Patterns of immune infiltration in gastric cancer and their clinical significance

Yin Jin
Wenzhou Medical University First Affiliated Hospital
Liping Tao
Wenzhou Medical University First Affiliated Hospital
Shengnan Li
Shanghai Jiao Tong University School of Medicine
Shuqing Jin (zjwzjsq@163.com)
Wenzhou Medical University First Affiliated Hospital
Weiyang Cai (caiweiyang@sjtu.edu.cn)
Wenzhou Medical University First Affiliated Hospital https://orcid.org/0000-0001-7733-4015

Research

Keywords: Gastric cancer, Tumor- infiltrating immune cells, Molecular subtypes, Immune score

Posted Date: August 5th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-41448/v1

Version of Record: A version of this preprint was published at Japanese Journal of Clinical Oncology on April 23rd, 2021. See the published version at https://doi.org/10.1093/jjco/hyab054.

Abstract

Background: The malignant phenotypes of cancer are defined not only by its intrinsic tumor cells but also by the tumor- infiltrating immune cells (TIICs) activated and recruited to the cancer microenvironment. However, a comprehensive introduction of gastric cancer (GC) immune cell infiltration has not been identified so far.

Reuslts: In this study, we comprehensively analyzed the TIICs abundance in GC for the first time by CIBERSORT. The fraction of TIICs subpopulations was also evaluated to determine the associations with clinical features and molecular subtypes. Unsupervised clustering analysis revealed there existed three distinct TIICs subgroups with distinct survival patterns. We also focused on analyzing the prognostic influence of TIICs in TP53, TTP and PIK3CA molecular subtypes.

Conclusions: Collectively, our data explored the differences of TIICs in GC, and these variations were likely to be important clues for prognosis and management of its future clinical implementation.

Introduction

Gastric carcinoma (GC) is the second most common cancer worldwide, which approximately accounts for 900,000 total cases and 700,000 deaths globally per annum[1]. More seriously, the overall survival (OS) for patients diagnosed with metastatic still less than 1 year[2]. Traditional tumor/lymph node/metastasis (TNM) stage has been used to assess the prognosis of GC for decades. However, due to heterogeneous group of tumor cells, the prognosis varied evidently among GC patients with the same TNM stage[3]. The malignant phenotypes of cancer are defined not only by the intrinsic cancer cells but also the TIICs which are activated and recruited to the cancer microenvironment. However, a comprehensive introduction of GC immune cell infiltration has not been identified so far. Thus, it is urgent to improve the comprehension of immune system in GC and management of its future clinical implementation.

GC develops as a consequence of chronic inflammation of the stomach lining caused by persistent H. pylori infection[2]. The bacteria seems to exert effect on the T-helper 1 response, which is characterized by T cell activation and IFN-γ production, leading to considerable tissue lesion[4, 5]. So far, data accumulated are consistent in the viewpoint that immunity plays critical role in controlling GC development and progression, and this complex process was defined as “immunoediting”[6]. In the immunoediting theory, growing cancer cells firstly are recognized and killed by innate and adaptive immune responses; next these specific surviving tumor cells and TIICs reach in a dynamic balance, which allow selective resistant tumor cells to shape “formidable” tumor clones; moreover, treg and specific immunosuppressive cytokines, including IL-10, IL-8, TNF-α, IL-1β and TGF- β would promote these resistant tumor cells growing and proliferation[6, 7].

In view of immunotherapeutic rather than anti-cancer strategies may be more effective than conventional therapy, several immune checkpoint inhibitors have revolutionized entering into clinical cancer treatment.

Breakthroughs in immune modulating therapies are currently changing the landscape of oncotherapy, particularly for GC. For example, the introduction of immune checkpoint inhibitors targeting CTLA-4 and the pathways of PD-1, have already conducted in the immunotherapy of GC[8]; adoptive immunotherapy targeted tumor-associated lymphocytes combined with chemotherapy showed a novel prognostic value over chemotherapy alone in advanced GC patients [9]. However, we ignored that there also exist many futile immune treatments and serious adverse reactions in patients with checkpoint inhibitors design. Building on this complex situation, a key step is urgent to provide an in-depth overview of GC TIICs, which brings about a profound understanding of the underpinning regulatory mechanism.

However, the traditional techniques analyzing the immune cell composition of tumors, including immunohistochemistry (IHC) and flow cytometry, have characteristic of many shortcomings. The contribution of TIICs subgroup to either the pathogenesis or protection in the stomach environment has been difficult to elucidate. Nowadays, the biology tool CIBERSORT employs deconvolution of gene expression data to estimate the fractions of 22 TIICs, which could precisely calculated the diversity and the landscape of TIICs[10, 11]. The method was successfully validated by FACS, and used for determination of the immune cell landscapes in several malignant tumors such as colon, melanoma, lung and breast cancers[12, 13]. In this study, we applied CIBERSORT, for the first time, to quantify the 22 TIICs subsets of immune response in GC in order to investigate the relationship with molecular subpopulation, survival, and clinical features. It is hoped that this immune landscape could provide a more accurate understanding for gastric cancer development and tumor molecular subtype therapy.

Materials And Methods

2.1 Data acquisition

GC gene expression data with relevant clinicopathological data were collected from the TCGA dataset up until June 2019. Only patients diagnosed with GC, with clinicopathological and survival information available, were included for follow-up study. RNAseq data was processed and quality control via limma package. Then, we screened GC microarray in GEO database (http://www.ncbi.nlm.nih.gov/gds/) up until June 2019. We searched “gastric cancer” and "Homo sapiens" term for the selection of the suitable datasets. The general work algorithm of the GEO database was summarized in the Fig S1. All studies with gene expression data (containing at least 20 samples) from primary human gastric tumours were considered eligible, with no specific exclusion criteria applied. GEO RNAseq data was also processed and qualitied control via limma package, setting $P \leq 0.01$, fold change ≥ 1.5 as the cutoff line. In total, we gathered 14 cohorts of samples for this study: GSE2685, GSE13911, GSE26899, GSE29272, GSE37023, GSE54129, GSE66229, GSE112369, GSE84426, GSE84437, GSE26942, GSE84787, GSE65801 and GSE79973 (detailed described in the table 1). Details of the design were illustrated in Figure 1 flowchart. Affymetrix SNP 6.0 arrays were downloaded from TCGA. SNP array data were normalized based on Corrected Robust Linear Model with Maximum Likelihood Distance” algorithm, setting 0.05 as the threshold[14].

2.2 Calculation of TIICs abundance and immune score

CIBERSORT is a gene expression-based deconvolution algorithm combined with LM22, which was used to characterizing immune cell composition via a series of barcode gene expression values. Thus, we calculated the relative proportions of 22 TIICs abundance via the CIBERSORT algorithm. CIBERSORT P-value represented the confidence value of each sample results. In order to estimate the effect of calculation bias, we randomly deleted gene group in increments of 10% until 10% of genes remained. The accuracy of the TIICs proportions were precise while variable was limited to cases with CIBERSORT $P \leq 0.05$ and barcode genes >40% (figure S3). The value of root mean squared error (RMSE) represented the geometric mean of GZMA and PRF1, which was another established measure for immune cytolytic activity assessment[15]. We also used ESTIMATE algorithm to assess tumor immune score, which quantified the immune activity (or immune infiltration level) as its gene expression profiles[16].

2.3 meta-analysis

We comprehensively analyzed and summarized characterizing TIICs composition in GC, so as to make more scientific conclusions. We conducted meta-analysis via Review Manager, which provided an overall effect estimate of the heterogeneity of effects across a series of chips. Continuous outcomes were estimated as standard mean difference with 95 % confidence interval. Continuous outcomes were estimated as standard mean difference with 95 % confidence interval (CI).

2.4 Statistical analyses

The enrichment scores of specific immune term were quantified by ssGSEA in the R package[17]. The specific markers for each immune gene set were listed in table S1. We also detailedly explored the relationship between TIICs and pro-inflammatory cytokines (MMP7, PTGES, PLAT, IL-33, LGNM, TLR2, CD276, SCARB1, ICAM1, CCND1, TNFRSF2A, ITGA2, TIRAP, CXCL10, CXCL9, AIM2, PLA2G2D), inflammatory cytokines (CD19, CXCL13, IL12A, IL12B, CCL5, CD8B, IFNG, CXCL10, CXCL9,GNLY, IRF1 and PRF1) and check point recognition (IDO2, TIM-3, IDO1, PDL-1, CTLA4, LAG3 and TIGIT). Associations between inferred proportions of TIICs and immunoreactions were tested using pearson correlation. GC patients with CIBERSORT $P \leq 0.05$ were included in the following survival analysis. The associations of TIICs and corresponding survival state were evaluated using Log-rank test in univariate Cox regression. To assess the association between immune infiltration and clinical features, we compared its expression via Rank sum test. Sample with qualitatively diverse TIICs patterns were also grouped using hierarchical agglomerative clustering. We combined the Elbow method and the Gap statistic to explore the likely number of distinct immune clusters in the data. This method rationally distributes each sample into a data frame, and finds the closest (most similar) pair of clusters and merge them into a single cluster. The associations between immune clusters and clinical efficacy were tested using log- rank test. Next, we detailed analyzed survival effect of each TIICs composition. All analyses were calculated using R version 3.5.2 and Graphpad Prism 6. All statistical tests performed were two sided, and the P values ≤ 0.05 were considered as statistical significance.

