Characterization of tumor microenvironment and Immune infiltration in head and neck squamous cell carcinoma

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Research article

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Abstract

Background: Head and neck squamous cell carcinoma (HNSCC) is a malignant tumor of the head and neck. Although it one of the most prevalent heterogeneous diseases, the role of tumor-related immune cells in HNSCC is not well understood, particularly in the current immunotherapy.

Method: We explored TME, TMB in HNSCC to evaluate 22 subsets of TIICs, using GEO and TCGA database, to deduce the relationship of immune subpopulation, survival of patients, function and expression difference to reveal potential targets and biomarkers for immunotherapy.

Results: GSE6631 database containing 22 HNSCC samples and 22 normal samples and TCGA database containing 111 HNSCC and 12 normal tissues were downloaded. M0 macrophages and resting memory CD4 +T cells were differentially expressed between tumor and normal cells, and therefore may plays an important role in regulating progression of cancer (P<0.05). Missense mutation was the most common alteration in the tumors, while SNP was the most common type of DNA polymorphism. The most common single nucleotide variation was C>T. There were on average 78 Variation per HNSCC tumor. Top 10 mutated genes related to TMB were *TP53, TTN, FAT1, MUC16, CDKN2A, CSMD3, SYNE1, LRP1B, NOTCH1* and *PIK3CA*.

Conclusion: There is an intricate connection between TIIC, TMB and genomic alterations. Our findings offer the basis for future research on enhanced tumor immunotherapy.

Background

Head and Neck Squamous Cell Carcinoma (HNSCC) is a head and neck malignancy affecting the lips, mouth, paranasal sinuses, larynx, nasopharynx, and other pharyngeal organs[1]. It is the sixth most common type of malignant tumor, accounting for more than 655,000 new cases and 90,000 deaths every year[2]. Smoking, drinking, and human papilloma virus infection are the risk factors for occurrence and prognosis of HNSCC[3]. Even with early detection, less than 50% of individuals with HNSCC survive for 5 years. The survival rate is further reduced to 35% in individuals with local recurrence and metastasis[4]. Occurrence and development of HNSCC is a complex process involving multiple molecules. The malignant phenotypes of cancers are characterized by intrinsic activities of tumor cells as well as by the immune cells activated and recruited at the site of infection[5]. Although it's a heterogeneous disease, the role of tumor-related immune cells in HNSCC, particularly in the current immunotherapy, is not well understood

Tumor microenvironment (TME) comprises of many types of cells such as immune, fibroblasts and endothelial cells as well as extracellular molecules such as cytokines, extracellular matrix and growth factors, all fed by a vascular network[6]. Tumor-infiltrating immune cells (TIIC) in TME, plays an indispensable role the initiation, progression and even metastasis of tumors and affects the efficacy of therapeutic interventions[7]. TIIC comprise of macrophages (M0/M1/M2 macrophages), 7 types of T-cell

types (resting memory CD4+ T cells, T follicular helper [Tfh] cells, activated memory CD4+ T cells, γδ T cells, Tregs, CD8+ T cells and naïve CD4+ T cells), resting natural killer cells, resting/activated mast cells, activated NK cells, resting dendritic cells, memory B cells, activated DC, monocytes, naïve B cells, plasma cells, eosinophils and neutrophils. TIIC at TME are targets for drugs in managing progression of cancers, and have shown positive prognostic value[8]. There is a complex immune interaction, which has to balance between over stimulation and controlling tumor progression[9]. Tumor cells avoids host immunosurveillance by upregulating expression of TIICs inhibitors, impacting on tumor mutational burden (TMB)[10-11]. TMB is the total number of mutations per megabase of a tumor tissue. It is an indicator of immunological reaction and tumor behavior[12-13] such as formation of mutations through activation or inactivation of related genes and pathways. As such, novel peptides that can animate immune reaction can be created[14]. High TMB may be driven by other underlying risk factors. Immune checkpoint inhibitors and has been appeared to be more significantly connected with reaction to PD-1 and PD-L1 blockade immunotherapy than PD-1 or PD-L1 expression[15]. Previous attempts to assess TIIC using flow cytometry and immunohistochemistry was limited by the quantity of fluorescent channels accessible as well as few types of immune cell available for immediate assessment[16-17]. In this study, we used (CIBERSORT) to explore the relationship between TME, TMB and the concentration of 22 TIICs based on Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) database on HNSCC, in relation to molecular subpopulation, survival, function and expression difference to reveal potential targets and biomarkers for immunotherapy.

