Tumor-infiltrating immune cell activated memory CD4+ T cells predicts prognosis in gastric cancer

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Tumor-infiltrating Immune Cell Activated Memory CD4+ T Cells Predicts Prognosis in Gastric Cancer

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Abstract

Background: Tumor-infiltrating immune cells (TIICs) play an important role in gastric cancer pathogenesis and are promising prognostic biomarkers. However, the prognostic value of activated memory CD4+ T cells remains unclear.

Methods: The aim of this study is to explore the value of activated memory CD4+ T cells and the risk model predicting the prognosis in GC. We analyzed the composition of 22 TIICs in GC by the CIBERSORT algorithm using RNA-seq data, we also evaluated the prognostic value of TIICs by constructing the risk model. Furthermore, we performed Kaplan-Meier survival and Cox regression analysis to evaluate the prognosis of GC.

Results: The results showed that the high expression of activated memory CD4+ T cells indicated a better overall survival. Risk score was identified as an independent prognostic

factor which means activated memory CD4+ T cells can predict prognosis well in gastric cancer.

Conclusions: Our study verified the expression of *IL23A* was associated with the overall survival of GC patients, and its low expression indicated a poor prognosis, which confirms the reliability of activated memory CD4+ T cells as a prognostic factor in gastric cancer.

Key words

Tumor-infiltrating Immune Cell; CD4+ T Cells; Gastric Cancer; Immunohistochemically

Highlights

1. The expression of *IL23A* was associated with the overall survival of GC patients, and *IL23A* low-expression had a poor overall survival in GC.

2. High expression of activated memory CD4+ T cells had a better overall survival in GC.

3. Activated memory CD4+ T cells can be an independent prognosis factor in GC.

Background

Gastric cancer (GC) is a heterogeneous tumor [1]. The incidence and mortality of GC in malignant tumor worldwide are increasing [2]. GC was the second most common cancer in China [3]. At present, the main treatment of GC is the most radical surgery. The 5-year survival rate with advanced gastric cancer remains only 5-20% in 10 months of median overall survival [4]. The mechanism of GC is still unknown, so novel therapeutic ways and prognostic biomarkers must be explored to improve prognosis in GC patients. The tumor microenvironment (TME) plays an important role in tumor epigenetics, tumor differentiation, immune escape, and infiltration metastasis [5]. Tumor-infiltrating immune cells (TIICs) are the

main components of TME of cancer which are associated with overall survival in pancreatic cancer [6], hepatic cancer [5], pulmonary neuroendocrine tumors, gastroenteropancreatic neuroendocrine tumors (GEP-NETs). TIICs may increase the response rate of immunotherapy. Targeting the tumor microenvironment to increase the CD4+ lymphocyte count and CD8+/FOXP3+ lymphocyte ratio could improve the clinical outcomes of PDAC patients [7]. M0 macrophages, M1 macrophages and activated memory CD4+ T cells were infiltrated significantly in CRC [8].

"Cell type Identification By Estimating Relative Subsets Of RNA Transcripts "(CIBERSORT) is a biology analytical tool that uses the deconvolution method to analyze bulky gene expression data of 22 immune cell types which using a signature matrix of 547 marker genes in amount of heterogeneous samples, and then fractions of immune cells infiltrated in the TME can be obtained. CIBERSORT has been successfully validated, and used for determining immune cell landscapes and their relations to treatment response and outcome in breast, lung and liver cancers [9, 10].

IL23A is representative markers of CD4 memory activated T cells and its receptor mainly expressed on activated memory CD4+ T cells. In the current study, based on a large scale bioinformatics analysis of The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) cohort using the CIBERSORT method, we analyzed the landscape of 22 subsets of TIICs, and evaluated the expression of activated memory CD4+ T cells and *IL23A* expression using immunohistochemistry in 40 GC patients for the first time. The results showed that activated memory CD4+ T cells may be expected to have a good prognosis.

Materials and methods

Data obtained from the GEO and TCGA cohort

Gene expression data were obtained from the TCGA and GEO datasets. Raw microarray expression data of GC was collected in GSE19826 including 15 GC tissues and 12 normal tissues from GEO database. 375 GC tissues and 32 adjacent non-tumorous tissues samples were collected from TCGA database. Relevant clinical characteristics of cancer cases were likewise collected. Patients with a follow-up time < 30 days or a lack of pathological diagnosis were not included in the study.

Identification of immune cells infiltration

A total of 19 553 genes were identified from TCGA database. 210 significantly differential expression genes were selected from GEO database using edgeR package. 22 immune cells were identified by using the CIBERSORT algorithm and selected for the next analysis, with CIBERSORT $P < 0.05$ considered significant [11].