Results

3.1 The landscape of characterizing TIICs composition in GC

CIBERSORT coupled with LM22 allows for highly sensitive and specific discrimination of human TIICs composition, and it has been applied in previous researches[18-20]. We firstly conducted CIBERSORT to systematically describe the constituent pattern of GC immune microenvironment. As shown in the figure 2A-2C and figure S2, the TIICs in GC tissues substantially differed from that of normal gastric tissue. In particular, plasma cells and T cells CD4 memory resting were most frequent in normal tissue; whether macrophages M0 and macrophages M1 were most frequent in GC tissue (figure 2B). In order to evaluate the effect of P-value and barcode genes, we then calculated the CIBERSORT outcomes where barcode genes were randomly removed in increments of 10%[20]. Obviously, the P- value was sensitive to lessen the selection bias of barcode genes (Figure 2D and figure S3).

3.2 meta-analyze of the proportions of TIICs

To confirm the accuracy of above results, we inferred its accuracy in other independent RCC datasets both containing adjacent normal and GC specimens. GEO dataset gene expressions were measured by various platforms, as detailed in Table 1(except GSE84426 and GSE84437). In total, 1542 GC and 416 normal cases were enrolled in the subsequent analyses. We pooled different platform datasets and wiped out binding batch effect. Bar charts summaried TIICs subpopulations and CIBERSORT p-value by study (figure S4). Obviously, although above chip profiles were obtained from different specimen sources and platforms, the abundance of TIICs subpopulation did not show evident cohort bias. Furthermore, the relative proportions of 22 TIICs subpopulation were also compared between these two independent datasets (containing TCGA and GEO), and their distribution showed a high degree level of consistence (Figure 2E-2F, $P < 0.001$).

meta-analysis is a efficient and effective standard method for summarizing the results of many studies than subjective judgment. So, we conducted meta-analyze for each significant TIICs composition. Notably, the plasma (SMD =-1.18; 95 % CI, − 1.32 to -1.04; $p < 0.0001$) and mast cells resting proportions (SMD =-0.71; 95 % CI, − 1.68 to 0.27; $p = 0.16$) exhibited a decrease trend in the case group of GC patients; the T CD4+ memory activated (SMD =1.30; 95 % CI, 0.63 to 1.97, $p < 0.0001$), T cells follicular helper (SMD =0.31; 95 % CI, -0.19 to 0.82, $p = 0.23$) , T cells regulatory (SMD =0.53; 95 % CI, 0.21 to 0.84, $p = 0.001$), macrophages M1 (SMD =2.69; 95 % CI, 1.62 to 3.76, $p < 0.0001$), macrophages M0 (SMD =6.42; 95 % CI, 4.23 to 8.62, $p < 0.0001$) and mast cells activated (SMD =0.98; 95 % CI, 0.46 to 1.50, $p = 0.0002$) proportions exhibited an increasing trend in the case group of GC patients. In brief, these meta-analyze outcomes combination with prior studies demonstrated that our CIBERSORT results were powerful enough to precisely discriminate TIICs subpopulation in GC patients.

3.3 CIBERSORT p-Values Reflect the Proportion of TIICs in GC

A greater proportion of TIICs would generate a corresponding smaller p-value, which could meticulously reflect the proportion of TIICs versus non-immune cells. Then we explored CIBERSORT P-Values against immune cytolytic activity. Rooney et al defined the geometric mean of GZMA and PRF1 as cytolytic activity [15]. As shown in the figure 4A, strong relation existed between cytolytic activity and different P-value thresholds in both the GEO and TCGA datasets. On the other hand, cytolytic activity also had moderate correlation with the proportions of different TIICs subpopulations (figure 4B). As concretely shown in the figure 4C, cytolytic activity was mostly correlated with the proportion of T cells CD4 memory activated (Pearson correlation = 0.59) and T cells CD8 (Pearson correlation = 0.52).

Considering the important role of the TIICs composition in cancer progress, we then investigated their role in carcinogenesis. Common cancer immune signature processes include MHC class, HLA expression, check point recognition, IFN response, APC recognition, inflammation promotion and parainflammation. We obtained the set of genes relative immune pathways from KEGG[21] , and applied the single-sample gene-set enrichment analysis (ssGSEA) to quantify these immune signatures. We surprisingly discovered that immune cells exerted multiple effects on different immune responses in GC (figure 4D). Then, we detailedly explored the relationship between TIICs and pro-inflammatory cytokines, inflammatory cytokines and check point recognition. As can be seen from figure 4D, T cells CD4 memory activated, T cells CD8 and macrophages M1 played significant functions in above immune reaction.

3.4 Distribution of Proportion of TIICs and their clinical characteristics

Immune scores calculated based on the ESTIMATE algorithm could facilitate the quantification of the immune cells. Based on ESTIMATE algorithm, we calculated each GC patient immune score, ranging from -2862 to 2826. Setting median immune scores as the cutoff line, we divided the patients into high- and low- immune groups. As shown in the figure 5A, high immune score group was associated with poorer survival (P = 0.064 in log-rank survival test). The average immune scores of diffuse subtype GC cases ranked the highest of all 5 subtypes, followed by that of mucinous subtype, and signet ring subtype (figure 5B). Since Hp infection is a powerful factor participating in GC development, we detailedly analyzed its effect. As shown in the figure 5B, HP infection strongly improved the immune scores of GC patients. To further explore the relationship between GC progression and immune scores, we plotted the distribution of immune scores based on the TNM stage. As shown in the figure 5C, immune score accumulates with the GC advanced progression. In a word, immune score may serve as newly developing biomarkers for GC subtype classification. Based on the classification provided by Asian Cancer Research Group, MSI, TTP and TP53 are the newly biological characteristics for GC classification and treatment. When the defective mismatch repair system is defective, it results in mismatched mutations in the genome, especially in the repeating DNA (microsatellite) region, which leads to microsatellite instability (MSI). Thus, we conducted exploratory subgroup analyses immune score by molecular subtype defined by TTP, TP53 and MSI. As shown in the figure 5D, mutation of TP53, TTP and MSS reduce GC patient immune scores.

To achieve a better overview of immune subpopulation clinical characteristics, stratification analyses were performed in the entire TCGA cohort of GC patients grouped by clinical characteristics. In view of smaller P- value represented the greater confidence of sample, we selected cases with a CIBERSORT $p \leq 0.05$ for follow-up analysis. As shown in the figure S5A, the proportion of mast cells resting, macrophages M1, T cells regulatory, T cells CD4 memory activated and T cells CD8 were increased accompany with advanced tumor grade; yet mast cells activated, macrophages M0 and neutrophils were down-regulated. Macrophages M1, macrophages M2 and eosinophils can also function in diagnosing GC T stage (figure S5B). To further confirm TIICs clinical value, we applied the validation cohort in GEO datasets provided with clinical information. We only found GSE26899 possess both mRNA expression data and clinical information. As shown fig S6A-S6B, there was a similar Macrophages M1 and Macrophages M2 component tendency in the validation cohorts (The proportion of eosinophils was too tiny to analyze).

3.5 Immune clusters associated with GC prognosis

On the base of many findings, the Proportion of TIICs subgroups partly reflects the GC prognosis. Thus, we performed distinct patterns of immune infiltration analyzed by hierarchical clustering of all samples. Restricting follow-up calculations to samples with CIBERSORT $P \leq 0.05$, there were 440 patients with a median follow-up of 493.78 days for GC. Detailed results were provided in Table S2. We firstly performed hierarchical clustering analysis for each sample and selected the optimal number of clusters by combining Elbow method and Gap statistic method. The scatter diagram identified three well independent clusters (figure 6A-6B). Cluster1 were defined by relative high level of T cells CD4 memory resting, low level of T cells CD4 memory activated, T cells CD8 and Macrophages M0. Moreover, these three immune clusters were closely correlated with distinct survival pattern(Figure 6C).