Methods

Data acquisition.

Freely access data on gene expression profiles and corresponding visualization were identified and downloaded. Crude information from the microarray data produced by Affymetrix was downloaded from the GEO using RMA algorithm for background adjustment. Information on gene expression, somatic mutations, copy number and corresponding clinical information were downloaded from level 4 gene-expression data (FPKM normalized) of TCGA- HNSCC cohort. Although HNSCC has multiple histologically and occur in many anatomical sites, larynx data was downloaded from TCGA. RNA-sequencing data (FPKM values) from this set were changed into transcripts per kilobase million values, to create uniformity with subsequent microarrays data, to ease comparison between samples18. Bio and clinicopathological data collected included sex, age, stage, grade, T-stage, M-stage, N-stage, survival status and survival duration in days.

Assessment of immune infiltration.

CIBERSORT metagene tool utilizes data on deconvolution of mass gene expression, whose reliability has been validated by fluorescence activated cell sorting. CIBERSORT is used to estimate the abundances of individual cell types in a mixed cell population, using data on gene expression. This enables large-scale analysis of mixed RNA for cellular biomarkers and therapeutic targets, especially in noise, unknown

mixture content and closely related cell types[19-20]. Thus, we used CIBERSORT to evaluate TIIC in TME and in measuring the proportions of immune cells and cell types in HNSCC heterogeneous samples.

Assessment of tumor mutational burden.

TMB is the total number of mutations per megabase of tumor tissue. In general terms, it's the mutation density of a tumor gene, including the total number of gene coding errors, base substitutions, gene insertions or deletion errors. The larger the TMB, the easier it is to be detected by immune cells, and the easier it is to be targeted for tumor immunity, thus the more likely it is to be contained by immunotherapy. We described the copy number and characteristics of somatic mutations using TCGA data base. We also analyzed the effect of TMB on survival. Type of TCGA workflow based on VarScan2 Variant Aggregation and Masking. Student's t-test was performed to analyze differentially expressed genes (DEGs) associated with TMB using R software[21]. DEGs among TMB were controlled using significance standards as applied in the R package limma. Adjusted P value for multiple testing was calculated using the Benjamini–Hochberg correction[22]. Function of the identified DEGs and biological processes were analyzed using GO (cellular components, biological processes and molecular functions) while enrichment and KEGG pathway analyses were performed using R software. Finally, we evaluated the relationship between tumor immune cells-infiltration and TMB.

Statistical analysis.

Statistical analyses have been conducted the use of R (version 3.5.3) and R Bioconductor software packages. Each dataset was handled in a weighted average approach to evaluate the differences in the composition of TIIC. Differences were illustrated using histograms, heatmap, corHeatmap and vioplot. Overall survival (OS) was characterized as the time interval from the date of diagnosis to the date of death Samples with missing data were excluded from the study. Wilcox test analysis was performed to evaluate statistical differences in expression of immune genes for checkpoint molecules and clinical information on TMB between tumor and normal tissues in TCGA. For each statistical analysis, a *P*-value<0.05 was considered as significant.

Results

Clinical information.

The GSE6631 database contained 22 HNSCC and 22 normal samples, but after filtering immune cell matrix, only 12 HNSCC and 10 normal samples matched our inclusion criteria. TCGA database on its part contained 111 HNSCC and 12 normal tissues, but after immune cell matrix filtration, 98 HNSCC and only 1 normal tissue fitted our inclusion criteria, the clinical patients contained 117 patients and the clinicopathological attributes of these samples are appeared in Table 1. The study cohort comprised of 20 (17.1%) female and 97 (82.9%) male patients. Two patients (1.7%) were at stage I of the disease, 10 (8.5%) at stage II, while 14 (12.0%) at stage III and 74 (63.2%) at stage IV. Tumor stage was found T1 in 7 patients (6.0%), T2 in 14 (12.0%), T3 in 26 (22.2%) and T4 in 55 (47.0%). Node stage contained N0 in 41

(35.0%), N1 in 12 (10.3%), N2 in 41 (35.0%), N3 in 2 (1.7%). 42 of 117 (35%) cases had non-distant metastases. In addition, TCGA-HNSCC contained 506 cases on simple nucleotide variation.

Distribution of tumor-infiltrating immune cells based on GEO and TCGA.

