS (G) in patients with HCC in TCGA datasets. (H) A forest plot demonstrates the prognostic value of MED8 for overall survival in various subgroups.

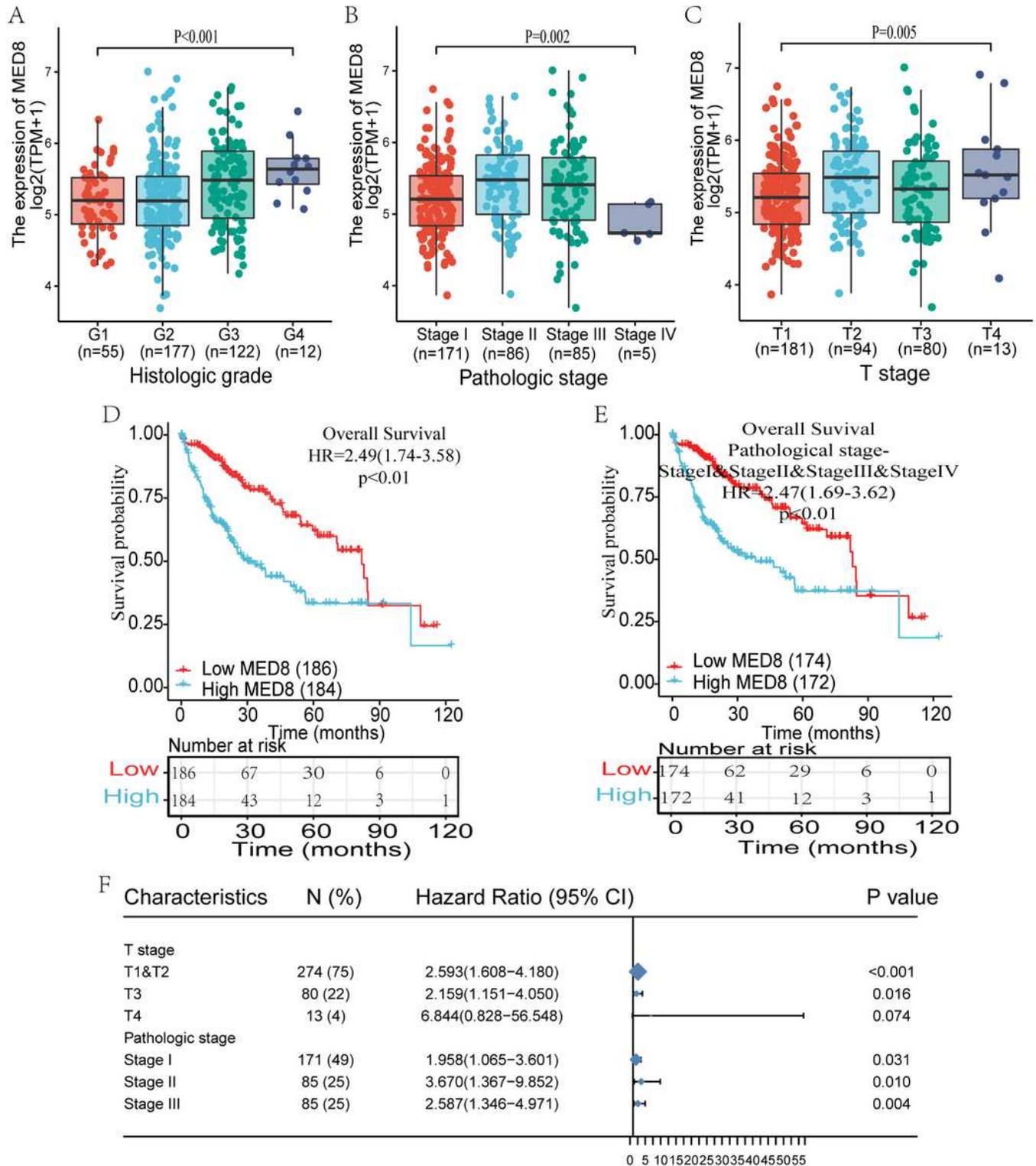


Figure 5

Relationship between the MED8 and other clinical factors. (A) Nomogram for predicting the probability of 1-, 3-, and 5-year OS for HCC patients. (B) Calibration curve of the nomogram in the TCGA datasets. (C)

ROC created by plotting the true positive rate against the false positive rate at various threshold settings with corresponding AUC labeled around the curve