Now that we have confirmed the close association with immune clusters with GC prognosis, we further concretely investigated the association of each TIICs subpopulation statistically with GC overall survival and PFS by Cox regression analysis. As shown in the figure 6D, T cells follicular helper (OR: HR=1.31, 95%CI=1.25-1.48, P=0.13; PFS: HR=1.47, 95%CI=1.05-2.18, P=0.048), T cells CD4 memory activated (OR: HR=1.55, 95%CI=1.11-2.17,P=0.0093; PFS: HR=1.73, 95%CI=1.16-2.58, P=0.00097) were significantly associated with poor overall survival and PFS, whereas B cells naïve (PFS: HR=0.57, 95%CI=0.36-0.88, P=0.011) was correlated with improved PFS. In line with the hierarchical clustering outcomes, TIICs subpopulation, specially for Macrophages subgroup, T cells follicular helper and T cells CD8, made great contributions to tumorigenesis

3.6 Prognostic Subsets of TIICs in GC molecular subtypes

Epstein-Barr virus infection and the microsatellite instability subtype (respectively accounting for 9% and 22% of cancers in TCGA cohort[22]), have been proven momentous immunological significance for GC clinical therapy. EBV-positive cancer is characterized for frequent PIK3CA mutations[22]; MSI tumor is characteristic of hypermutated, chromosomal instability and TP53 mutation[23]. It has been proven that SNPs were closely associated with GC risks and prognosis. As shown in the figure S7, TTN and TP53 were the most characteristic SNP gene in GC. Combined with the above conclusions, we conducted

exploratory PI3K, TTN and TP53 subgroup analyses of the prognostic effect of 22 TIICs subgroups. As shown in the figure S8A, there was a significant different proportion of T cells CD8, T cells CD4 memory resting, T cells CD4 memory activated, T cells follicular helper, T cells regulatory, T cells gamma delta, NK cells activated, Macrophages M0, Macrophages M1 and neutrophils in PI3KCA mutant and wildtype ($P \leq 0.05$, table S3). There was also a significantly diverse distribution of TIICs in TP53 and TTN wild and mutant GC patients (Figure S7B-7C and Table S4-S5).

We next carried out subgroup exploratory analyses of the prognostic effect of 22 TIICs subsets by molecular subgroup defined by PIK3CA, TP53 and TTP based on TCGA classier. As shown in the figure 7, T cells follicular helper showed the strong association with poor outcome in TTP mutant (HR=1.75, 95% CI 1.04–2.94) and TP53 wildtype (HR=1.57, 95% CI 1.01–2.34). In contrast, dendritic cells activate and T cells regulatory respectively associated with poorer outcome in the TTP wildtpye and TP53 mutant. Most strikingly, monocytes and polarization macrophages have the most effect in SNP-related gastric patients, with the largest effect observed monocytes in the TP53 mutant GC (HR=0.51, 95% CI 0.3–0.86). Similarly, B cells naïve show association with better outcome in the TP53 wildtype (HR=0.65, 95% CI 0.42–0.96). Collectively, these results indicated that there existed considerable variability in the nature of the immune system across GC—partly determined by key molecular characteristics of the primary cacner—and that this exert significant effect clinical outcome.

Discussion

Approximately 20% of tumor deaths worldwide are associated with unresolved infection or chronic inflammation[24]. Chronic inflammation has been proven associated with pancreatic cancer, colorectal cancer, hepatocellular carcinoma, cervical carcinoma, gastric cancer and etc[24, 25]. In addition to malignant cancer cells, immune cells have also been regarded as essential components in the tumour progression [26]. The improved survival was proven in tumor treated with immunotherapeutic strategies, which highlighted the importance of the immune cell in cancer microenvironment. For example, immunotherapeutic specially for PD-1 by pembrolizumab monotherapy, have achieved significant effects on prolonging the survival of solid tumours, such as renal cell carcinoma, melanoma, non-small-cell lung carcinoma, cervical cancer and triple-negative breast cancer [27-29]; immunological marker has been used as a prognostic marker and targeted with specific immunotherapies in colorectal cancer, melanoma and breast cancer [30, 31] .

As for GC, the majority of patients are developed as a consequence of persistent infection with H. pylori[32]. H. pylori -induced chronic gastritis is characterized by Th2 immune response to revitalite B cells in the pathogen-driven gastritis[33];It has been found that H. pylori could be recognized by CD4 activated T cell and CD8 activated T cell and then cause IFN-γ production, leading to considerable tissue damage[5]. It is well recognized that there exists marked different types of TIICs in GC, which are significantly associated with tumor progression and survival. For example, it has found that the percentages of NK cells in GC were significantly decreased, and lower percentages of tumor-infiltrating NK cells were positively correlated with poor survival and disease progression[34]; Foxp3 + CD4 + ICOS +

effector Tregs (eTregs), which was characteristic of suppressive functions, was abundant in advanced GCs [35]; the study concluded that the numbers of CD8 + , FOXP3 + , CD3 + , CD57 + , CD20 + , CD45RO + , Granzyme B + and T-bet + infiltrating lymphocytes were closely related with improved survival in GC[36]. These large body of evidences suggest pivotal roles for However, the results obtained in GC immunotherapies are still unsatisfactory, and the majority of novel immunotherapies are still in the early phases of clinical investigation. Several factors block the development of immunotherapeutic strategies in GC. Owing to the technical limitation, immunohistochemistry and flow cytometry could not precisely identify TIICs subpopulation and frequently lead to inconsistent results in clinical researches. The contribution of TIICs to either the pathogenesis or protection in the GC has been difficult to elucidate. Thus, a comprehensive systematic assessment of the immune landscape is needed to make more accurate understanding for gastric cancer development, compared with single-factor predictors.

By conducting computer-based analytical method to public genomic data, it is now possible to overcome the shortcomings of traditional immunohistochemistry- based method, and to precisely figure out TIICs subpopulation[15]. CIBERSORT, as an emerging technologies, has been conducted in breast cancer, lung cancer, colorectal cancer, melanoma and leukocyte. We firstly performed of CIBERSORT in GC, with the order to assess the immune landscape. We downloaded overall data from TCGA and GEO dataset, it was possible generate a precise investigation of the immune response in GC. Our study firstly revealed the detail of infiltration of 22 TIICs subgroups in GC. The most common TIICs in gastric cancer tissues were macrophage M0 and macrophage M1; whereas plasma cells occupied the biggest proportion in normal gastric tissues (figure 2A). Researchers also found that the macrophages constitute one of the most abundant immune cell populations in the GC microenvironment[37]. In total, there exists 8 different TIICs subpopulation in GC development. We also obtained the same TIICs proportion alteration in systematical meta-analyze (figure 3). Surprisingly, these above conclusions are consistent with the previous results reported in the relevant study. Previous experiments have shown that M1 macrophages activating by IFN-γ and microbial components, contributes to the pathogenesis of the gastric mucosa via Wnt/β-catenin signaling[23, 38]. Tregs was more abundant in late stage GCs at the tumour margin and inside itself[38]. B-cell has been proven produce antibodies against tumor antigens, and activates specific B-cell subgroup that secrete anti-inflammatory factors, thus resting B cells could inhibit the development of gastric cancer[39].

As increasing evidences have suggested the clinical importance of immune infiltration in GC development. Thus, we also made comprehensive analysis of the clinical impact of the immune cell, which may help clinicians predict patient outcomes more reliably and precisely. We discovered Tfh and T cells CD4 memory activated were associated with poorer overall survival and PFS, but that B cells naïve was opposite for PFS. Treg mainly functions in maintaining immunological tolerance to self-antigens and suppress excessive immune responses, which inhibits cytotoxic lymphocytes and natural killer (NK) cells[38]. Previous experiments have shown that T cells follicular helper promotes tumour progression by secreting TGF-β and IL-17[40] , however, tumor-infiltrating Tregs in gastric cancer tumors have been reported to express little TGFb1[34]. Further study associating with the exact role and relative regulation mechanism of Tregs is required. It has been reported that 1macrophages may either pro-tumorigenic

effects accelerating cancer development, or anti-tumourigenic effects according to differentiation patterns into M1 or M2 subtypes [41]. Macrophages have been found may influence the aggressiveness and prognosis of GC patients. Macrophages increasing in the GC is associated with poor outcome[42, 43]. Essentially the same results were obtained in our analysize. GC is likely to contain different differentiation pattern of macrophages along with the cancer development (figure 5). Higher proportions of macrophages M1 and M2 were associated with advanced GC, suggesting that they may have discernible effect on GC development. Collectively, these findings demonstrated the potential function of tumour-associated macrophages as marked biomarkers in GC. Treatments combating the tumour macrophages are already in early phase clinical trials, which block CSF1 signalling by targeting colony stimulating factor 1 receptor [44].

We also found that Treg is strongly associated with GC progress and shorter overall survival. Tregs play key role in adaptive immune response and are also involved in H. pylori-related inflammation and bacterial persistence[22, 45]. Tregs normally maintain immunological tolerance and suppressing excessive immune responses by inhibiting cytotoxic lymphocytes, helper T activity and NK cells[36]. Whether Tregs exerted tremendous effect in GC-specific immunity-dependent mechanisms is uncertain, but there were some evidences for cancer specific responses triggered by Tregs, as well as observations of an association with clinical outcome across solid tumours. Recent study demonstrated that Treg was related with advanced GC progression and reduced patient survival[46]. Tumor infiltrating Tregs is abundant in advanced GC tissues, which produce IL-10, and further inhibit the proliferation of responder CD8 activated T cells[47]. Treg could also suppress the epithelial cell-initiated inflammatory response, which leads to bacterial overgrowth and ulcers. The accumulation of Tregs function in preventing carcinogenesis, but it may contribute GC progression and metastasis in already established tumors[47]. Thus, modulation of the regulatory function of Treg cells may be an important avenue to improve antitumor immune activity.