Immunohistochemical validation of activated memory CD4+ T cells

40 GC patients were collected from Tianjin Medical General Hospital (Tianjin, China) from October 2009 to October 2019 in this study. GC patients were undergoing curative resection with no neoadjuvant therapy before surgery. All tissue slides of each GC sample were examined and confirmed by two experienced pathologists. Tissue microarray was performed according to previous study [11]. Immunohistochemical (IHC) slides were analyzed using Image-proplus 6.0 software (Medi a Cybernetics, USA). All results were tested by two pathologists. To validate the expression of activated memory CD4+ T cells, the protein expressions of *IL23A*

which were respectively representative markers for CD4 memory activated T cells were analyzed by using relevant primary antibodies (Bs-18146r, 1:200; Bioss) for quantitative IHC. A H-score（histochemistry score） was calculated by using the reported formula for *IL23A* expression[12].

Statistical analysis

Immune cells with CIBERSORT $P < 0.05$ were taken into the subsequent analysis. TIICs correlation and clinical factors in the TCGA and GEO cohorts were analyzed by Wilcox test and visualized by corrplot package. Survival package was used for overall survival analysis, including univariate and multivariate analyses based on Cox regression. Then risk model was constructed according to previous survival analysis. Survival rate of ROC analysis was calculated by time ROC R package. The expression of *IL23A* was assessed by the two-tailed paired t-test using GraphPad Prism 5 software. $P < 0.05$ were considered as statistically significant. All analyses were performed using R software (version 3.3.2).

Results

The landscape of TIICs in the GEO and TCGA databases

GSE19826 from GEO database and TCGA GC genes expression data were analyzed by using CIBERSORT algorithm [12] and the edgeR package of R software. The landscapes of 22 subsets of TIICs were shown in Table 1 and Figure 1A, C. 22 subsets of TIICs expression in every samples were revealed in heatmap in Figure 1B, D. The highest negative correlation was CD8+ T cells and resting memory CD4+ T cells. Neutrophils showed a positive correlation with mast cells activated as showed in Figure 2A from TCGA cohorts. Among TIICs, NK cell

resting and T cell gamma delta had opposite expression changes in the GEO cohorts, activated NK cells and monocytes showed the highest positive correlation (Figure 2B). In Figure 2C, monocytes, plasma cells, activated memory CD4+ T cells, macrophages M0, M1 and M2 were differentially expressed. M0, M1 macrophages, mast cells activated, T cells CD4 memory resting, T cells CD4 naive were differentially expressed in GSE19826, the results were presented in Figure 2D.

TIICs expression and clinical factors with prognosis

R packages ('survival') and ("survminer") were used to analyze the relationship of prognosis with TIICs and clinical factors. We found that age (P=0.004) and tumor-infiltrating immune cell activated memory CD4+ T cells (P=0.008) were associated with patients' prognosis (Figure 3A). In this study, the high-expression of activated memory CD4+ T cells and Tregs had a better overall survival (Figure 3B, C).

To determine a novel prognostic index based on TIICs, we used activated memory CD4+ T cells and T cells gamma delta to construct a risk model (Figure 3D). According to a previous study, survival risk of each GC patient was evaluated as follows: Risk score = (-4.989)×expression of activated memory CD4+ T cells + (20.165)×expression of T cells gamma delta[13]. GC patients were ranked according to the risk score from low to high, and we showed the population follow-up time and genes heatmap by the ranking (Figure 3E, 3F).

Relationship of prognostic risk model with clinical factors

Based on the median value of the risk score, GC patients were divided into high-risk group and low-risk group. We found that the survival curves of the high-low risk group were significantly different, and the low-risk group had a better survival period (Figure 4A). To verify the value

of this model, the ROC curves were described and the area under the ROC of risk score demonstrated that the risk score meets basic criteria for potential consideration for the detection of gastric carcinoma in humans (Figure 4B). Univariate Cox regression analysis identified clinical factors statistically significantly associated with OS were age (HR = 1.039, 95% CI = 1.013-1.067; P = 0.004), risk score (HR = 1.747, 95% CI = 1.292-2.363; P < 0.001). Multivariable analyses adjusted for known prognostic factors revealed that age (HR = 1.029, 95% CI = 1.005-1.053; P = 0.017), stage (HR = 1.460, 95% CI = 1.069-1.993; P = 0.017) and risk score (HR = 1.640, 95% CI = 1.221-2.204; P = 0.001) were identified as independent prognostic factors (Table S1, Figure 4C and 4D). So, the risk score model (P=0.001) can predict prognosis well. This study showed that the expression of activated memory CD4+ T cells was associated with a better prognosis.

