

MCEMP1 as a Tumor Microenvironment and Immune Infiltration Related Gene for Prognostic Prediction in Advanced Gastric Cancer

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Research Article

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Abstract

Background Tumor microenvironment (TME) has displayed profound clinical significance in cancer progression, prognosis and the efficacy of immunotherapy. However, the overall characteristics of TME in patients with advanced gastric cancer (AGC) have not been intensively studied. In order to get a more comprehensive understanding, this study aimed to investigate TME-related prognostic genes in patients with AGC based on bioinformatics, combined with histological verification.

Methods Transcriptome and clinical data on stage III/IV GC were obtained from The Cancer Genome Atlas (TCGA) database. The data of stromal, immune scores and 22 infiltrating immune cells from AGC samples were evaluated by ESTIMATE and CIBERSORT algorithms. Then, mast cell-expressed membrane protein 1 (MCEMP1) was focused by integrated protein-protein interaction (PPI) network and Cox regression. The survival and expression analysis of MCEMP1 was evaluated and verified in tissues by immunohistochemistry (IHC) and quantitative real-time PCR (qRT-PCR).

Results There was a positive correlation between TME scores and pathological grades. A total of 666 TME-related differential genes were screened. MCEMP1 was identified as a predictive factor related to the prognosis of AGC both in TCGA database and tissue samples. Further analysis indicated that MCEMP1 was involved in regulating pathways of immune activities. The results of CIBERSORT demonstrated that MCEMP1 expression was significantly correlated with the proportion of 8 kinds of infiltrating immune cells.

Conclusion As a TME-related prognostic gene, MCEMP1 might play a crucial role in remodeling immune infiltrates in AGC patients, which might be a potential immunotherapy target for patients with AGC.

Introduction

Gastric cancer (GC) is the fifth most common diagnosed cancer and the third leading cause of cancer death in the world[1]. Although the incidence and mortality of GC have shown a decreasing trend in recent years, it is still a major public health burden. Most patients diagnosed with GC are at an advanced stage, with a median survival time of less than 1 year[2]. Despite the tremendous progress in biotherapy of advanced GC (AGC), there are still no substantial survival benefits[3, 4]. This emphasizes the importance of identifying new biomarkers and therapeutic targets for AGC.

In recent years, immunotherapy has revolutionized the treatment of various cancers, including GC[5, 6]. Previous clinical studies have proved that patients with AGC benefit from immunotherapy[7, 8]. However, accumulating evidence indicates that the efficacy of immunotherapy may be limited to a subset of patients with AGC[9]. JAVELIN 300 Trail confirmed that compared with chemotherapy, the monoclonal antibody Avelumab that blocks PD-1/PD-L1 has not shown better efficacy in patients with AGC [10]. This mainly due to the complexity and heterogeneity of the tumor microenvironment (TME) which contribute to differences in the efficacy of immunotherapy in patients with GC. TME is the environment composed of extracellular matrix, tumor vasculature, stromal cells, immune cells and the acidic hypoxic environment

of the tumor. TME has been widely regarded as a critical role in the occurrence and development of tumors [11–13]. Cancer cells typically reprogrammed TME to support their survival and escape immune surveillance. It has been reported that tumor-infiltrating immune cells (TICs), such as M2 macrophages and NK cells, were significantly related to the prognosis of malignant tumors[14, 15]. In pancreatic cancer, the stromal components of TME secreted a variety of factors affecting angiogenesis to overcome hypoxia and acidic state, which further facilitated tumor invasion[16]. Succinate derived from TME controlled the polarization of tumor-associated macrophages (TAMs) to promote tumor progression and metastasis[17]. The immunosuppression and fibrosis status of TME affect the efficacy of immunotherapy. Cancer-associated fibroblasts (CAFs) in TME maintain the fibrotic environment and exclude T cells from the cancer cells, leading to limiting effective immunotherapy[18]. TAMs maintain tumor growth in TME, reducing the effectiveness of immunotherapy[19]. Therefore, mining the prognostic genes related to TME and exploring the correlation with TICs may be an effective method to optimize tumor immunotherapy and improve outcomes for AGC.

In this study, we performed ESTIMATE and CIBERSORT algorithms to calculate the TME scores and the composition of TICs in TME. According to univariate Cox regression analysis and PPI network, MCEMP1 was identified as a prognostic gene related to TME. We further validated the expression and evaluate the prognostic value of MCEMP1 in the tissues of patients with AGC. MCEMP1 was thought to be involved in the regulation of mast cell differentiation and immune inflammatory responses[20]. In this study, we demonstrated that MCEMP1 was associated with immunoactivity and immune infiltration, suggesting that MCEMP1 may be a potential biomarker in TME of AGC.

Materials And Methods

Datasets and tissues acquisition

Expression data of transcriptome and corresponding clinical data of AGC samples were obtained from TCGA data portal (<https://portal.gdc.cancer.gov/>). Thirty-one paired AGC and normal tissues were collected from patients undergoing surgery at Lanzhou University Second Hospital. All samples were confirmed by clinicopathological. This research protocol was granted by the Lanzhou University Second Hospital Ethics Committee. Details of baseline characteristics of AGC patients in TCGA and validation cohort are listed in Table 1.

Identification of differentially expressed genes

The algorithm of ESTIMATE was performed to calculate Stromal-, Immune- and ESTIMATE scores of AGC. AGC data were classified into different groups according to the median immune score and stromal score respectively. With a “limma” package, differentially expressed genes (DEGs) in the group of Immune- and Stromal score were separately screened in line with the cut-off value of $|\log \text{ fold change}| > 1$ and $\text{adj. } p < 0.05$.

Functional analysis

Functional analysis of DEGs, including Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional analyses, were presented with the ggplot2, enrichplot and cluster Profiler packages. The items with p value less than 0.05 were considered significantly enriched.

enriched.

Cox regression analysis and Protein-protein interaction network

To further identify DEGs related to the prognosis of AGC, Univariate Cox regression analysis was performed. The Search Tool for the Retrieval of Interacting Gene (STRING) database was applied to conduct the Protein-protein interaction (PPI) network of DEGs, and then the network was reconstructed using Cytoscape 3.8.1 software to screen hub genes with a degree of connection of more than 15. To visualize the intersection targets gene between the prognostic-related DEGs and hub genes in PPI network, venn diagram was performed.

Gene Set Enrichment Analysis

The survival package was used for gene survival analysis. Gene expression, survival and clinical correlation analysis were verified in Gene Expression Profiling Interactive Analysis database. The gene set enrichment analysis (GSEA) was down with the Hallmark and C7 gene sets downloaded from GSEA 4.1.0 software. The criteria were set on NOM $p < 0.05$ and FDR < 0.25 .