The wide-range apply of immunotherapy is revolutionizing the classification and treatment paradigms for GC. Evidently, the classification of GC is progressively transforming from a histological to a more pinpoint molecular clustering. For example, TCGA proposed to classify GCs into four main subgroups, basing on six molecular biology approaches: EBV-positive cancers featured by frequent PIK3CA mutations and PD-L1/PD-L2 expression level; microsatellite instability tumors frequently hypermutated; chromosomal instability tumors that are frequently TP53 mutated with RTK-RAS activation, with a high rate of CNV; and genomically stable cancers frequent altered motility and mutations in the adhesion molecules[48]. Asian Cancer Research Group also provided newly biological characteristics for Asian GC patients: Mesenchymal subgroup, MSI subgroup, Microsatellite Stable TP53 positive and MSS TP53- tumors[49]. Thus, we focused on analyzing the prognostic influence of immune cells in TP53, TTN and PIK3CA molecular mutant subtypes. As shown in the figure S7, immune infiltration cells have a sweeping effect of the survival of TP53 and TTN mutant subgroup. The TP53 gene is by far the most frequently mutated gene in human cancer[50]. However, the majority of TP53 mutations in cancer (75%) are missense mutations[50]. The exact mechanisms of mutant p53-mediated tumour development are not fully understood. We speculate that immune infiltration cells may function in reactivating mutant p53 and

restore normal p53 function[51, 52]. Also immune infiltration cells may regulate tumour cell environment to potentiate the reactivation of mutant p53 function (Targeting mutant p53 for efficient cancer therapy). Immunotherapy may be a promising therapeutic approach for these specific subtypes of GC patients.

In general, improvements in the GC immune cells characterization may also function in identifying patients who could benefit from targeted therapy, and novel data suggest that immunotherapeutic approaches might represent an emerging opportunity. In summary, our analysis of 22 immune cell subsets in GC has revealed significant relation with cancer progress, as well as highlighting possible targets for future immunotherapy study. This was a large analysis of GC from a great breadth of studies in which we enumerated the immunoreaction in detail. Hence, our conclusions are confidently reliable and credible. Despite abundant outcomes obtained form this present study, there still exist several disadvantages. Although systematically combined TCGA and GEO genomic data, we still could not eliminate cohort bias and heterogeneity exist in this analyze, which reduces the reliability of results. On the other hand, though the results were statistically compelling, all findings and conclusions were derived from database. We have started to validate its confidence in GC tissues and cell lines. We have taken effect to build specimen bank of GC, and to explore the function and distribution of tumour-infiltrating immune cells.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets analysed during the current study are available in the the TCGA (https://cancergenome.nih.gov/) and GEO dataset(https://www.ncbi.nlm.nih.gov/geo/).

Competing interests

The authors declare that they have no competing interests

Funding

This study was supported by National Natural Science Foundation of China (81672354) and the Scientific Research Foundation of the First Affiliated Hospital of Wenzhou Medical University (Grant No. FHY2019002)

Authors' contributions

Weiyang Cai and Shuqing Jin conceived and designed the experiments. Liping Tao interpreted the patient data. Weiyang Cai analyzed the data. YinJin and Shengnan Li wrote the manuscript.

Acknowledgements

Not applicable

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A: Global cancer statistics, 2012. *CA: a cancer journal for clinicians* 2015, 65(2):87-108.
2. Bilici A: Treatment options in patients with metastatic gastric cancer: current status and future perspectives. *World journal of gastroenterology* 2014, 20(14):3905-3915.
3. Wong SS, Kim KM, Ting JC, Yu K, Fu J, Liu S, Cristescu R, Nebozhyn M, Gong L, Yue YG *et al*: Genomic landscape and genetic heterogeneity in gastric adenocarcinoma revealed by whole-genome sequencing. *Nature communications* 2014, 5:5477.
4. Ito T, Kobayashi D, Uchida K, Takemura T, Nagaoka S, Kobayashi I, Yokoyama T, Ishige I, Ishige Y, Ishida N *et al*: Helicobacter pylori invades the gastric mucosa and translocates to the gastric lymph nodes. *Laboratory investigation; a journal of technical methods and pathology* 2008, 88(6):664-681.
5. Wen S, Felley CP, Bouzourene H, Reimers M, Michetti P, Pan-Hammarstrom Q: Inflammatory gene profiles in gastric mucosa during Helicobacter pylori infection in humans. *Journal of immunology* 2004, 172(4):2595-2606.
6. Finn OJ: A Believer's Overview of Cancer Immunosurveillance and Immunotherapy. *Journal of immunology* 2018, 200(2):385-391.
7. Deng B, Zhu JM, Wang Y, Liu TT, Ding YB, Xiao WM, Lu GT, Bo P, Shen XZ: Intratumor hypoxia promotes immune tolerance by inducing regulatory T cells via TGF-beta1 in gastric cancer. *PloS one* 2013, 8(5):e63777.
8. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB *et al*: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *The New England journal of medicine* 2012, 366(26):2443-2454.
9. Kono K, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, Sekikawa T, Matsumoto Y: Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. *Clinical cancer research : an official journal of the American Association for Cancer Research* 2002, 8(6):1767-1771.
10. Liu X, Wu S, Yang Y, Zhao M, Zhu G, Hou Z: The prognostic landscape of tumor-infiltrating immune cell and immunomodulators in lung cancer. *Biomedicine & pharmacotherapy = Biomedecine & pharmacotherapie* 2017, 95:55-61.
11. Bense RD, Sotiriou C, Piccart-Gebhart MJ, Haanen J, van Vugt M, de Vries EGE, Schroder CP, Fehrmann RSN: Relevance of Tumor-Infiltrating Immune Cell Composition and Functionality for

Disease Outcome in Breast Cancer. *J Natl Cancer Inst* 2017, 109(1).

12. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, Hoang CD, Diehn M, Alizadeh AA: Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015, 12(5):453-457.
13. Angelova M, Charoentong P, Hackl H, Fischer ML, Snajder R, Krogsdam AM, Waldner MJ, Bindea G, Mlecnik B, Galon J *et al*: Characterization of the immunophenotypes and antigenomes of colorectal cancers reveals distinct tumor escape mechanisms and novel targets for immunotherapy. *Genome biology* 2015, 16:64.
14. Carvalho B, Bengtsson H, Speed TP, Irizarry RA: Exploration, normalization, and genotype calls of high-density oligonucleotide SNP array data. *Biostatistics* 2007, 8(2):485-499.
15. Rooney MS, Shukla SA, Wu CJ, Getz G, Hacohen N: Molecular and genetic properties of tumors associated with local immune cytolytic activity. *Cell* 2015, 160(1-2):48-61.
16. Yoshihara K, Shahmoradgoli M, Martinez E, Vegesna R, Kim H, Torres-Garcia W, Trevino V, Shen H, Laird PW, Levine DA *et al*: Inferring tumour purity and stromal and immune cell admixture from expression data. *Nature communications* 2013, 4:2612.
17. Foroutan M, Bhuva DD, Lyu R, Horan K, Cursons J, Davis MJ: Single sample scoring of molecular phenotypes. *BMC Bioinformatics* 2018, 19(1):404.
18. Ali HR, Chlon L, Pharoah PD, Markowetz F, Caldas C: Patterns of Immune Infiltration in Breast Cancer and Their Clinical Implications: A Gene-Expression-Based Retrospective Study. *PLoS medicine* 2016, 13(12):e1002194.
19. Edgar R, Domrachev M, Lash AE: Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic acids research* 2002, 30(1):207-210.
20. Xiong Y, Wang K, Zhou H, Peng L, You W, Fu Z: Profiles of immune infiltration in colorectal cancer and their clinical significant: A gene expression-based study. *Cancer Med* 2018, 7(9):4496-4508.
21. Kanehisa M, Sato Y: KEGG Mapper for inferring cellular functions from protein sequences. *Protein science : a publication of the Protein Society* 2019.
22. Cancer Genome Atlas Research N: Comprehensive molecular characterization of gastric adenocarcinoma. *Nature* 2014, 513(7517):202-209.
23. Wang M, Busuttil RA, Pattison S, Neeson PJ, Boussioutas A: Immunological battlefield in gastric cancer and role of immunotherapies. *World journal of gastroenterology* 2016, 22(28):6373-6384.
24. Balkwill F, Mantovani A: Inflammation and cancer: back to Virchow? *Lancet* 2001, 357(9255):539-545.
25. Selleri S, Rumio C, Sabatino M, Marincola FM, Wang E: Tumor microenvironment and the immune response. *Surgical oncology clinics of North America* 2007, 16(4):737-753, vii-viii.
26. Choi Y, Kim JW, Nam KH, Han SH, Kim JW, Ahn SH, Park DJ, Lee KW, Lee HS, Kim HH: Systemic inflammation is associated with the density of immune cells in the tumor microenvironment of gastric cancer. *Gastric cancer : official journal of the International Gastric Cancer Association and the Japanese Gastric Cancer Association* 2017, 20(4):602-611.