Relationship of prognosis between IL23A expression and clinical factors in GC tissues

Interleukin 23 (IL23) is a heterogeneous and important proinflammatory cytokine, composed of IL23A and the p40 subunit of interleukin 12 (IL12B), and mainly acts on activated memory CD4+ T cells, by promoting its proliferation. IL23A receptor is mainly present in activated memory CD4+ T cells. IL23A can be used as the main surface marker of activated memory CD4+ T cells. Identification of expression of IL23 in cancer tissues and adjacent tissues of gastric cancer patients proved the content of activated memory CD4+ T cells in cancer patients at different stages and the accuracy as an independent prognostic factor. Furthermore, it also clarified that IL23 played an important role in cancer development and prognosis. *IL23A* expression was evaluated by IHC using a tissue microarray including paired GC samples. This set of experiments showed that *IL23A* was upregulated in 50% (20/40) of GC tissues,

respectively (Figure 5A). We found that *IL23A* was differentially expressed between in 40 GC tissue samples and 40 adjacent non-tumorous tissues, moreover, the expression of IL23A was significantly upregulated in gastric cancer tissues (Figure. 5B, p =0.0015). To further analyze the expression of *IL23A* in gastric cancer, we used TCGA data to analyze the expression of *IL23A* from the UALCAN database (http://ualcan.path.uab.edu/analysis.html). The result indicated that the expression of *IL23A* was significantly increased in STAD (stomach adenocarcinoma) than in normal patients (Figure 5C). The results of gastric cancer tissue IHC were divided into high scores and low scores according to the level of H-score, and the survival analysis was performed. The results showed that the *IL23A* high expression group had significantly better survival (Figure 5D).

We found that *IL23A* gene was differentially expressed between in 40 GC tissue samples and 40 adjacent non-tumorous tissues. The result indicated that the expression of *IL23A* was significantly increased and was higher in STAD than in normal patients. To evaluate the relationship of clinical factors and *IL23A* expression with overall survival, we analyzed the clinical data using GraphPad prism 5 software. The results showed that low expression of *IL23A* was associated with a poor prognosis.

Discussion

TIICs play an important role in tumorigenesis, tumor immunity, tumor invasion and drug resistance [14, 15]. TIICs interacting with cancer cells can change the TME and affect clinical outcomes. In gastric cancer, TIICs expression can predict poor survival [16], but memory T cell (Tm) and its subsets were good immune indicators for gastric cancer [17]. However, the

role of tumor-infiltrating immune cell activated memory CD4+ T cells in gastric cancer is still unclear.

To evaluate the prognostic value of TIICs, we revealed the levels of 22 immune cells infiltrated in the TME of GC and their associations with clinical outcome. Our results showed that monocytes, plasma cells, activated memory CD4+ T cells, macrophages M0, M1 and M2 significantly infiltrated in tumors in TCGA database. However, in the GEO database, M0, M1 macrophages, mast cells activated, T cells CD4 memory resting, T cells CD4 naive were differentially expressed in GSE19826. Significantly expressed cell types are somewhat different from TCGA while the overall trend is consistent, which maybe probably due to the small sample size. We further evaluated the prognostic value of TIICs in gastric cancer. The results showed that activated memory CD4+ T cells and Tregs were associated with OS. Activated memory CD4+ T cells and age were independent prognostic factors for GC.

We constructed the prognostic risk model to estimate the relationship between clinical factors with risk score. The results show that activated memory CD4+ T cells can be an independent prognosis factor. To verify this conclusion, we performed IHC on IL23A and examined its expression in cancer tissues and confirmed that the expression of activated memory CD4+ T cells in clinical tissues was consistent with the analysis. At the same time, our research found that *IL23A* was closely related to the development and prognosis of gastric cancer. Interaction between IL23 and macrophages has a significant impact on the immunotherapy of cancer[18, 19]. Blogowski showed that patients with all types of gastric neoplasms have significantly lower *IL23A* levels[20]. Wang suggested that low concentrations of IL23 promoted bladder tumor progression and high expression of IL23 had a better prognosis[21]. Hussain found that

tumors highly expressing IL23 were associated with long-term survival in pancreatic ductal adenocarcinoma, while IL23 may delay the metastatic potential of pancreatic cancer[22]. In summary, our results are suggestive and congruent with previous studies. It's warranted to explore the mechanism of IL23 in regulating tumor associated macrophages in GC in our future work.

Conclusions

Taken together, our study verified the relationship between immune cells and clinical factors and proved the high expression of activated memory CD4+ T cells had a better overall survival, IL23A low-expression had a poor overall survival. Activated memory CD4+ T cells may be an independent prognosis factor.

Availability of data and materials

The original data of the present study can be found at TCGA database (https://portal.gdc.cancer.gov/) and GEO dataset (https://www.ncbi.nlm.nih.gov/geo/).