CIBERSORT

With CIBERSORT algorithm, the infiltration ratio of tumor-infiltrating immune cells (TICs) was then evaluated. The correlation between different subgroups of TIC was analyzed by the corrplot package. The correlation between MCEMP1 expression level and TIC was carried out by limma, ggplot, ggpubr and ggExtra packages.

Quantitative real-time PCR (qRT-PCR)

Total RNA extraction from tissues was implemented with TRIzol reagent (TaKaRa, Japan), which was then reversely transcribed to cDNA. qRT-PCR was carried out on CFX96 Touch qRT-PCR system (Bio-Rad) using SYBR Green Premix (ACCURATE BIOLOGY, China). The primer sequences were as follows: MCEMP1, 5'-ATTTATCTCCGTACATGTGGGT-3' (forward) and 5'-CGGCACACTCATAATGACAC-3'(reverse), GAPDH was used as the endogenous reference. The relative mRNA expression was quantified by $2^{-\Delta\Delta Ct}$ method.

Immunohistochemistry (IHC)

The protein expressions of MCEMP1 in AGC tissues and adjacent normal tissues were detected by IHC. After deparaffinization and hydration, the sections were subjected to antigen retrieval in citrate buffer, which were then incubated overnight with anti-MCEMP1 antibody (1:50, Bioss, China) at 4°C. After

incubating with secondary antibody, 3,3'-diaminobenzidine (DAB) was used to detect the section immunoreactivity. Finally, hematoxylin is used for dyeing and dehydration. The sections were analyzed with an optical microscope.

Statistical analysis

R 4.0.2, SPSS 20.0 software and Graphpad Prism 9.0 were applied for statistical analysis. Chi-square test was used for nonparametric variables, and Student's t-test was applied for parametric variables. $P < 0.05$ was regarded as statistically significant.

Results

Relationship between TME scores and the grade of AGC patients

To evaluate the correlation between Immune-, Stromal-, ESTIMATE score and clinicopathological characteristics, we then obtained clinical data. As shown in Fig. 1a-c, ESTIMATE score ($p < 0.001$), Immune score ($p < 0.001$) and Stromal score ($p < 0.001$) of AGC patients with G3 histological grade were notably higher than those with G2 histological grade. These results indicated that the stromal and immune components in TME were involved in the malignant progression of AGC

Identification of DEGs related to TME

To identify the accurate discrepancy of gene profile in TME in relation to immune and stromal components along with the role it plays in AGC, the comparison analysis between normal samples and AGC samples was implemented. "Limma" package was conducted to extract TME-related DEGs. According to stromal and immune scores, heatmaps of DEGs between normal samples and AGC were shown in Fig. 1d and e. A total of 1587 up-regulated and 151 down-regulated DEGs were obtained between the high and low stromal score groups. Compared with the low immune score group, 874 DEGs in the high immune score group were up-regulated, and 355 DEGs were down-regulated. The Venn diagram showed that a total of 575 up-regulated genes and 91 down-regulated genes sharing by stromal score group and immune score group (Fig. 1f, g), the DEGs (total 666 genes) may be the determinants of TME status. Subsequently, DEGs proceeded for functional enrichment analysis. GO analysis revealed that DEGs were significantly mapped on immune-related activities, such as T cell activation, lymphocyte activation regulation, and immune receptor activity (Fig. 2a). The results of KEGG analysis indicated that DEGs were significantly enriched in pathways related to immune response (Fig. 2b). These results suggest that the participation of TME is a dominating characteristic of AGC patients.

Screening of overlapping gene via univariate Cox regression and PPI network

We performed Cox regression analysis to investigate the prognostic genes for patients with AGC, and four genes were determined (Fig. 3a). The PPI network was constructed to visualize interactions between DEGs from the STRING database (Fig. 3b). Cytoscape 3.8.1 software was used to reconstruct the PPI network and calculate the connectivity of each node in the network to determine hub genes (Fig. 3c).

Interaction analysis between univariate Cox regression and PPI network revealed that MCEMP1 was the only overlapping gene (Fig. 3d).

The correlation of MCEMP1 expression with survival and clinical stage in AGC patients

AGC cases were divided into high and low expression groups in TCGA cohort, with the median of MCEMP1 expression. The Kaplan-Meier survival curve exhibited that overall survival of AGC patients with low expression of MCEMP1 was better than that of the high expression group (Fig. 4a). Based on the GEPIA database, the expression of MCEMP1 was closely related to TNM stage (Fig. 4b). Survival curve indicated that high expression level of MCEMP1 was associated with poor prognosis in patients with GC (Fig. 4c).

Validation of the expression and prognostic value of MCEMP1 in AGC tissues

A total of 31 AGC patients with complete follow-up clinical data were included. The protein expression level of MCEMP1 in AGC tissues was higher than that in adjacent normal tissues detecting by IHC (Fig. 5a, b). The results of relative mRNA expression of MCEMP1 were consistent with the results of IHC (Fig. 5c). Survival analysis revealed that high expression of MCEMP1 in AGC patients led to the poor prognosis (Fig. 5d). The experimental results provide strong support for the above conclusions.

MCEMP1 participate in the modulation of immune activity in AGC

In view of MCEMP1 is highly expressed in AGC and the expression levels of MCEMP1 were positively related with the prognosis of AGC patients, GSEA was implemented in the high-expression group. The results of GSEA suggested that MCEMP1 was significantly related to immune-related signaling pathways, including the interaction of cytokines and cytokine receptors, and natural killer cell-mediated cytotoxicity (Fig. 6). The results revealed that MCEMP1 may play a role in the regulation of immune activity in AGC and is of certain significance to assess the state of TME.

Correlation of MCEMP1 with the proportion of TICs

To further verify the role of MCEMP1 and TME, CIBERSORT algorithm was performed to analyze the proportion of TICs in AGC microenvironment (Fig. 7a-c). MCEMP1 expression was significantly correlated with 8 infiltrating immune cells (Fig. 8a). Among them, the expression of MCEMP1 was negatively correlated with CD8 T cells, regulatory T cells, and resting mast cells, while it was positively correlated with resting NK cells, M0 macrophages, activated mast cells, eosinophils, and neutrophils (Fig. 8b). The results further supported the effect of MCEMP1 act on the TME.

Discussion

GC is a common malignant tumor globally. Patients with GC are often diagnosed at an advanced stage, accompanied by extensive tumor invasion and distant metastasis. Due to *Helicobacter pylori* infection, the occurrence and development of GC are closely related to chronic inflammation with a state of

immune tolerance[21, 22]. Accumulating evidence indicates that the progression of GC is the result of the joint evolution of cancer cells and TME[23, 24]. Therefore, it is essential to explore the crucial markers that regulate TME of AGC for reversing the carcinogenic effects of TME. In this study, we identified TME-related genes that are beneficial to the survival of AGC patients via ESTIMATE algorithm from TCGA database. A series of bioinformatics analyses and histological verification determined that MCEMP1 participated in immune activities, which may be an indicator of TME status of AGC patients.