Histograms visualization showed the Relative percentage of immune cells and heatmap showed the cluster information of the 22 TIICs subsets in each sample based on GEO (odd number is normal, even number is the tumor) Figure 1A, 1C. CorHeatmap revealed that M1macrophages and resting memory CD4 +T cells had the highest interaction (0.86), indicating that these two immune cells play a synergistic role in GEO-HNSCC patients. In contrast, activated mast and CD8 +T cells had the lowest co-expression coefficient (-0.71) as shown in Figure 1B, indicating that these two immune cells may antagonize other immune cells in controlling HNSCC.

Based on vioplot analysis, there was a significant difference in the 22 TIICs between normal and tumor cells as shown in Figure 1D. The expression of macrophages M0 and resting memory CD4 + T cells was significant suggesting that these cells may play an important role in regulating cancer progression (P<0.05). Similarly, the co-expression of resting NK cells and activated memory and T cells was highest in tumor patients while, the co-expression of M0 macrophages and CD8+ T cells was lowest (0.32) as shown in Figure 2.

Tumor TMB variability among cancers.

Missense mutation was the most common somatic genetetic variation, while SNP was the most common DNA sequence polymorphism, with C>T being the most common single nucleotide variants class. The median variation per HNSCC tissue was 78. Top 10 genes mutations contributing to TMB were *TP53, TTN, FAT1, MUC16, CDKN2A, CSMD3, SYNE1, LRP1B, NOTCH1* and *PIK3CA* as shown in Figure 3A. CorHeatmap analysis revealed a co-expression relationship among many mutated genes Figure 3B. *TTN* gene had the most mutations, with alterations observed in 478 (94.47%) of 506 samples as shown in Figure 3C-3D. There was no association between TMB and OS (P=0.87), which may be due samples size bias.

TMB and differentially expressed genes.

To evaluate the underlying biological characteristics of TMB, we divided individuals into high or low tumor mutational burden based on TMB median value, and analyzed 249 DEGs using limma[23]. GO enrichment and KEGG pathway analyses were performed using R clusterProfiler. GO analysis showed that biological processes in DEGs were significantly enhanced in response to acid chemical, connective tissue and cartilage development. Molecular function was mainly enhanced in structural constituent of extracellular matrix. DEGs cellular components were elevated in extracellular matrix, presynapse and endoplasmic reticulum lumen. KEGG analysis revealed that DEGs were enhanced in glutathione metabolism. Vioplot revealed differences between the 22 TIICs in TMB subgroups. Here, the expression of

T regulatory cells (Tregs) was significantly different, and thus these cells may play an important role in regulating tumor cells (*P*=0.026) Figure 4.

Discussion

In this study, we used CIBERSORT to assess TME, TMB and characterized 22 subsets of TIICs based on GEO and TCGA database in individuals with HNSCC, to explore molecular subpopulation, survival, function and expression difference of these cells in tumor conditions. We found that the composition of TIIC subtypes differs substantially in different HNSCC. Tumor mutations generates novel epitopes. Understanding the immunogenicity of these epitopes may deepen our knowledge on the clinical immunology and prognosis of HNSCC tumors. Based on TCGA data TME was associated with TMB, supporting the hypothesis that TMB is a strong proxy for TME. This is an interesting revelation, particularly in the face of immunomodulatory therapies, and may uncover potential biomarkers for immunotherapy or prognosis of HNSCC tumors.

Infiltration of immune cells has been postulated to be a significant factor influencing the prognosis of tumors. In this study, we first explored the distribution of TIICs in HNSCC patients based on GEO and TCGA datasets. Macrophages which mediate phagocytosis and inflammation aid in controlling tumor progression, by facilitating invasion of tumor tissues and angiogenesis[24-26]. Macrophages are mainly classified into M0/M1/M2 Under specific conditions, M0 macrophages can be polarized to M1/M2 types, Liu *et al*[27] found that RhoA pathway could obstruct elongation (hummingbird phenotype) of M0 macrophages, suggesting that M0 may be important in bone-marrow-derived macrophages. As subpopulations of T cells, resting memory CD4+ T cells can differentiate to perform other functions (separate to those mediated by memory CD8 + T cells, but aiding in suppressing growth of tumors)[28-29]. Resting memory CD4 + cells must be activated by IL-7 and IL-15, yet not MHC class II, for their survival and intermittent homeostatic proliferation[30]. So, we hypothesized that M0 macrophages and resting memory CD4 T + cells may play a critical role in immunotherapy and therefore further research was needed. Meanwhile, we also analyzed the interaction between the 22 different TIICs and elucidated on co-expression of immune cells in HNSCC, to open up future research on the same. This analysis though, may be subject to biasness, because the interactions may be influence by the other factors in the TME.