List of abbreviations

TIICs: Tumor-infiltrating immune cells;

GC: Gastric cancer;

TME: tumor microenvironment;

GEP-NETs: gastroenteropancreatic neuroendocrine tumors;

CIBERSORT: Cell type Identification by Estimating Relative Subsets of RNA Transcripts;

TCGA: The Cancer Genome Atlas;

GEO: Gene Expression Omnibus;

IHC: Immunohistochemical;

IL23: Interleukin 23;

STAD: stomach adenocarcinoma.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Part of the data used in this study are from The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) and the Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/).

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

JW analyzed the data and generated the manuscript. YQ performed IHC experiment and revised the manuscript. XG collected and interpreted the data, TL revised the manuscript. YK designed

and supported the study and revised the manuscript. All authors read and approved the final manuscript.

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Not applicable.

Supplementary material

Table S1. Statistics of TIIC expression in TCGA and GEO databases.

Table S2. Univariate and multivariate regression analysis of clinical factors and risk score with OS in GC.

Table S3. H-score statistics of IHC of 40 gastric cancer patients.

Table S4. Clinical information statistics of 40 GC patients for IHC.

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Figure legends

Figure 1. Composition of 22 TIICs selected in the TCGA and GEO databases with CIBERSORT $p < 0.05$. (**A**)Fractions of 22 TIICs in 375 tumor and 32 normal samples in TCGA. (**B**) Fractions of 22 TIICs in 26 tumor and 28 normal samples in GEO. (**C**)Expression of 22 TIICs in heatmap in TCGA. (**D**) Expression of 22 TIICs in heatmap in GEO.

Figure 2. Correlations and Comparisons of 22 TIICs in the TCGA and GEO databases. (**A**) Correlations of 22 TIICs in 375 tumor and 32 normal samples in TCGA. (**B**) Correlations of 22 TIICs between 26 tumor and 28 normal samples in GEO. (**C**) Comparisons of immune cells in 375 tumor and in 32 normal tissues in TCGA. (**D**) Comparisons of immune cells in 26 tumor and in 28 normal tissues in GEO.

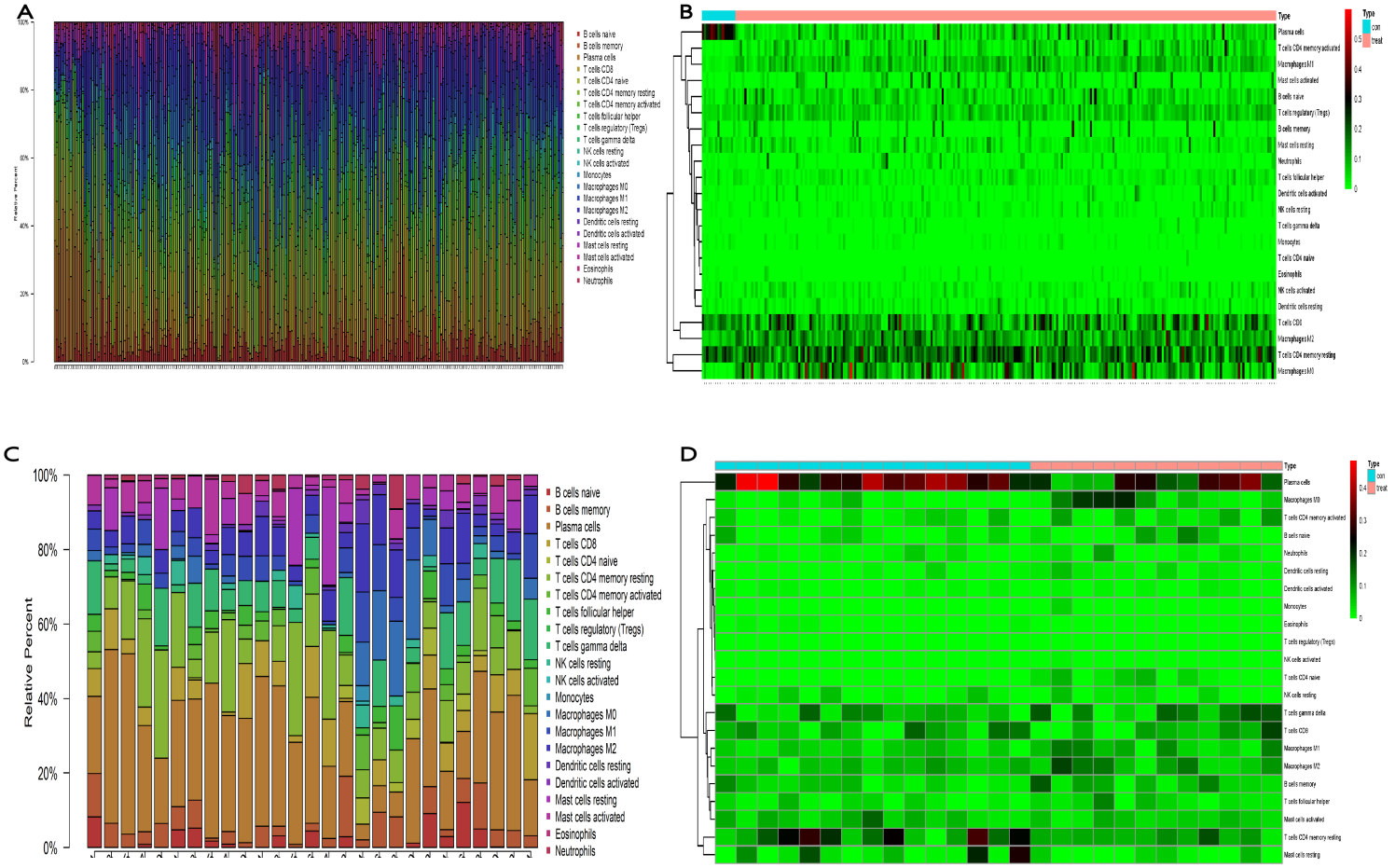
Figure 3. Over survival and multivariate Cox regression analysis between activated memory CD4+ T cells and clinicopathological factors and risk score analysis of the prognostic model in gastric cancer. (**A**). Multivariate Cox regression analysis (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (**B**). Kaplan-Meier curve showing the overall survival based on high expression and low expression patients for activated memory CD4+ T cells. (**C**). Kaplan-Meier curve showing the overall survival based on high expression and low expression patients for T cells gamma delta. (**D**) Expression heatmap of activated memory CD4+ T cells and T cells gamma delta in gastric cancer. (**E**) The low-score and high-score groups of gastric cancer patients based prognostic signature. (**F**) Survival status and duration of gastric cancer patients.