In recent years, immunotherapy, as an emerging therapy, has shown profound impacts on a variety of cancers including AGC. However, due to the complex and highly heterogeneous microenvironment of tumors, not all patients can benefit from it. TME was involved in cancer reprogramming through direct or indirect interaction with tumor cells, which affected the prognosis of patients with immunotherapy[25, 26]. The present study revealed that the stromal and immune components in TME were positively correlated with the grade of AGC, which indicated that remodeling TME might foster the progression of AGC. This was consistent with many previous studies. The M2 type macrophages in TME suppressed the immune response by promoting angiogenesis and epithelial-mesenchymal transition (EMT), thereby promoting cancer progression[27]. Tumor-associated fibroblasts, as one of the main components of TME, enhanced the metastasis and invasion ability of GC cell lines in vitro by inhibiting the anti-tumor effect of T cells[28]. Subsequently, we identified TME-related DEGs. The results of GO and KEGG analysis revealed that DEGs were closely related to the immune response of AGC patients, and participated in the proliferation and activation of immune cells and the interaction of cytokines. These evidences suggested that TME may play a vital role in the immunotherapy of AGC patients.

To evaluate the prognostic value of DEGs in AGC, we performed Cox regression analysis and PPI network. The results demonstrated that MCEMP1 was the most important gene related to the prognosis of AGC patients. MCEMP1 gene encodes a type I membrane protein expressed by mast cells. Studies have shown that MCEMP1 was mainly associated with regulating mast cell differentiation, inflammation and various immune responses[20]. There are few studies on investigating the role of MCEMP1 in cancer until now. This study identified MCEMP1 as a TME-related prognostic gene in AGC for the first time. Characterized by GEPIA database, MCEMP1 was overexpressed in AGC samples. Kaplan-Meier plot exhibited that high expression of MCEMP1 was substantially relevant to the poor prognosis of AGC patients, while IHC and qRT-PCR in tissues confirmed this result. These results suggest that MCEMP1 may take part in promoting the progression of AGC, therefore MCEMP1 can be used as a prognostic marker. Previously, it has been reported that based on the TCGA database, MCEMP1 was an independent prognostic predictor of high risk of GC[29], which was consistent with the results of our finding. In addition, we examined the biological mechanism of MCEMP1 in TME. The results of GSEA analysis demonstrated that high expression of MCEMP1 was enriched in pathways related to cell metabolism and immune activities, indicating that MCEMP1 may be involved in the regulation of immune activity of the AGC microenvironment.

TICs were related to the clinical efficacy of immunotherapy, and the infiltration degree of TICs was also an important indicator for tumor progression. According to previous study, PD-L1 and TICs were

associated with the outcome of GC immunotherapy[30]. To further investigate the mechanism by which MCEMP1 participated in the regulation of TME, we analyzed the infiltration ratio of TICs in AGC microenvironment and the correlation between MCEMP1 expression and TICs using CIBERSORT algorithm. The results indicated that CD8 T cells, regulatory T cells, NK cells resting, M0 macrophages and mast cells resting have significant differences in AGC. Besides, high expression of MCEMP1 was relevant to the low-level infiltration state of a variety of immune cells, including CD8 T cells, regulatory T cells and resting mast cells. These evidences indicated that MCEMP1 was likely to be a TME-related regulatory factor in AGC, and affect the prognosis of AGC through TME, especially TICs. Studies have reported that the increase of activated mast cells in GC TME can promote angiogenesis and lymph node metastasis by releasing angiogenic factors and lymphangiogenic factors, which are related to the survival of GC patients[31]. The increase in the proportion of quiescent mast cells may be related to improving the prognosis of GC patients. Studies have shown that MCEMP1 was positively correlated with the secretion of serum TNF- α , IL-1B, and IL-6, and inhibited the vitality of T cells, thereby inhibiting immune function[29, 32]. This suggested that MCEMP1 was related to the activity of T cells. CD8 T cells play a key role in the anti-tumor immune response. Studies have reported that the increasing infiltration level of CD8 T cells in TME can improve the prognosis of patients with GC and colon cancer[33]. In this study, MCEMP1 up-regulated was related to the poor prognosis of patients with AGC, which may be attributed to lower CD8 T cell infiltration. It can provide a reference for follow-up research. Therefore, MCEMP1 may affect the prognosis of patients with AGC by regulating immune cell infiltration, which requires further experimental studies to confirm. At present, the potential role and biological function of MCEMP1 in AGC are still unclear, and follow-up studies are still needed.

In summary, the study is based on ESTIMATE algorithm to screen prognostic-related genes in the AGC microenvironment. We found that the selected MCEMP1 gene plays a key role in affecting the progress and overall survival of AGC by regulating the immune activity of TME, and is expected to become a potential biomarker and immunotherapy target for AGC patients.

Abbreviations

TME tumor microenvironment

AGC advanced gastric cancer

TCGA The Cancer Genome Atlas

MCEMP1 mast cell-expressed membrane protein 1

PPI protein-protein interaction

IHC immunohistochemistry

qRT-PCR quantitative real-time PCR

GC Gastric cancer

TICs tumor-infiltrating immune cells

TAMs tumor-associated macrophages

CAFs Cancer-associated fibroblasts

DEGs differentially expressed genes

GO Gene Ontology

KEGG Kyoto Encyclopedia of Genes and Genomes

STRING Search Tool for the Retrieval of Interacting Gene

GSEA gene set enrichment analysis

EMT epithelial-mesenchymal transition

Declarations

Ethics approval and consent to participate

Approval for the research study was obtained from the Lanzhou University Second Hospital Ethics Board.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the TCGA repository. www.tcga.org.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

The subject design was completed by Yumin Li. Daijun Wang and Yanmei Gu performed the bioinformatics analysis and experiments. The final article was reviewed by Yumin Li and Yang Zhao. All authors read and approved the final manuscript.

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Not applicable

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Tables

Table 1. Baseline characteristics of AGC patients in TCGA and validation cohort

Characteristics	TCGA cohort (n=210)	Validation cohort (n=31)
Age (year)		
≤65	99(47.14)	27(87.10)
>65	111(52.86)	4(12.90)
Gender		
Male	133(63.33)	24(77.42)
Female	77(36.67)	7(22.58)
Differentiation		
Well	4(1.90)	2(6.45)
Moderate	62(29.52)	7(22.58)
Poor	144(68.57)	22(70.97)
Lauren classification		
Intestinal	—	2(6.45)
Diffuse	—	4(12.90)
Mixed	—	25(80.65)
T_stage		
T1	0	0
T2	15(7.14)	0
T3	105(50.00)	0
T4	90(42.86)	31(100.00)
N_stage		
N0	7(3.33)	1(3.23)
N1	56(26.67)	20(64.52)
N2	68(32.38)	9(29.03)
N3	79(37.62)	1(3.23)
M_stage		
M0	183(87.14)	30(96.77)
M1	27(12.86)	1(3.23)

Tumor stage		
III	169(80.48)	30(96.77)
IV	41(19.52)	1(3.23)

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Abbreviation: AGC , advanced gastric cancer.