The role of varying TMB in HNSCC was also investigated. Missense mutation we found to be the most common somatic alterations, while SNP was the most common DNA sequence polymorphism with C>T single nucleotide substitution being the most frequent. Changes that modify amino acid sequences are known as Missense mutations. These changes may influence the structure and stability of proteins, which may interfere their interactions with other biomolecules, translation of functional proteins and enhance progression of tumours[31]. For example, *BRAF* mutation in melanoma and *KRAS G12D* mutations in colorectal cancer[32-33]. Establishing impacts of cancer missense mutations may help in identifying drivers of mutations as well as illustrating molecular mechanisms for developing HNSCC. SNP have been identified in nearly all types of cancers, such as colorectal[34], breast[35], prostate[36], and HNSCC[37]. SNP is a

third-generation genetic marker for detecting numerous phenotypic differences in human, and susceptibility to diseases and drugs may be associated with SNP[38]. Top 10 mutated genes that related to TMB were *TP53, TTN, FAT1, MUC16, CDKN2A, CSMD3, SYNE1, LRP1B, NOTCH1* and *PIK3CA*. Kubesova *et al*[39] reported that *TP53* mutations with low variant allele frequency irrespective of disease subtype, driver gene status and cytoreduction during myeloproliferative neoplasm. Mukhopadhyay *et al*[40] suggested that ESR2-mutant *TP53* combination prognosticates survival in triple negative breast cancer. But in this study, there was no statistical difference between TMB and clinical survival time (P=0.87) which may have been influenced by too small samples size in TCGA database. We finally divided TMB into high and low groups to analyze the functions of DEGs. Analysis of biological processes performed by DEGs suggested that these genes may play a significant role in tissue development and response to acidic chemicals. Molecular function was enhanced in structural constituents in the extracellular matrix, and affected cellular structure as well. KEGG analysis revealed that for most part, metabolism of glutathione is enhanced in DEGs. Glutamine is a significant metabolite utilized in the development of malignant cells. Reducing the level of GLN through chemotherapy and radiotherapy has been found to restores diminished glutathione, enhancing the recovery of intestine epithelium and immunological system[41]. Our results also suggested that Tregs was differently expressed between high and low TMB group indicating these genes are essential in tumor immune response.

Meanwhile, our study had several limitations. First, our results were not validated in clinical settings and may not provide precise data. We also analyzed samples from relatively few patients. Second, due to multiple types of histology and anatomical sites for HNSCC, tumor-infiltrating immune cells may vary widely. Finally, the interacting of different immune cells is affected by environmental conditions, thus ours corHeatmap analysis may be subject to bias. We will do follow-up studies to develop new, accurate interventions for cancer medicine. Here, we described TME in relation to immune system, revealing variable infiltration of various HNSCC subtypes. The intricate connection between TIIC, TMB and genomic alterations was also elucidated the mechanism of immune response revealed in this study provides a strong foundation for future research that can enhance tumor immunotherapy.

Abbreviations

HNSCC: Head and neck squamous cell carcinoma; TME: the tumor microenvironment; TIIC: tumor-infiltrating immune cells; TMB: tumor mutational burden.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors contributed significantly to this work. W.Z.H. designed the research study; W.Z.H. and N.X performed the research study and extracted the data and analyzed the data; W.Z.H., N.X., X.X.Y.; W.Z.H. and C.X. wrote and revised the manuscript. In addition, all authors approved the final draft.

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Not applicable

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Table

TABLE 1. Primary tumor characteristics of HNSCC in TCGA.