Figure 4. Univariate and multivariate Cox regression analysis of the prognostic model in gastric cancer and relationship of the expression of activated memory CD4+ T cells and T cells

gamma delta with clinicopathological factors. (**A**) Kaplan-Meier curve showing the overall survival based on high risk and low risk patients based on the prognostic model. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (**B**) Receiver operating characteristics (ROC) curves analysis of survival prediction by the prognostic model for risk score and clinicopathological factors. (**C**) Univariate Cox regression analysis of the risk score and clinicopathological factors. (**D**) Multivariate Cox regression analysis of the risk score and clinicopathological factors.

Figure 5. Immunohistochemical staining and expression analysis of *IL23A* in GC. (**A**) a-d: IHC for *IL23A* (activated memory CD4+ T cells) in high expression; e-h: IHC for *IL23A* (activated memory CD4+ T cells) in low expression. (**B**) The expression levels of *IL23A* in GC and adjacent normal tissues from Tianjin hospital. (**C**) The expression levels of *IL23A* in GC and adjacent normal tissues from TCGA data. (**D**) The expression levels of *IL23A* with overall survival.

Figures



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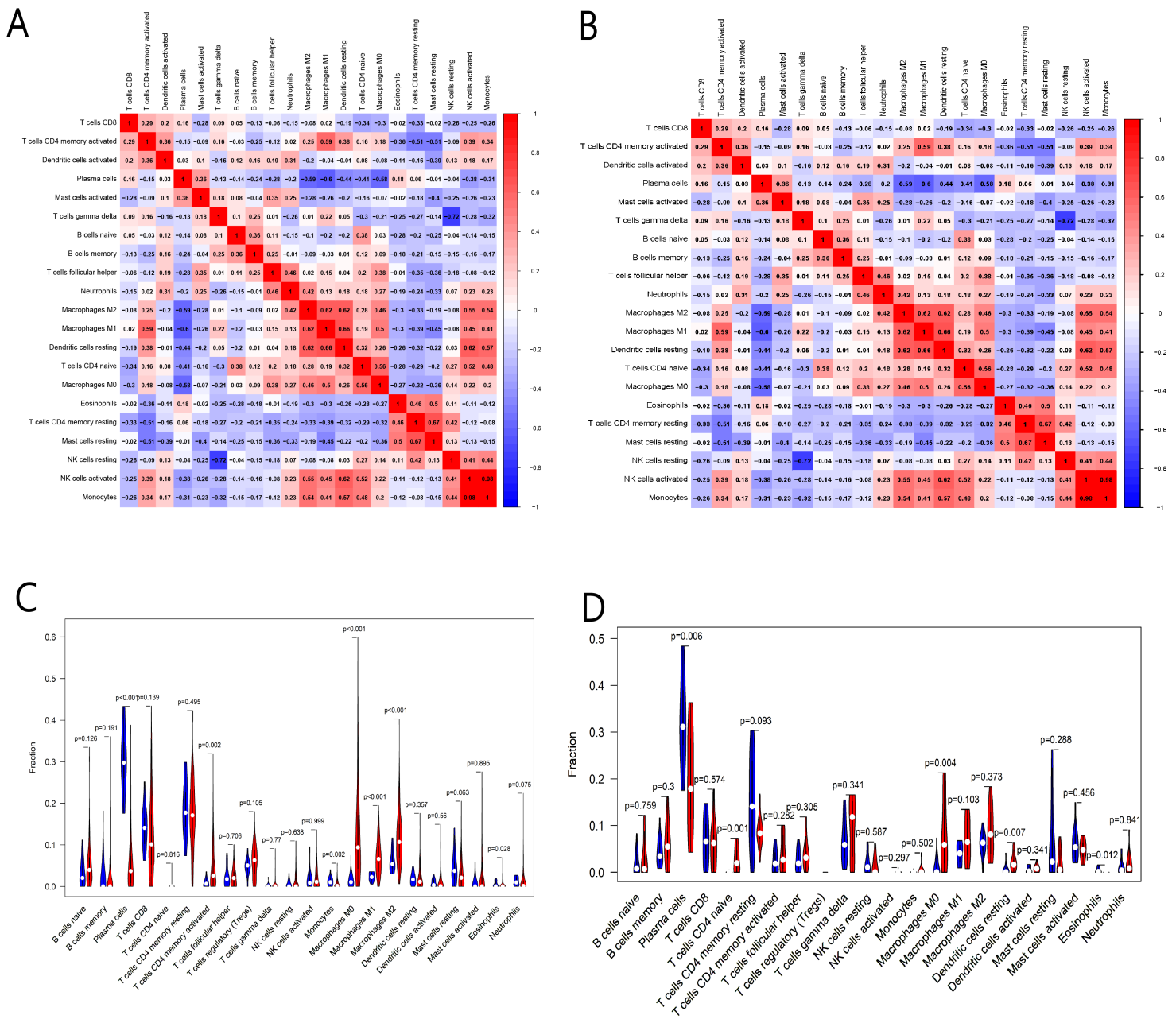

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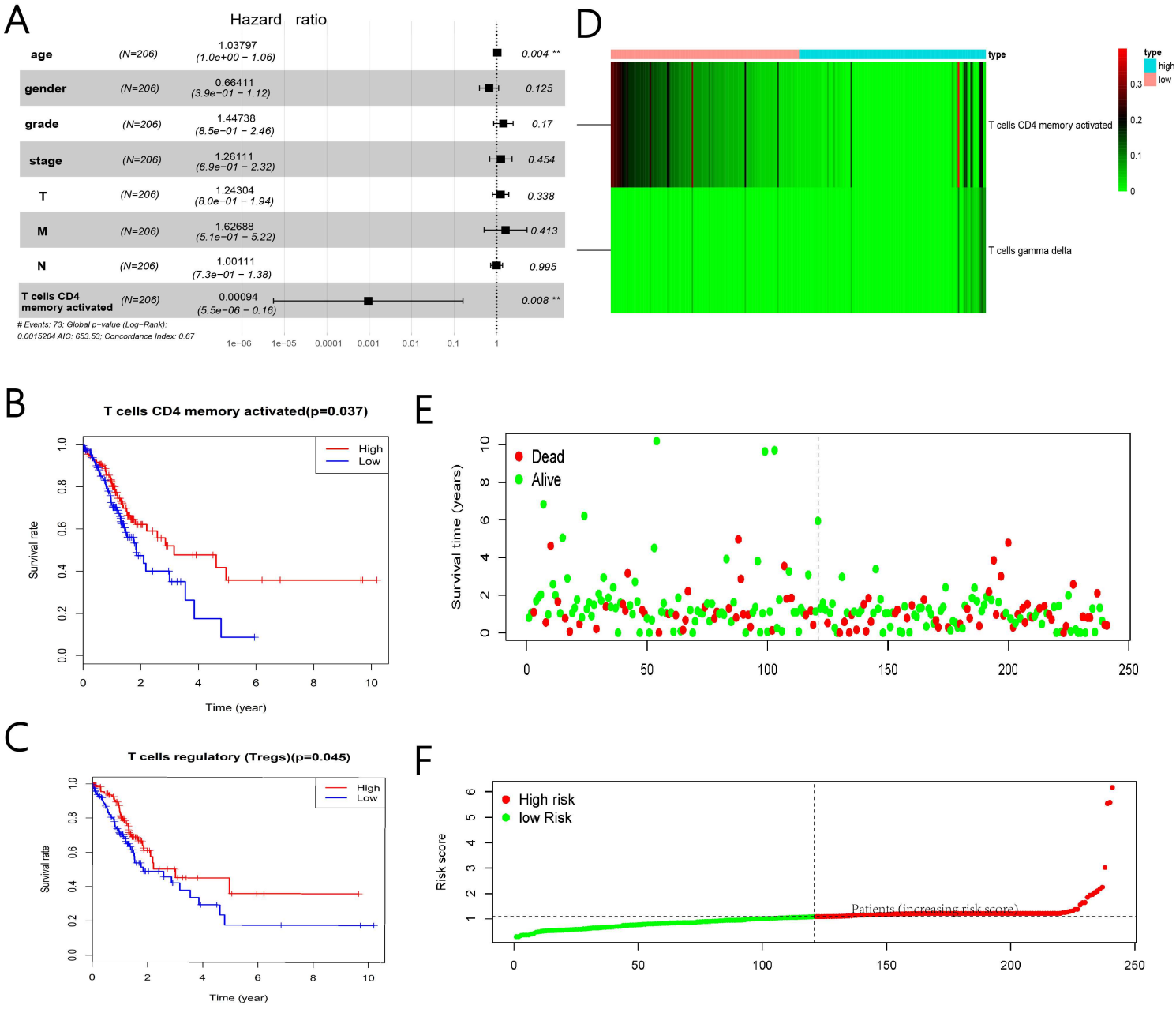