27. Fuchs CS, Doi T, Jang RW, Muro K, Satoh T, Machado M, Sun W, Jalal SI, Shah MA, Metges JP *et al*: Safety and Efficacy of Pembrolizumab Monotherapy in Patients With Previously Treated Advanced Gastric and Gastroesophageal Junction Cancer: Phase 2 Clinical KEYNOTE-059 Trial. *JAMA oncology* 2018, 4(5):e180013.
28. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, Gottfried M, Peled N, Tafreshi A, Cuffe S *et al*: Pembrolizumab versus Chemotherapy for PD-L1-Positive Non-Small-Cell Lung Cancer. *The New England journal of medicine* 2016, 375(19):1823-1833.
29. Bonotto M, Garattini SK, Basile D, Ongaro E, Fanotto V, Cattaneo M, Cortiula F, Iacono D, Cardellino GG, Pella N *et al*: Immunotherapy for gastric cancers: emerging role and future perspectives. *Expert review of clinical pharmacology* 2017, 10(6):609-619.
30. Mlecnik B, Bindea G, Angell HK, Maby P, Angelova M, Tougeron D, Church SE, Lafontaine L, Fischer M, Fredriksen T *et al*: Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* 2016, 44(3):698-711.
31. Church SE, Galon J: Tumor Microenvironment and Immunotherapy: The Whole Picture Is Better Than a Glimpse. *Immunity* 2015, 43(4):631-633.
32. Velin D, Michetti P: Immunology of Helicobacter pylori infection. *Digestion* 2006, 73(2-3):116-123.
33. Kido M, Tanaka J, Aoki N, Iwamoto S, Nishiura H, Chiba T, Watanabe N: Helicobacter pylori promotes the production of thymic stromal lymphopoietin by gastric epithelial cells and induces dendritic cell-mediated inflammatory Th2 responses. *Infection and immunity* 2010, 78(1):108-114.
34. Peng LS, Zhang JY, Teng YS, Zhao YL, Wang TT, Mao FY, Lv YP, Cheng P, Li WH, Chen N *et al*: Tumor-Associated Monocytes/Macrophages Impair NK-Cell Function via TGFbeta1 in Human Gastric Cancer. *Cancer immunology research* 2017, 5(3):248-256.
35. Nagase H, Takeoka T, Urakawa S, Morimoto-Okazawa A, Kawashima A, Iwahori K, Takiguchi S, Nishikawa H, Sato E, Sakaguchi S *et al*: ICOS(+) Foxp3(+) TILs in gastric cancer are prognostic markers and effector regulatory T cells associated with Helicobacter pylori. *International journal of cancer* 2017, 140(3):686-695.
36. Dolcetti R, De Re V, Canzonieri V: Immunotherapy for Gastric Cancer: Time for a Personalized Approach? *International journal of molecular sciences* 2018, 19(6).
37. Locati M, Mantovani A, Sica A: Macrophage activation and polarization as an adaptive component of innate immunity. *Advances in immunology* 2013, 120:163-184.
38. Lee K, Hwang H, Nam KT: Immune response and the tumor microenvironment: how they communicate to regulate gastric cancer. *Gut and liver* 2014, 8(2):131-139.
39. Shah S, Divekar AA, Hilchey SP, Cho HM, Newman CL, Shin SU, Nechustan H, Challita-Eid PM, Segal BM, Yi KH *et al*: Increased rejection of primary tumors in mice lacking B cells: inhibition of anti-tumor CTL and TH1 cytokine responses by B cells. *International journal of cancer* 2005, 117(4):574-586.
40. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, Taniguchi K, Yu GY, Osterreicher CH, Hung KE *et al*: Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. *Nature* 2012, 491(7423):254-258.

41. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T *et al*: Macrophage activation and polarization: nomenclature and experimental guidelines. *Immunity* 2014, 41(1):14-20.
42. Park JY, Sung JY, Lee J, Park YK, Kim YW, Kim GY, Won KY, Lim SJ: Polarized CD163+ tumor-associated macrophages are associated with increased angiogenesis and CXCL12 expression in gastric cancer. *Clinics and research in hepatology and gastroenterology* 2016, 40(3):357-365.
43. Ishigami S, Natsugoe S, Tokuda K, Nakajo A, Okumura H, Matsumoto M, Miyazono F, Hokita S, Aikou T: Tumor-associated macrophage (TAM) infiltration in gastric cancer. *Anticancer research* 2003, 23(5A):4079-4083.
44. Cassier PA, Italiano A, Gomez-Roca CA, Le Tourneau C, Toulmonde M, Cannarile MA, Ries C, Brillouet A, Muller C, Jegg AM *et al*: CSF1R inhibition with emactuzumab in locally advanced diffuse-type tenosynovial giant cell tumours of the soft tissue: a dose-escalation and dose-expansion phase 1 study. *The Lancet Oncology* 2015, 16(8):949-956.
45. Kandulski A, Malfertheiner P, Wex T: Role of regulatory T-cells in H. pylori-induced gastritis and gastric cancer. *Anticancer research* 2010, 30(4):1093-1103.
46. Mao FY, Kong H, Zhao YL, Peng LS, Chen W, Zhang JY, Cheng P, Wang TT, Lv YP, Teng YS *et al*: Increased tumor-infiltrating CD45RA(-)CCR7(-) regulatory T-cell subset with immunosuppressive properties foster gastric cancer progress. *Cell death & disease* 2017, 8(8):e3002.
47. Bagheri N, Azadegan-Dehkordi F, Rahimian G, Rafieian-Kopaei M, Shirzad H: Role of Regulatory T-cells in Different Clinical Expressions of Helicobacter pylori Infection. *Archives of medical research* 2016, 47(4):245-254.
48. Alexandrov LB, Nik-Zainal S, Wedge DC, Aparicio SA, Behjati S, Biankin AV, Bignell GR, Bolli N, Borg A, Borresen-Dale AL *et al*: Signatures of mutational processes in human cancer. *Nature* 2013, 500(7463):415-421.
49. Oh SC, Sohn BH, Cheong JH, Kim SB, Lee JE, Park KC, Lee SH, Park JL, Park YY, Lee HS *et al*: Clinical and genomic landscape of gastric cancer with a mesenchymal phenotype. *Nature communications* 2018, 9(1):1777.
50. Kandoth C, McLellan MD, Vandin F, Ye K, Niu B, Lu C, Xie M, Zhang Q, McMichael JF, Wyczalkowski MA *et al*: Mutational landscape and significance across 12 major cancer types. *Nature* 2013, 502(7471):333-339.
51. Bykov VJN, Eriksson SE, Bianchi J, Wiman KG: Targeting mutant p53 for efficient cancer therapy. *Nature reviews Cancer* 2018, 18(2):89-102.
52. Martins CP, Brown-Swigart L, Evan GI: Modeling the therapeutic efficacy of p53 restoration in tumors. *Cell* 2006, 127(7):1323-1334.

Table

Table 1 The detailed information of GEO chips

Accession	Platform	Number of normal samples	Number of cancer samples
GSE2685	GPL80	8	22
GSE13911	GPL570	31	38
GSE26899	GPL6947	12	96
GSE29272	GPL96	134	134
GSE37023	GPL96	40	114
GSE54129	GPL570	21	111
GSE66229	GPL570	100	300
GSE112369	GPL15207	6	36
GSE84426	GPL6947	0	76
GSE84437	GPL6947	0	358
GSE26942	GPL6947	12	205
GSE84787	GPL17077	10	10
GSE65801	GPL14500	32	32
GSE79973	GPL570	10	10
Total	1958	416	1542