Figures

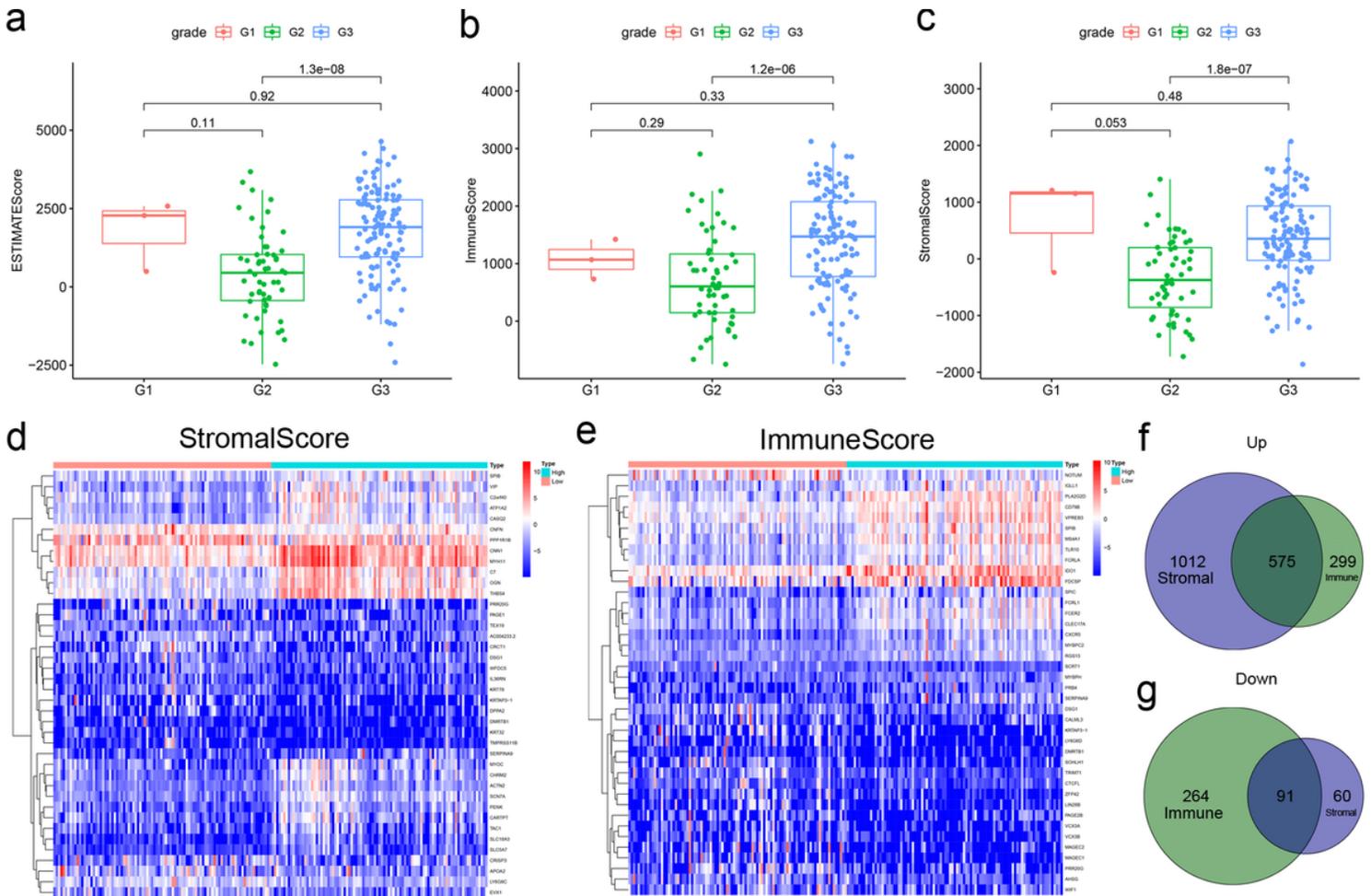
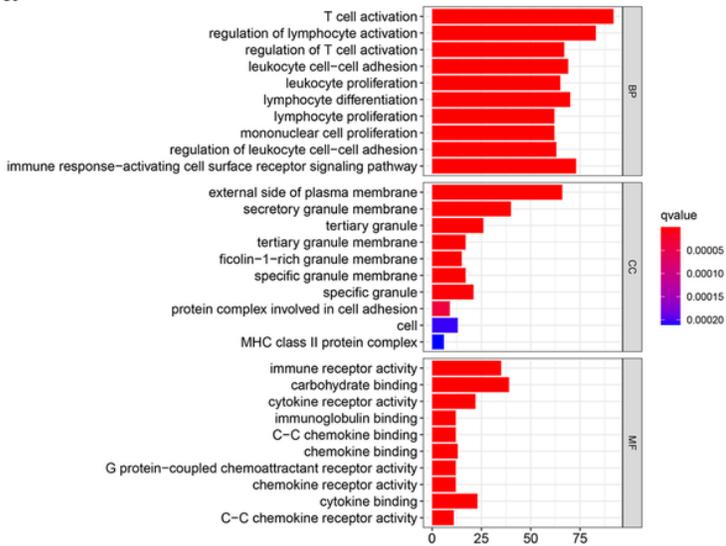


Figure 1

Associations of scores with clinicopathological characteristics and identification of DEGs. (a-c) The relationship between ESTIMATE/Immune/Stromal scores and grade. (d) Heatmaps of DEGs based on stromal scores. (e) Heatmaps of DEGs based on immune scores. (f) Upregulated DEGs of both stromal and immune scores by Venn diagram. (g) Downregulated DEGs of both stromal and immune scores by Venn diagram.

a



b

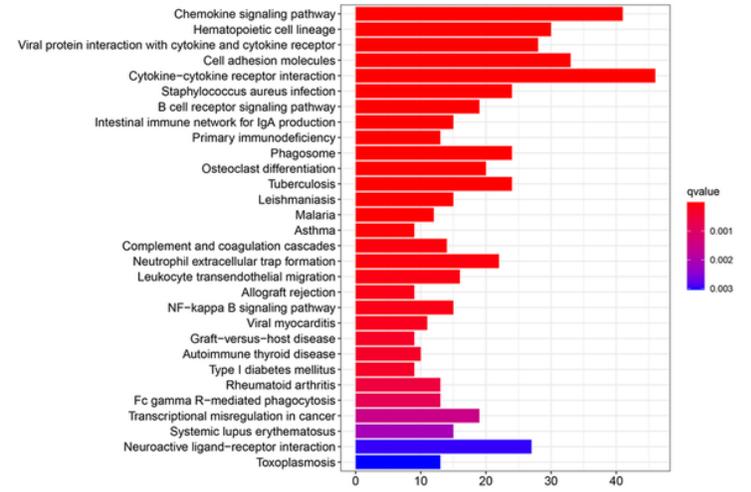


Figure 2

Functional enrichment analysis of TME-related DEGs. (a) GO analysis. (b) KEGG enrichment analysis.