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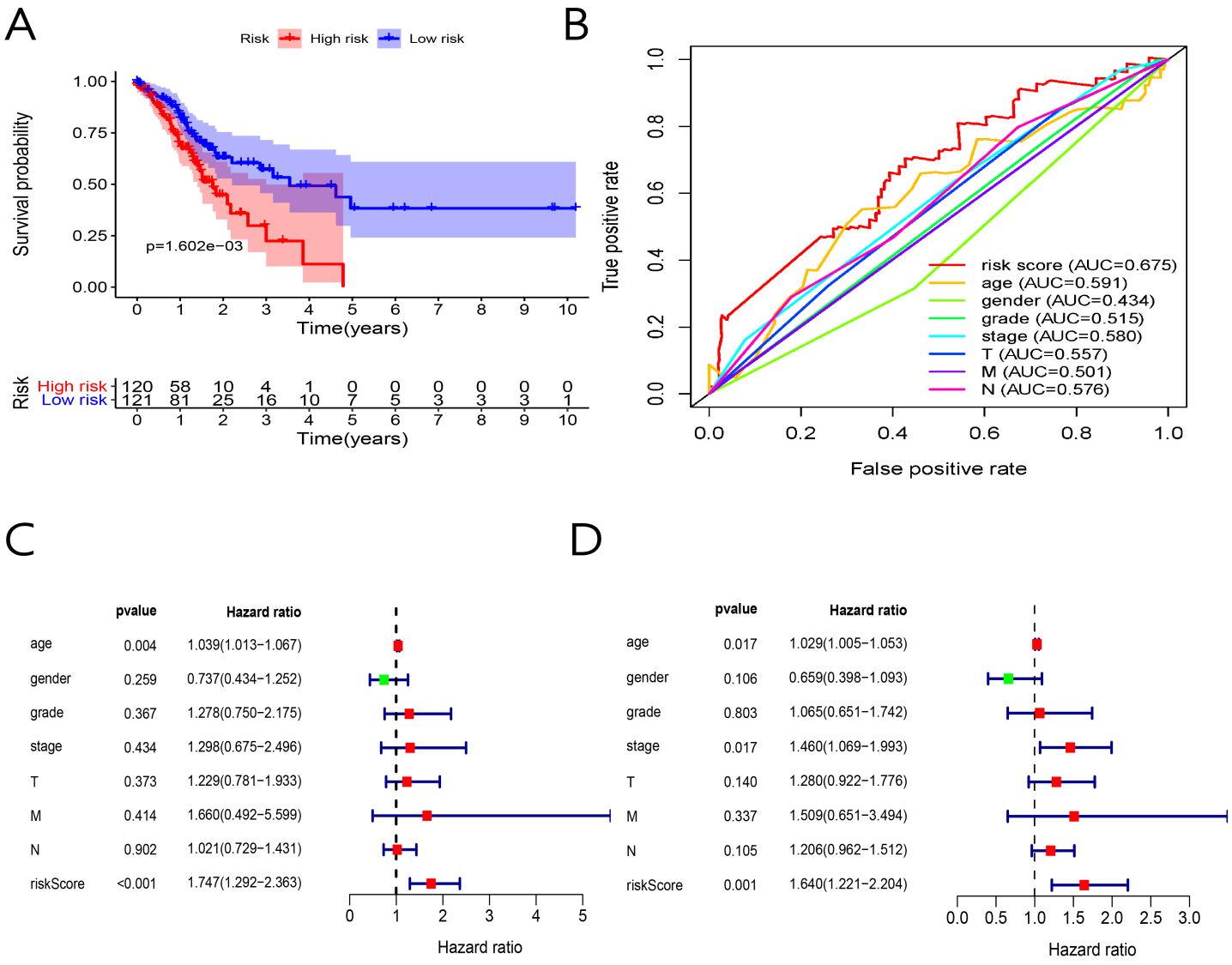