Figures

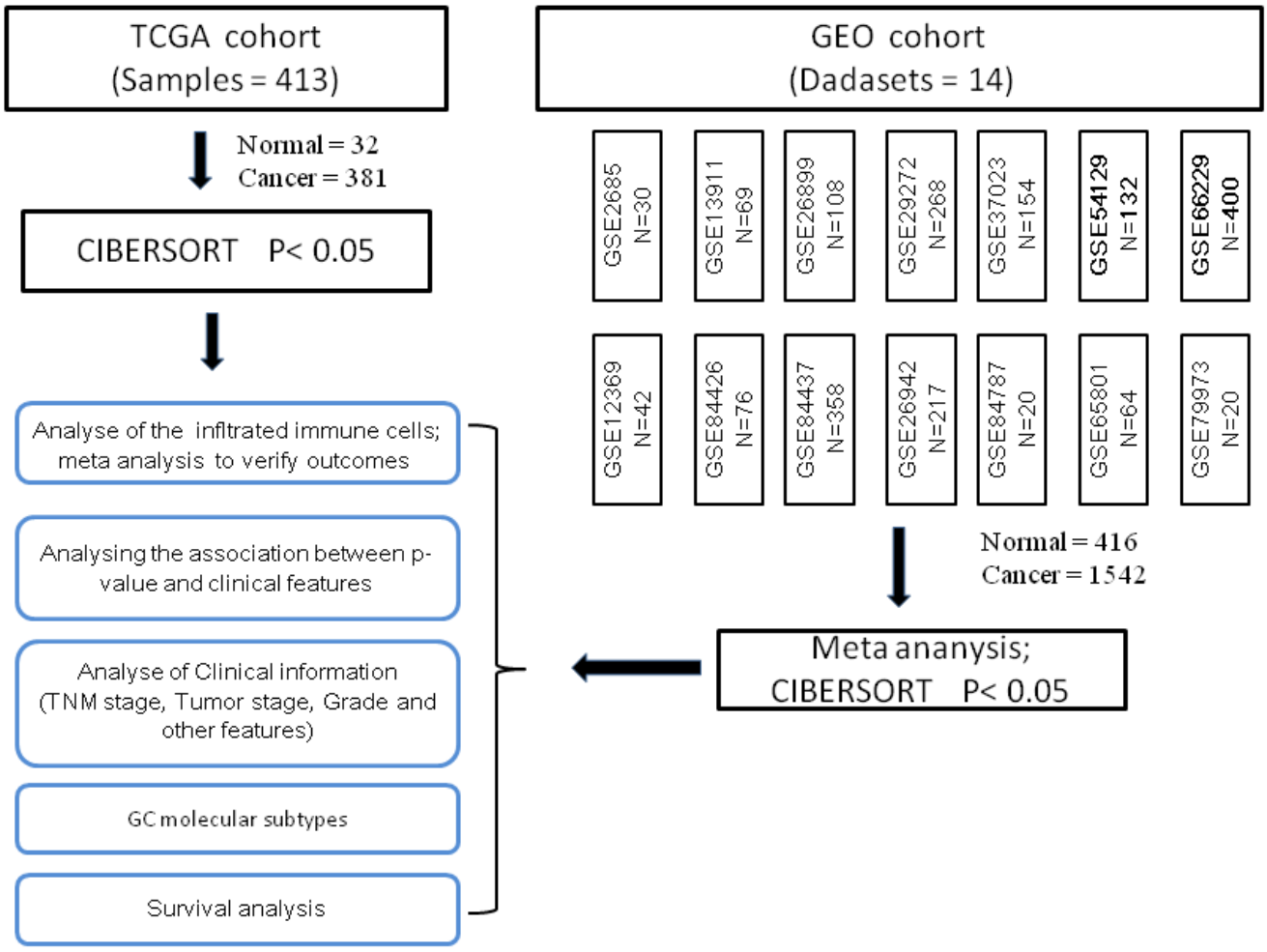

Figure 1

Flowchart of the overall study design.

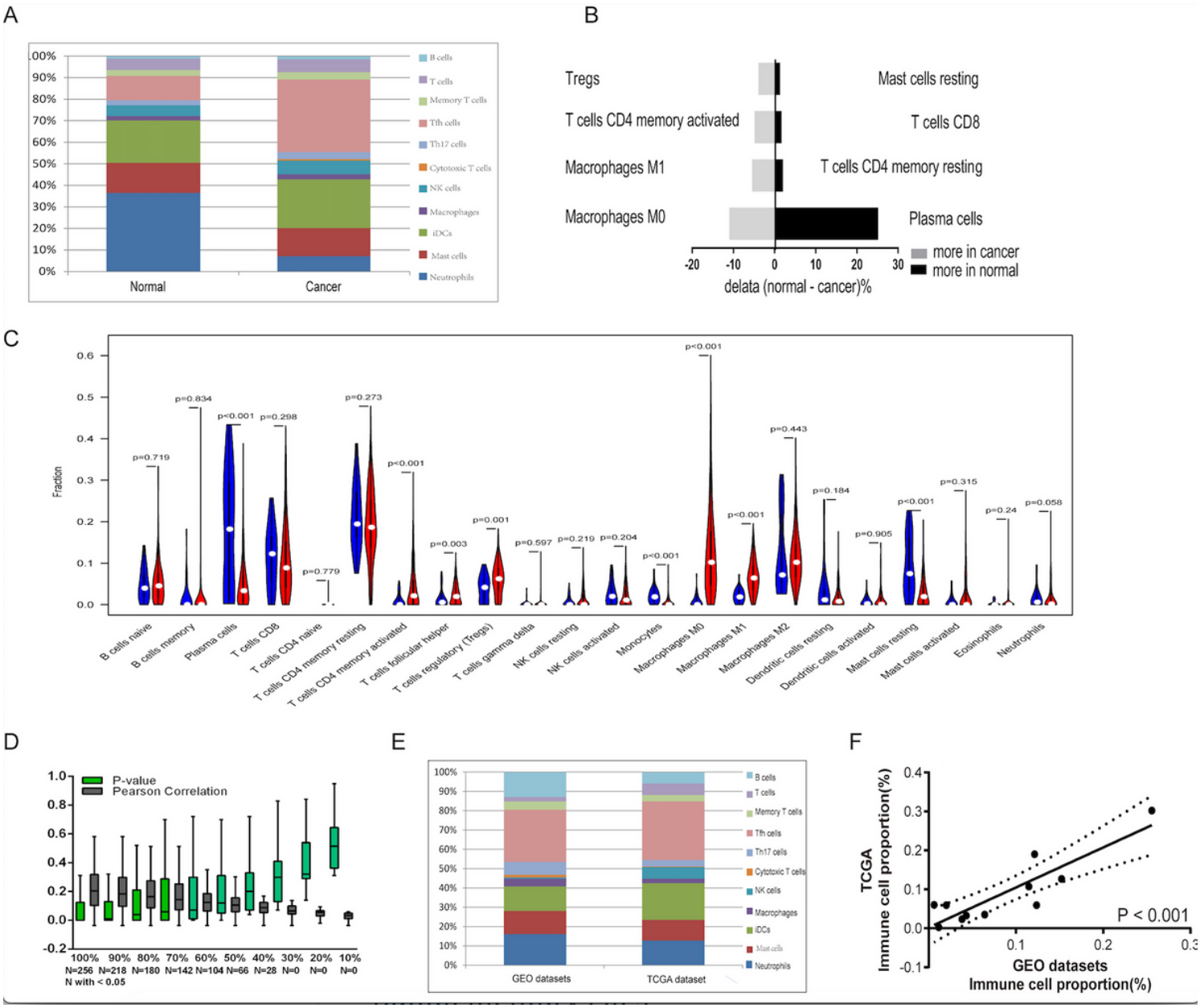

Figure 2

The performance of CIBERSORT for characterizing TIICs composition in GC. A. The proportions of TIICs subpopulation in normal and gastric cancer tissues. B. Quantified changes of infiltrating immune cell composition in normal and gastric cancer tissues. C. Violin plot of 22 TIICs subpopulation in normal and gastric cancer tissues. D. Box plot of the distribution of CIBERSORT P- value and Pearson's correlation using datasets with progressively fewer (10% increment) cases. E. The proportions of TIICs subpopulation in TCGA and GEO gastric cancer tissues. F. Comparison of TIICs subpopulation in two independent datasets