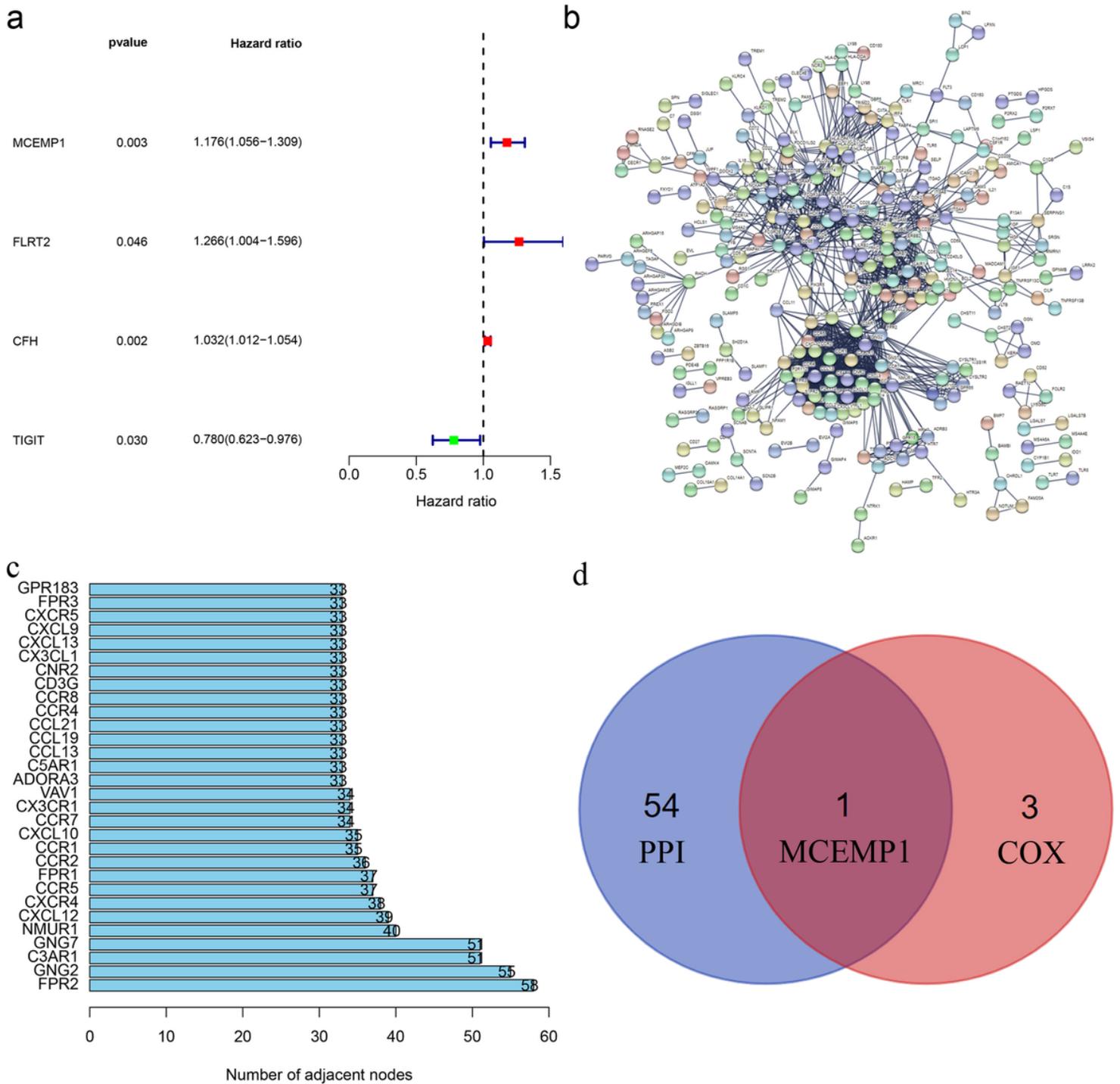


Figure 3

PPI network and univariate Cox regression analysis. (a) Forest plot of univariate Cox regression analysis. (b) PPI network of DEGs. (c) Top 30 DEGs according to the node connectivity in PPI network. (d) Venn diagram showing MCEMP1 shared by prognostic genes in Cox and hub genes in PPI network.

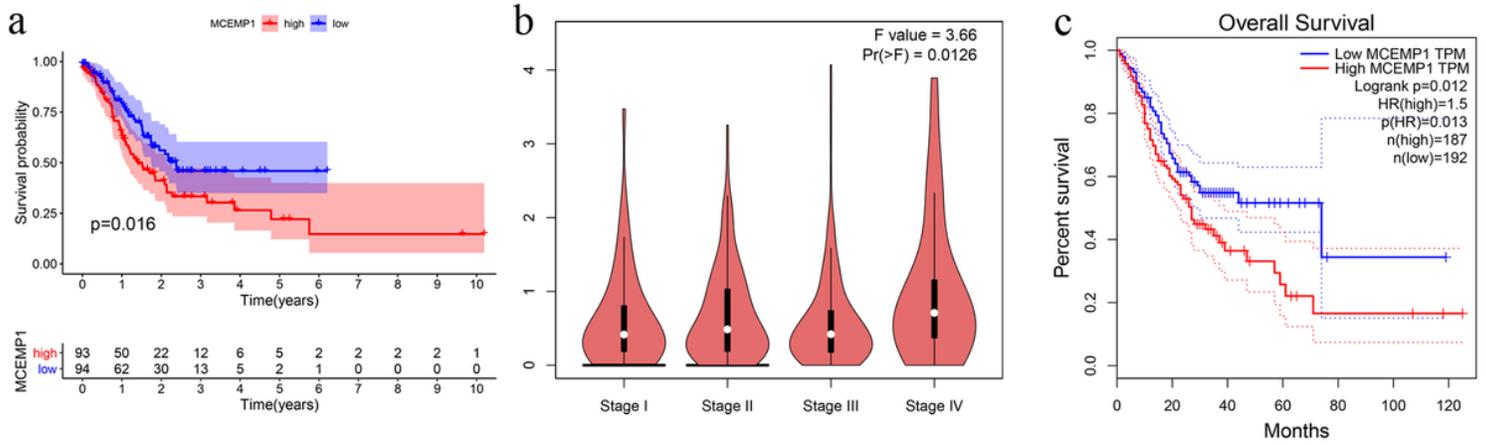


Figure 4

MCEMP1 survival and clinicopathological characteristic analysis in TCGA and GEPIA databases. (a) Survival analysis of AGC patients with high and low MCEMP1 expression in TCGA database. (b) Correlation analysis of MCEMP1 expression and TNM stage in GEPIA database. (c) Validation of MCEMP1 survival of AGC in GEPIA database.