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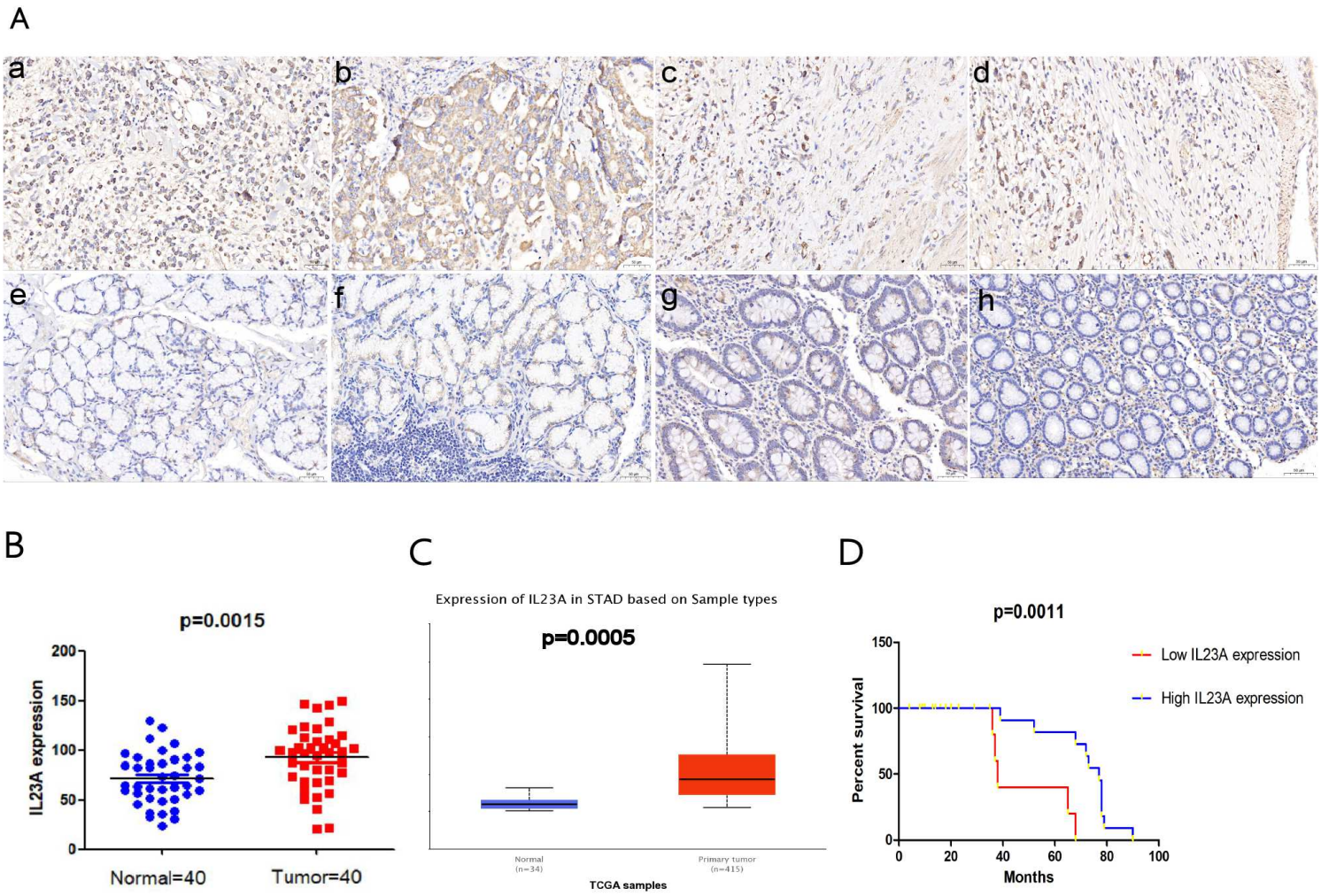

Figure 5

Immunohistochemical staining and expression analysis of IL23A in GC. (A) a-d: IHC for IL23A (activated memory CD4+ T cells) in high expression; e-h: IHC for IL23A (activated memory CD4+ T cells) in low expression. (B) The expression levels of IL23A in GC and adjacent normal tissues from Tianjin hospital. (C) The expression levels of IL23A in GC and adjacent normal tissues from TCGA data. (D) The expression levels of IL23A with overall survival.

Supplementary Files

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- TableS3.xlsx
- TableS2.xlsx