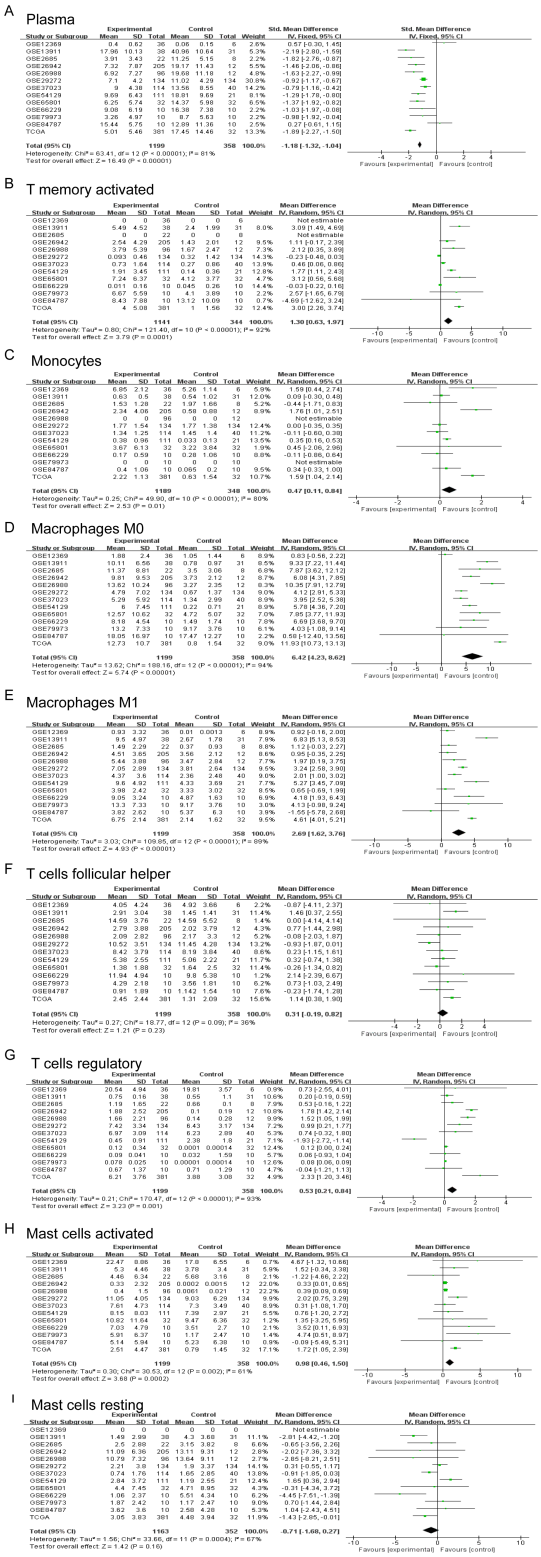

Figure 3

meta-analyze verified aberrant TIICs composition in GC

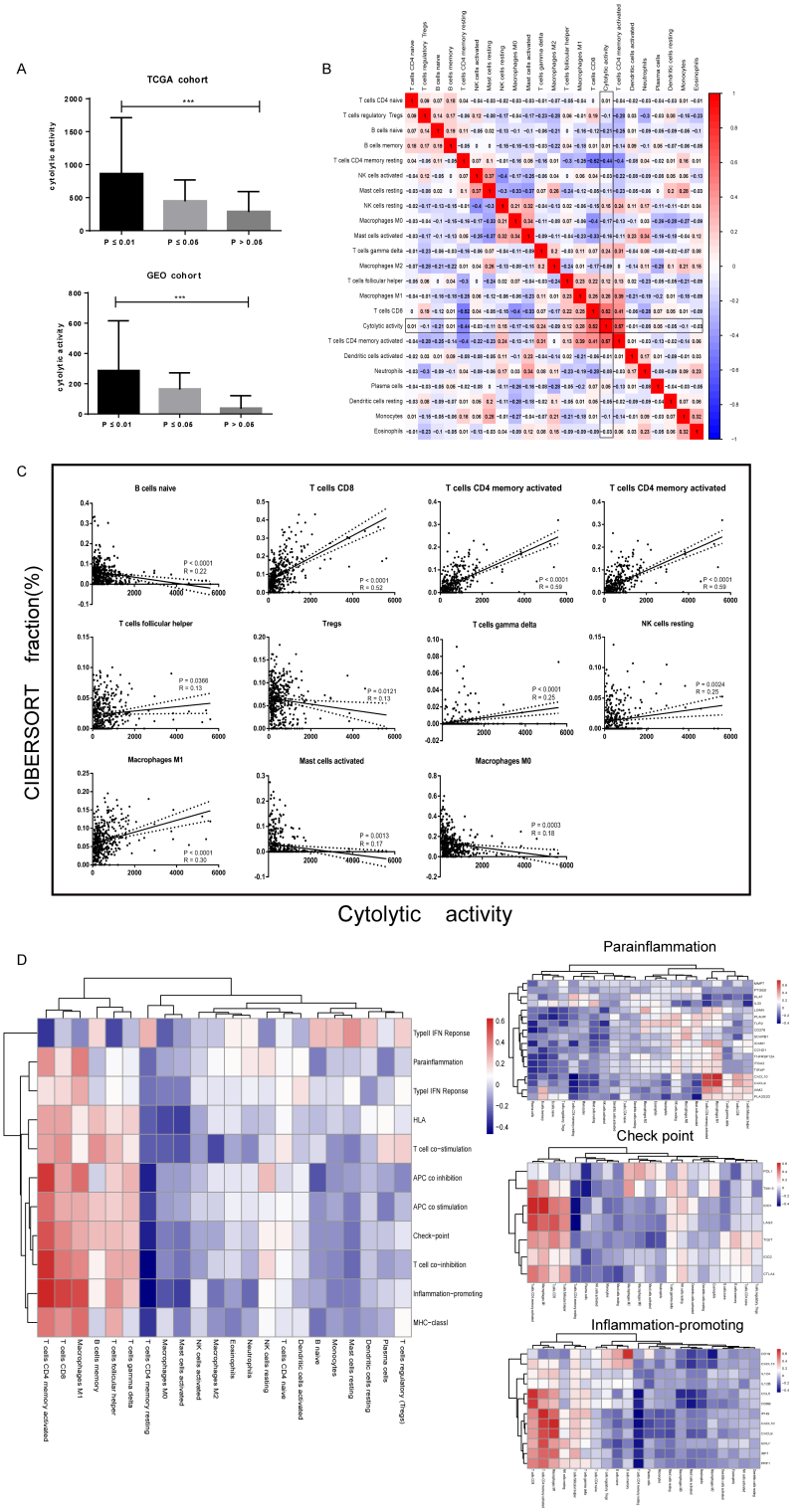

Figure 4

CIBERSORT P- values reflect the immune cytolytic activity A. The association between immune cytolytic activity and CIBERSORT P- value. B-C. Correlation bewteen immune cytolytic activity and 22 immune cell proportions in the TCGA cohort. D. ssGSEA quantify diverse immune signatures: Correlation of matrix of all 22 immune cell proportions and immune signatures.

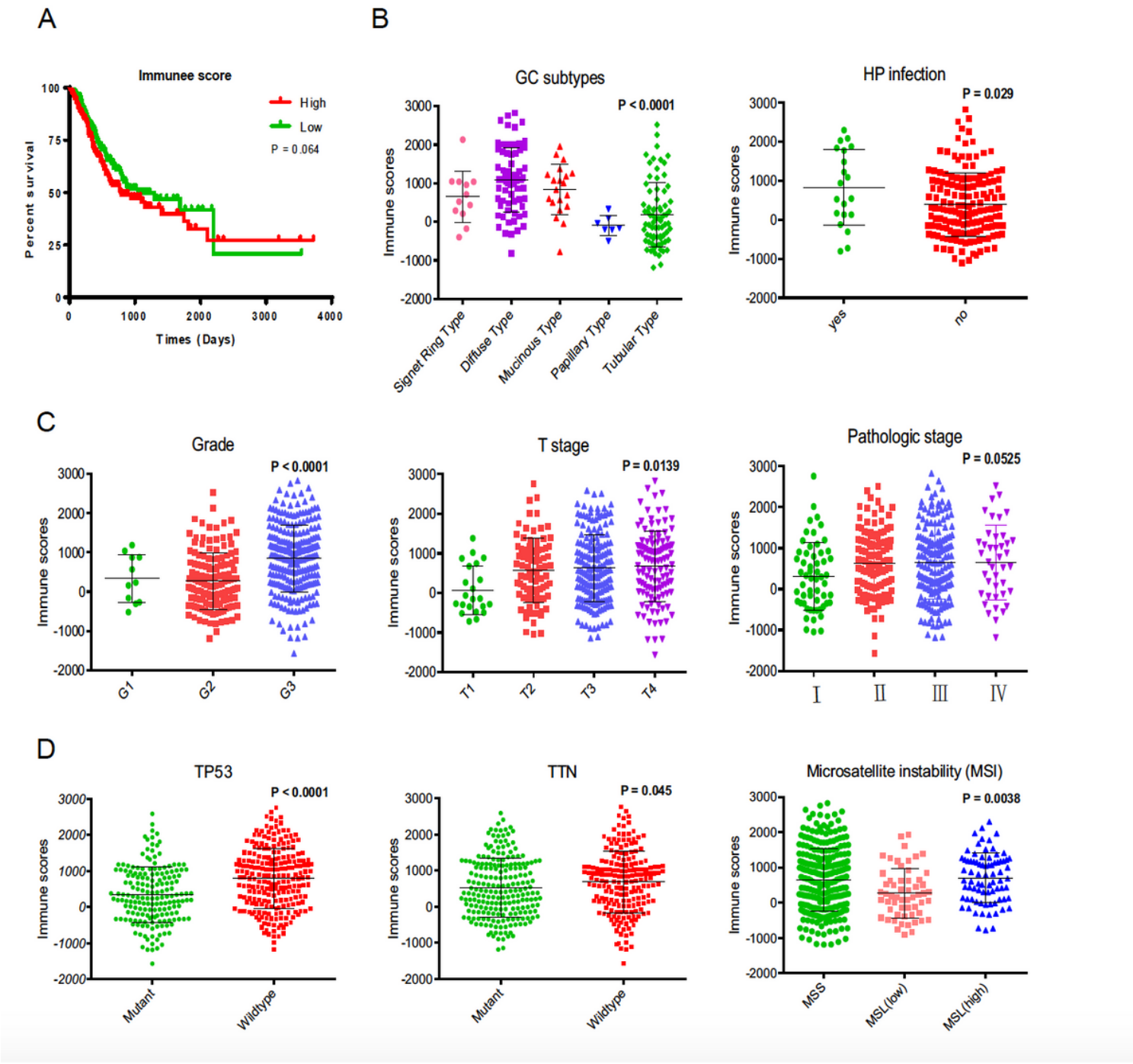

Figure 5

Immune scores were correlated with GC survival and subgroups A. The Kaplan-Meier survival curve of GC patients' overall survival (OS) based on immune scores; B. the distribution of immune scores of GC subtypes and HP infection; C. the distribution of immune scores of grade, T stage and pathological stage; D. the distribution of immune scores of specific mutant.