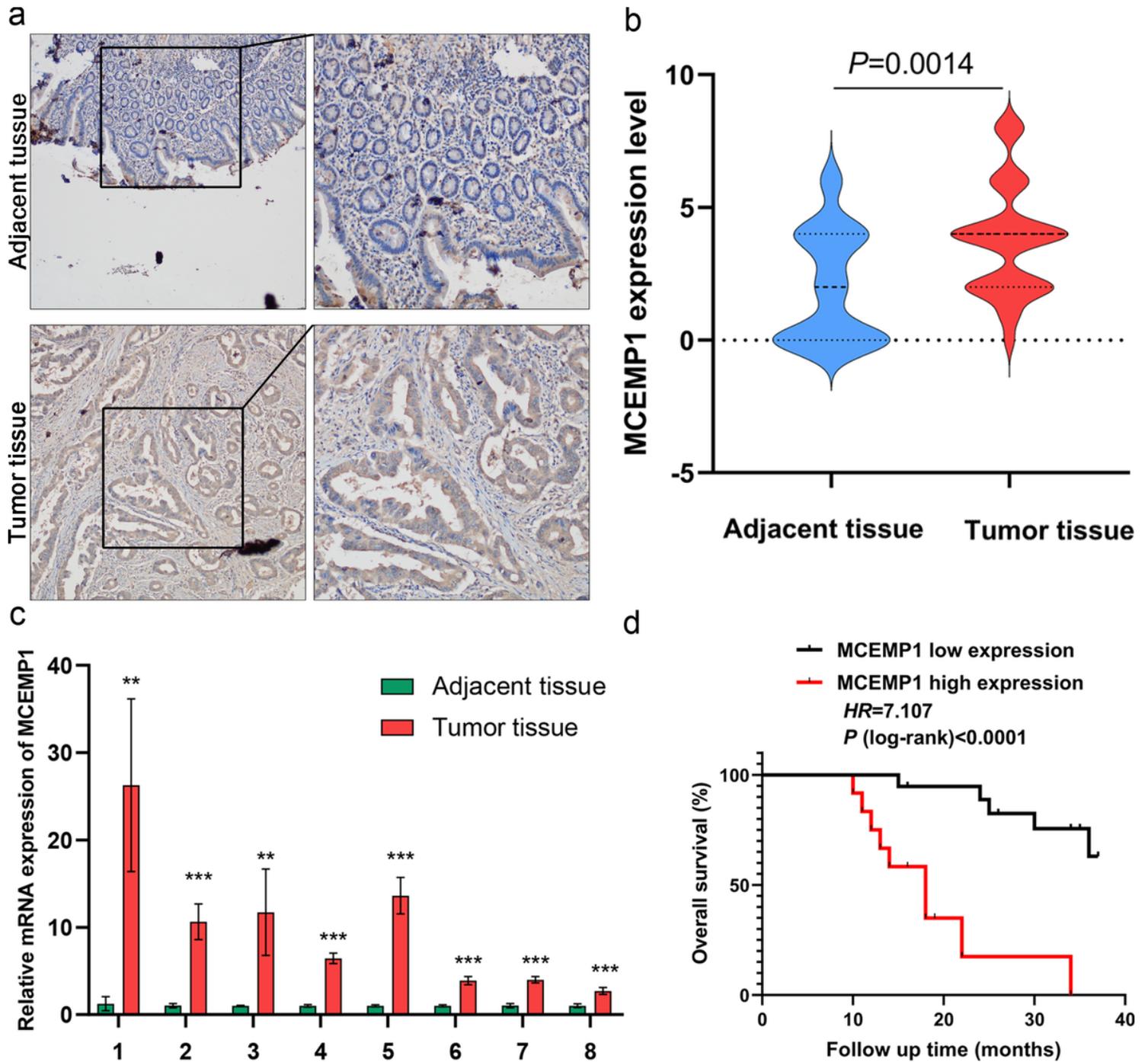


Figure 5

Validation of MCEMP1 expression and survival in tissues. (a, b) Immunohistochemistry analysis of MCEMP1 protein expression in AGC and normal tissues. (c) The mRNA expression of MCEMP1 in AGC and normal tissues by qRT-PCR. (d) The Kaplan-Meier survival analysis of AGC patients with high and low MCEMP1 expression.