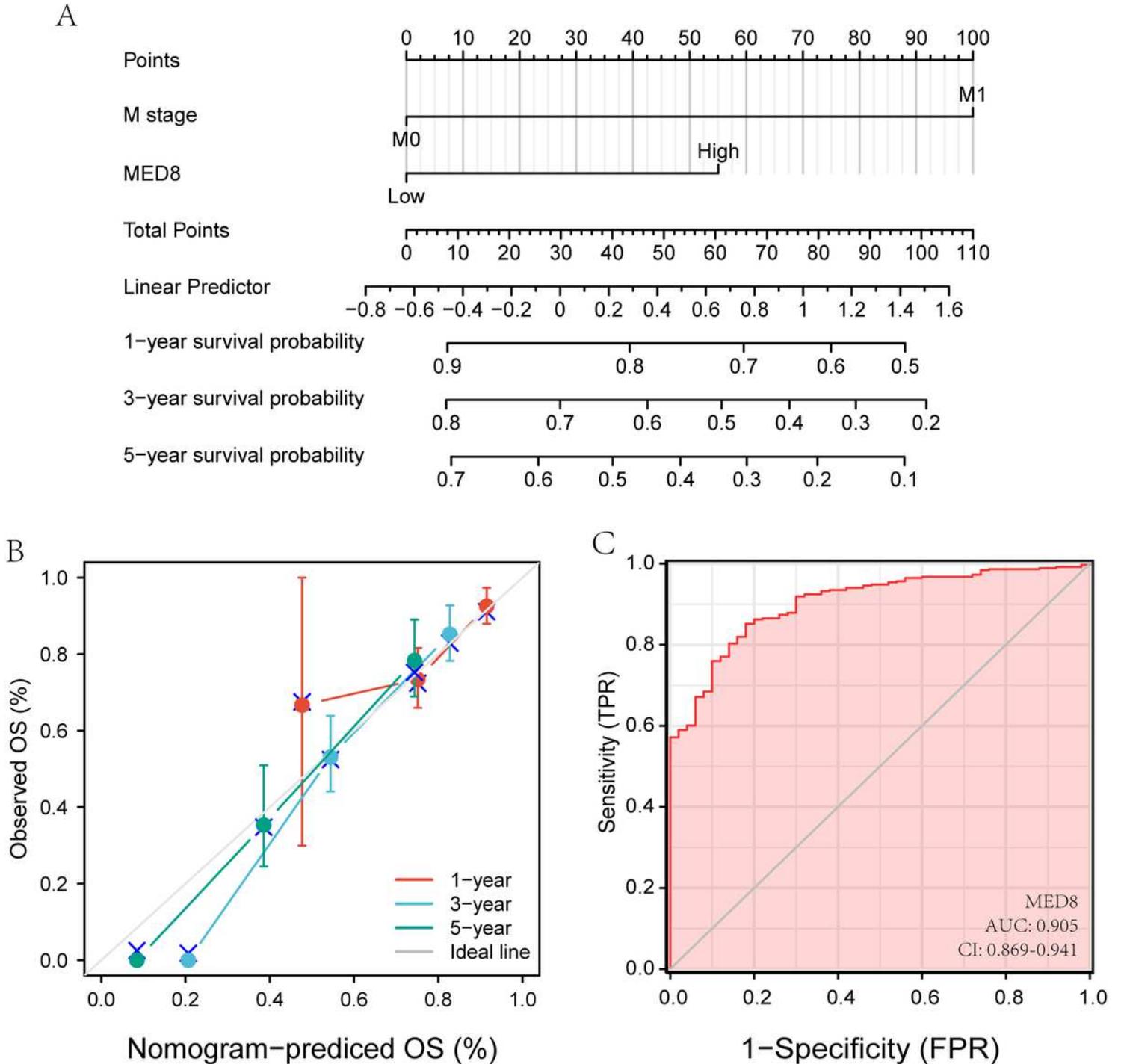


Figure 6

Univariate and multivariate Cox regression analyses were used to evaluate whether MED8 is an independent prognostic indicator for HCC. Variables with $P < 0.1$ in single-factor Cox regression were included in a univariate Cox regression, including T stage ($P < 0.001$), M stage ($P = 0.018$), pathologic stage ($P < 0.001$), and MED8 ($P < 0.001$). Further, multivariate Cox regression showed that M stage ($P = 0.026$) and MED8 ($P < 0.001$) were independent prognostic factors for overall survival ($P < 0.05$), as

shown in Table 3. A nomogram was MED8rated to evaluate the prognostic model, as shown in Figure 6A, including M stage and MED8 (c-index: 0.650 (0.624–0.677)). A calibration curve was used to verify the performance of models including M stage and MED8, as shown in Figure 6B. We analysed the diagnostic efficacy of MED8 in HCC by an ROC analysis, and the area under the curve (AUC) was 0.905, suggesting that MED8 is a potential diagnostic marker, as shown in Figure 6C.