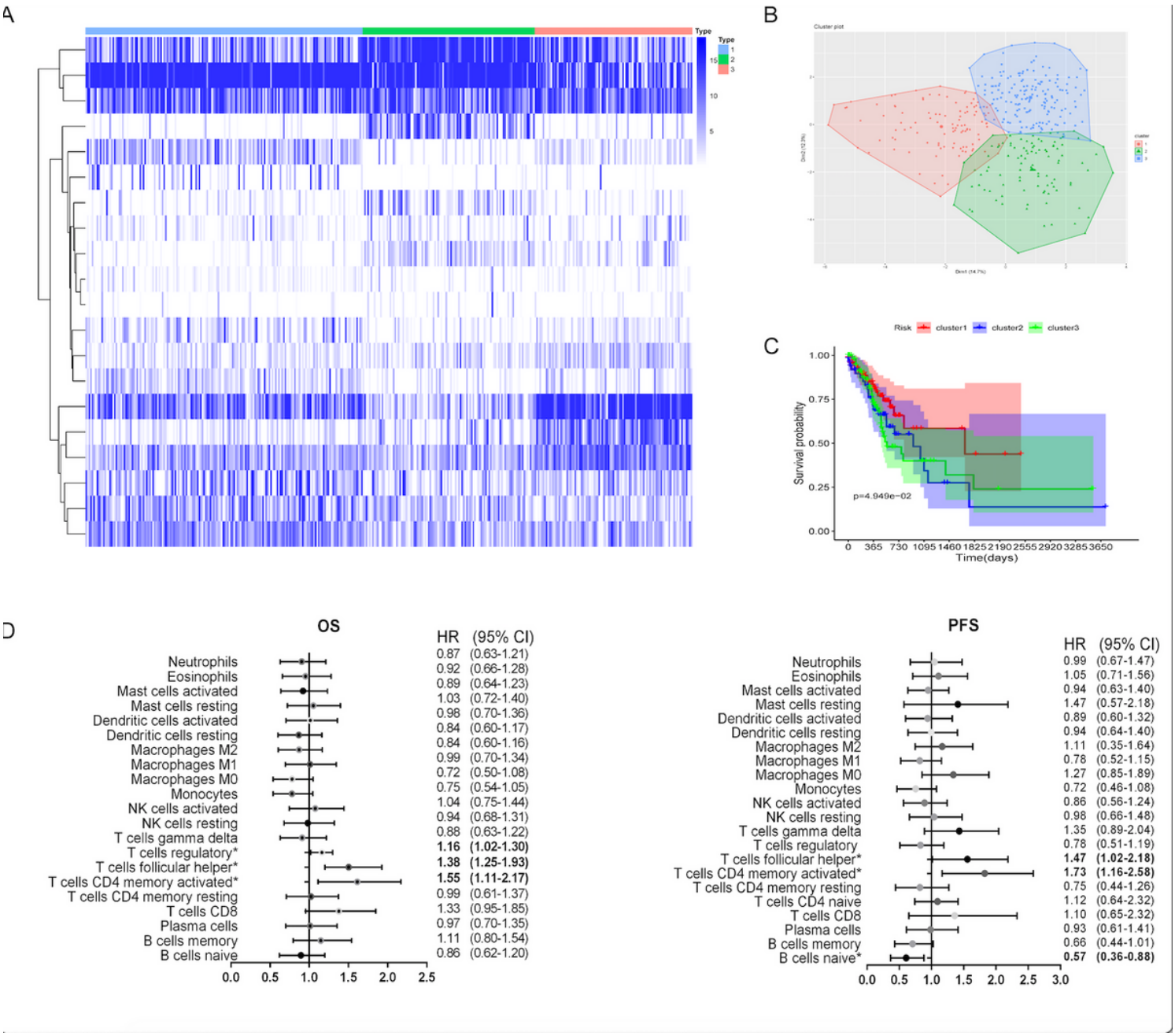

Figure 6

Immune clusters associated with GC prognosis A. Consensus matrix heatmap defined three clusters of samples for which consensus values; B. Scatter diagram showed the principal components variation of each patient, according to the cohort to which they belong; C. Kaplan-Meier survival analysis of patients within different clusters; C. Prognostic associations of TIICs subpopulation. D. Forest plot summary of analyses of TIICs subpopulation in overall survival and disease- free survival.

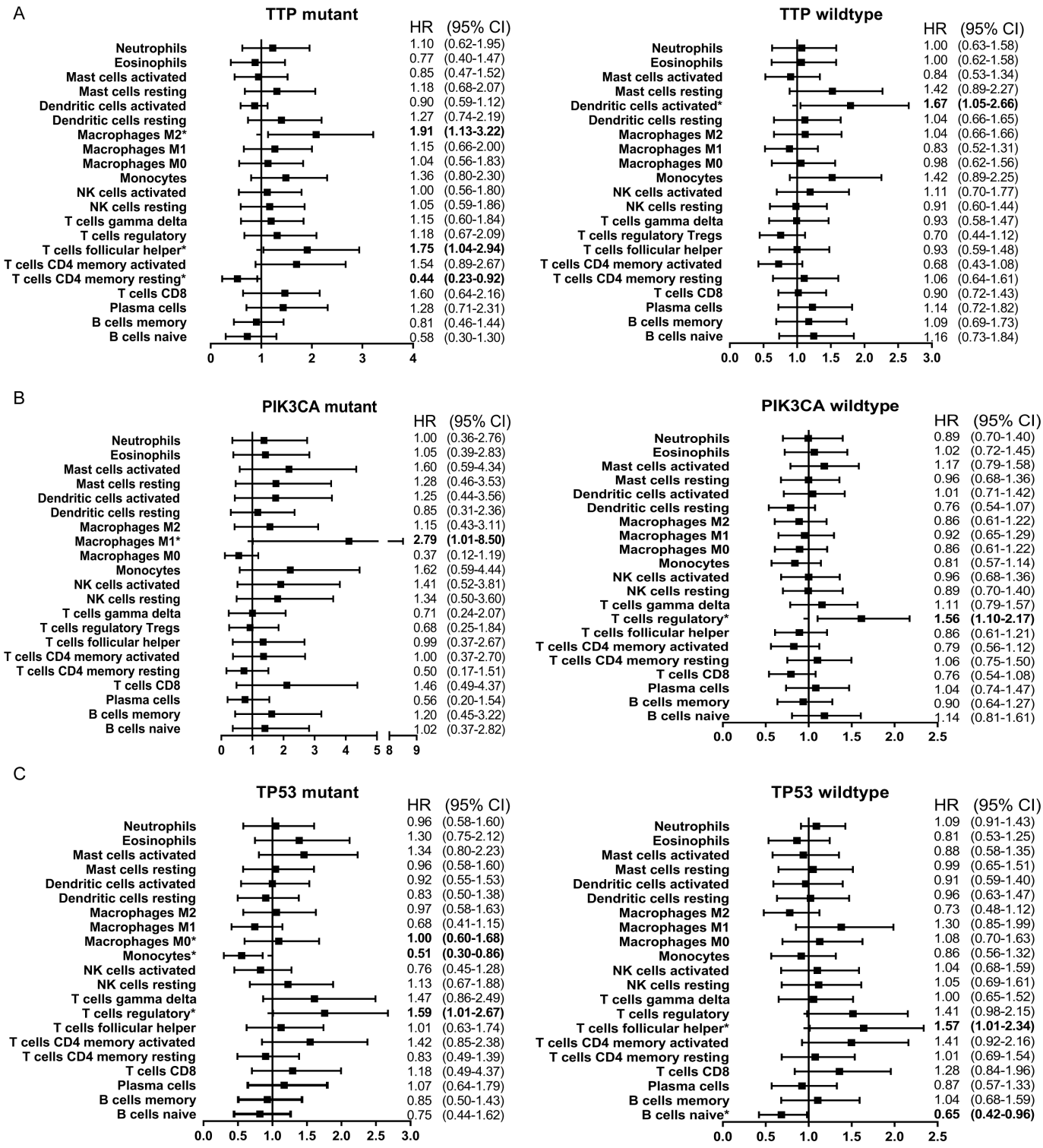

Figure 7

Prognostic associations of TIICs subpopulation Forest plot summary of analyses of TIICs subpopulation overall survival in TTN(A), PI3KCA(B) and TP53(C) subgroup.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- figures1.tif
- figures2.tif
- figures3.tif
- figures4.tif
- figures5.tif
- figures6.tif
- figures7.pdf
- figures8.pdf
- Supplementlytabel.docx