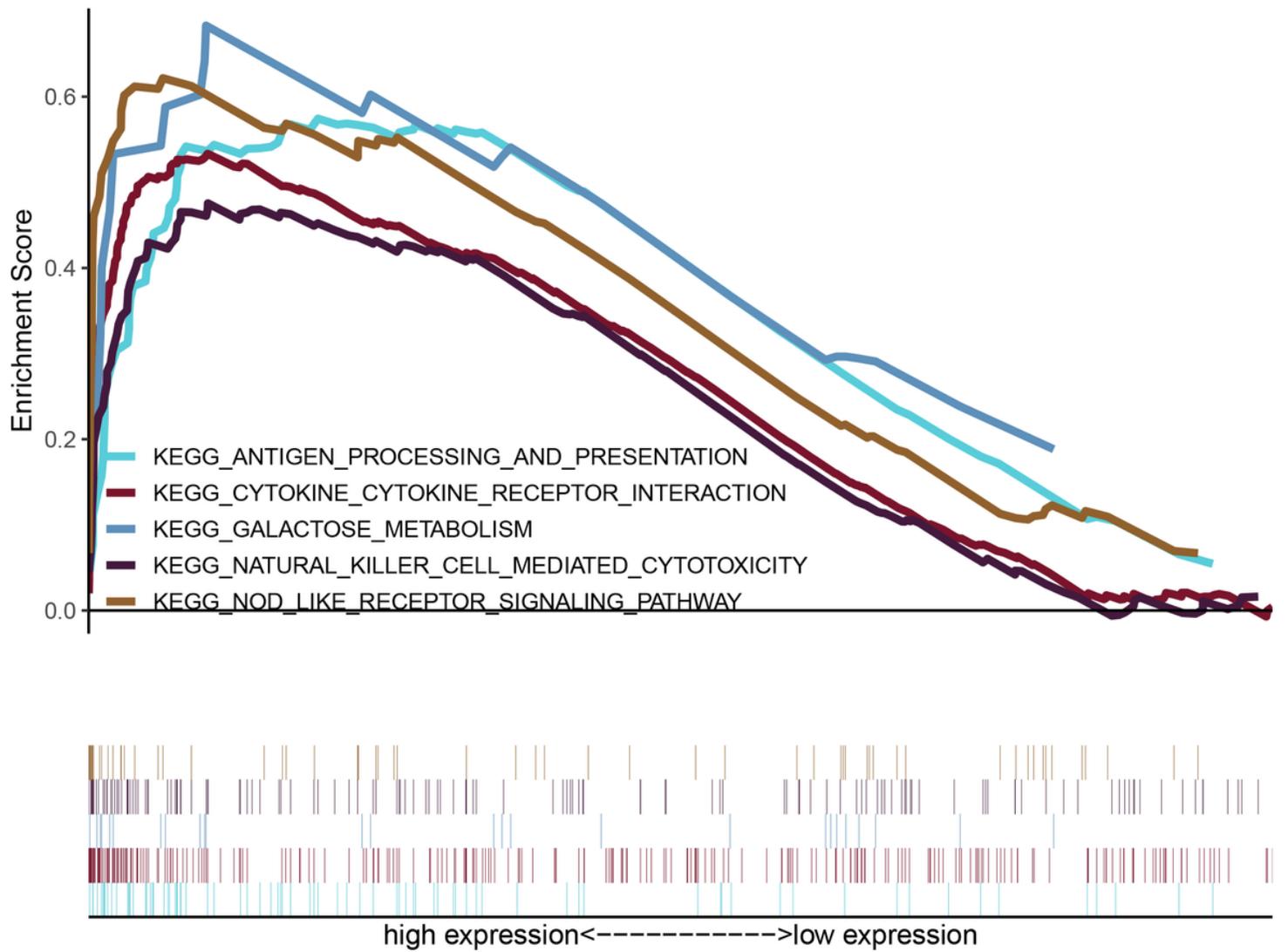


Figure 6

GSEA of samples with high MCEMP1 expression.

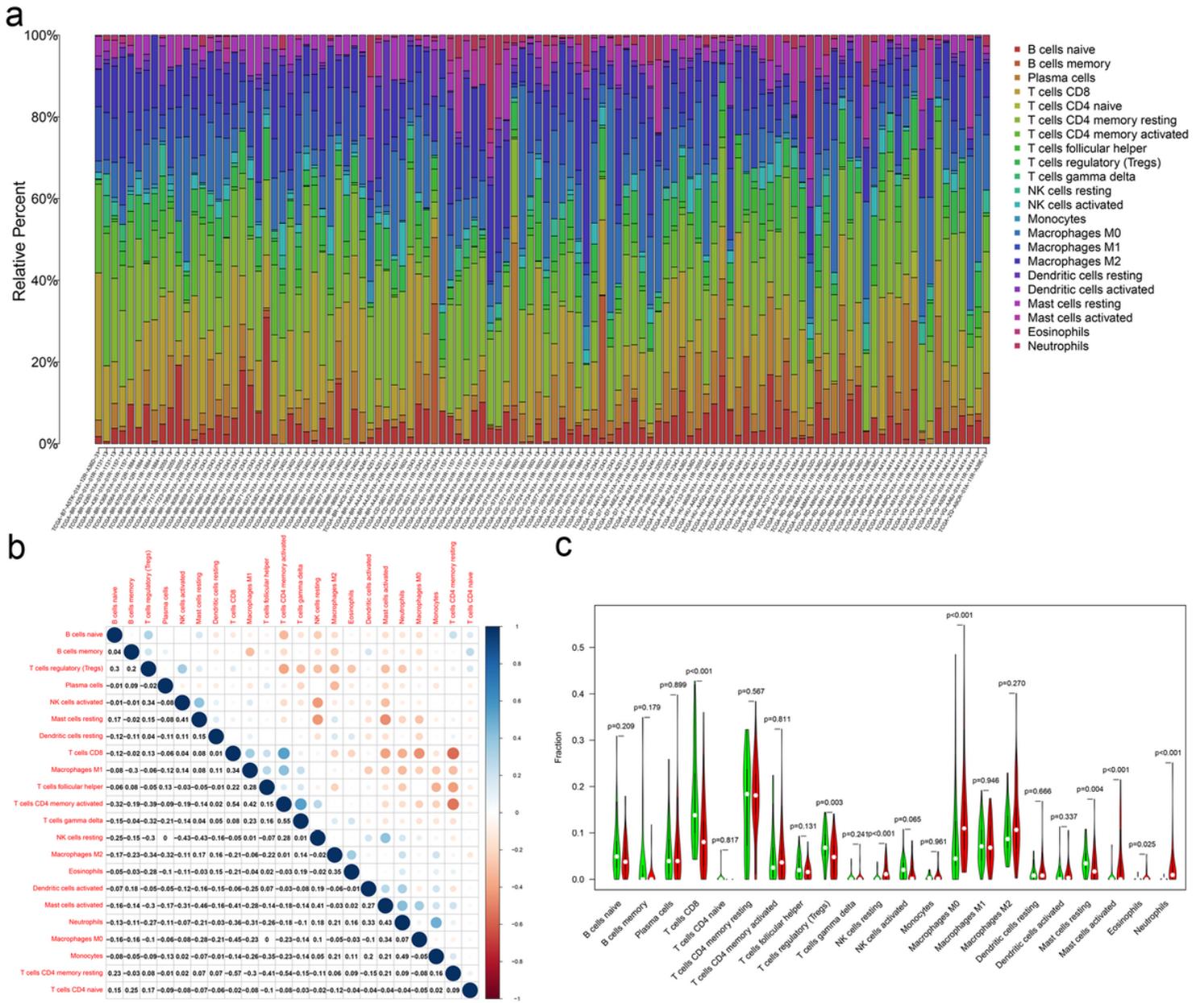


Figure 7

Analysis of tumor infiltrated immune cells in AGC with CIBERSORT. (a) The fractions of 22 kinds of infiltrating immune cells in AGC. (b) The correlation between 22 kinds of infiltrating immune cells. (c) Violin diagram for the levels of immune cells between AGC and normal samples.

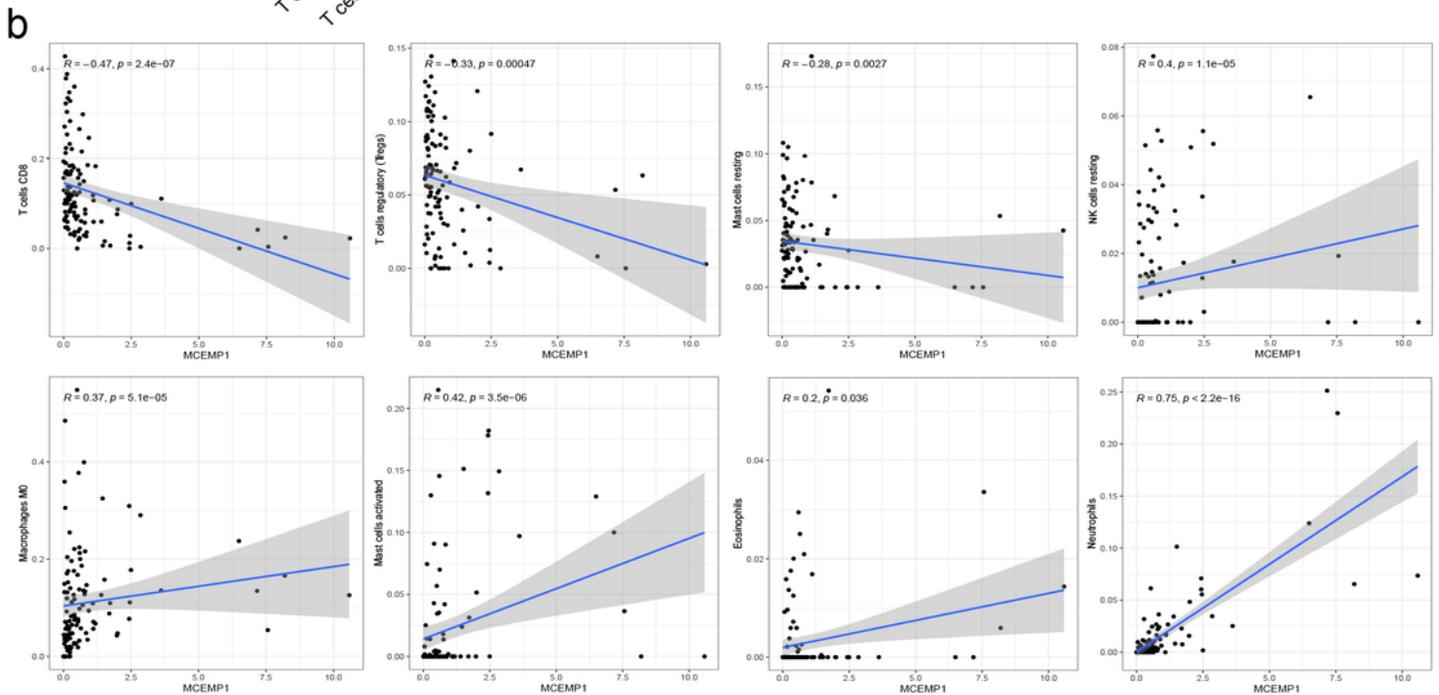
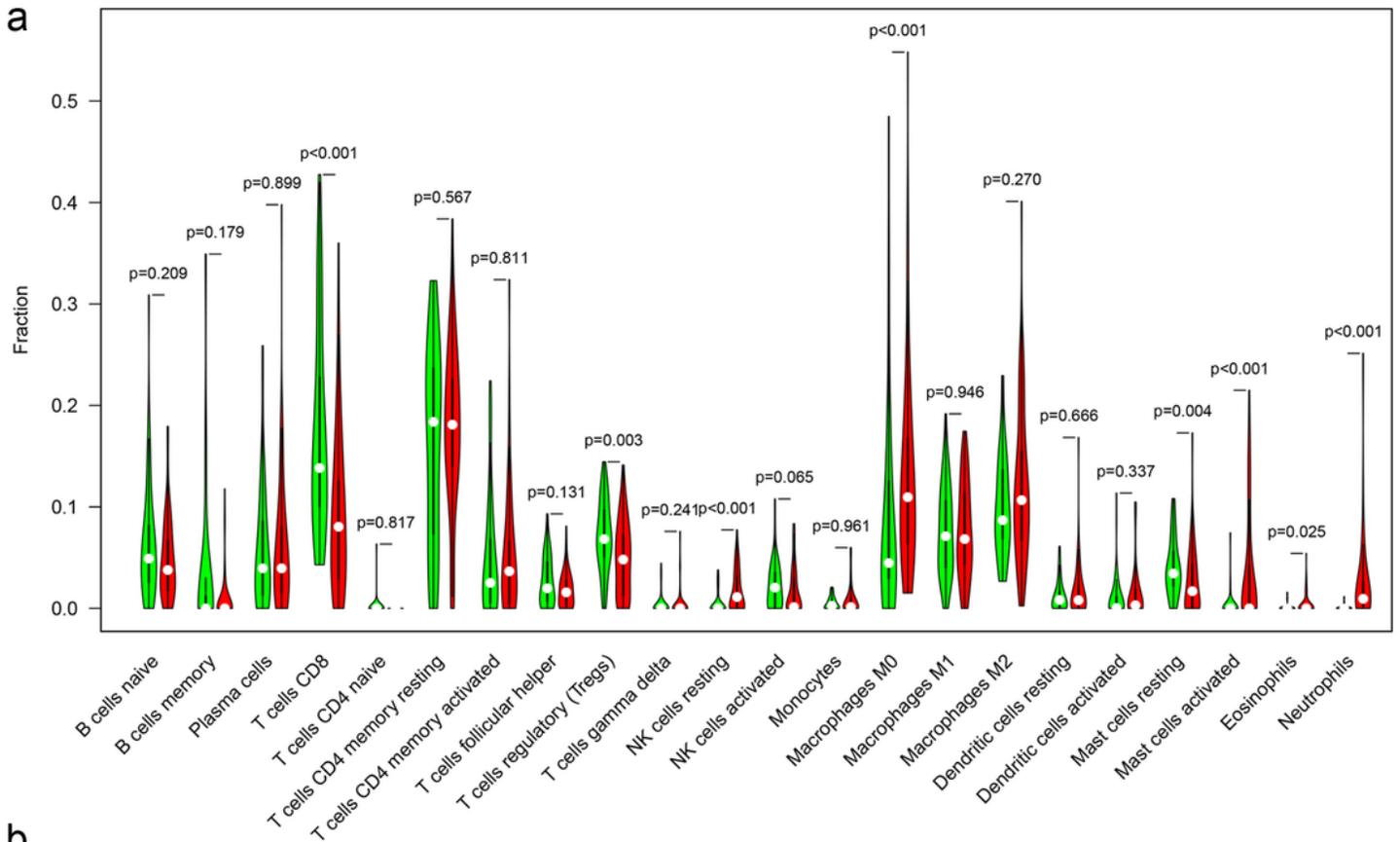


Figure 8

Association between the fractions of infiltrating immune cells and the expression levels of MCEMP1. (a) Violin plot showed the ratio differentiation of infiltrating immune cells with the expression high MCEMP1 expression and low MCEMP1 expression. (b) The correlation between 8 kinds infiltrating immune cells and the expression of MCEMP1.