Figures

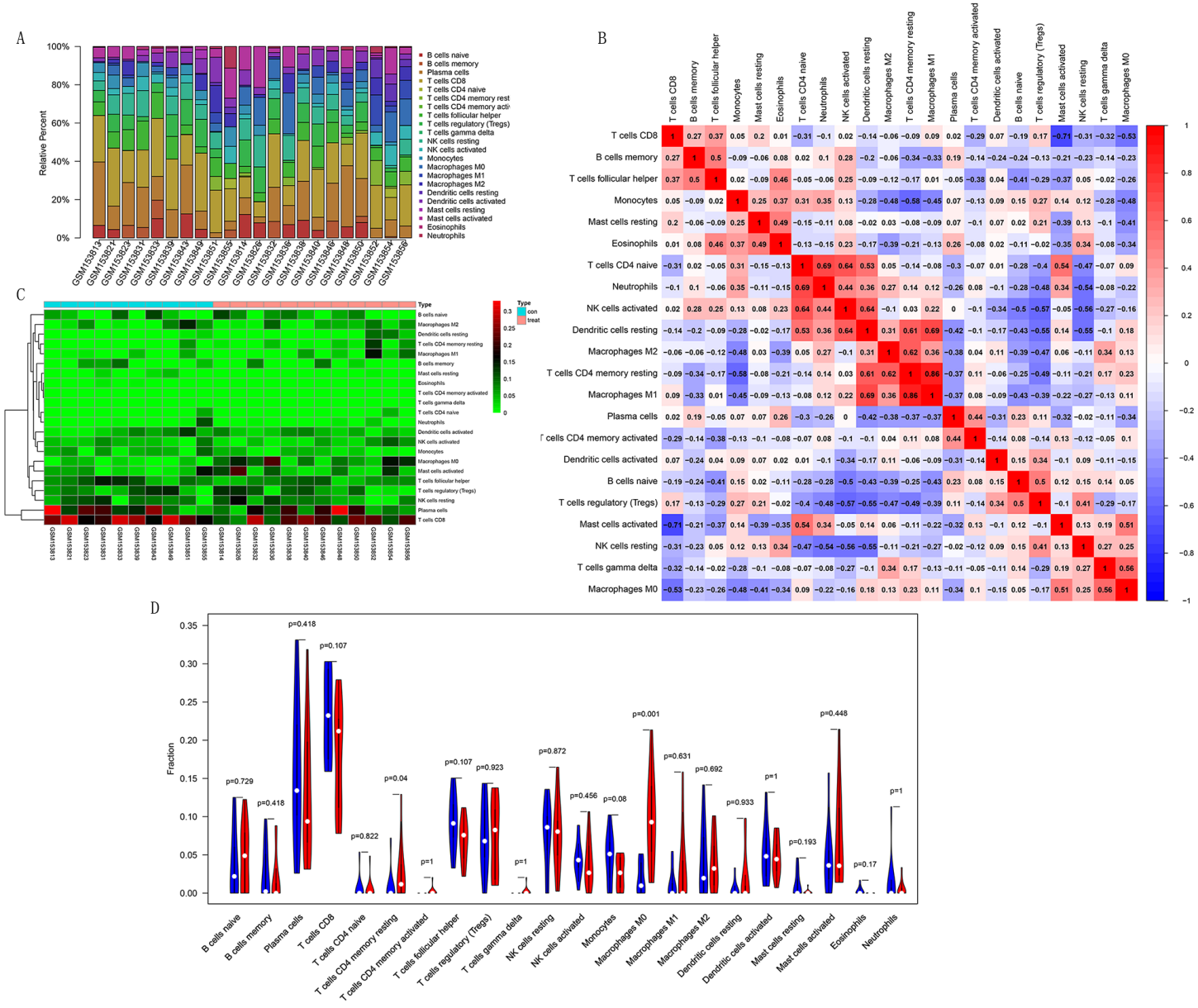

Figure 1

Variable	Number of samples	%
Gender		
Male	97	82.9
Female	20	17.1
Age at diagnosis		
≤65	77	65.8
>65	40	34.2
T-Stage		
T1	7	6.0
T2	14	12.0
T3	26	22.2
T4	55	47.0
Missing	15	12.8
Grade		
G1	8	6.8
G2	72	61.5
G3	32	27.4
G4	1	0.8
Missing	4	3.4
Stage		
I	2	17.1
II	10	8.5
III	14	3.4
IV	74	63.2
Missing	17	14.5
M-stage		
M0	41	35.0
M1	1	0.8
Missing	75	64.1

Boxplot, heatmap, corHeatmap and vioplot showed 22 TIICs subsets of immune response based on GEO (odd number is normal, even number is the tumor). The results suggested that the expression of macrophages M0 and T cells CD4 memory resting was significant difference and may plays an important role in regulate cancer progression ($P<0.05$).

N-stage		
N0	41	35.0
N1	12	10.3
N2	41	35.0
N3	2	1.7
Missing	21	17.9
HNSCC, head and neck squamous cell carcinoma.		

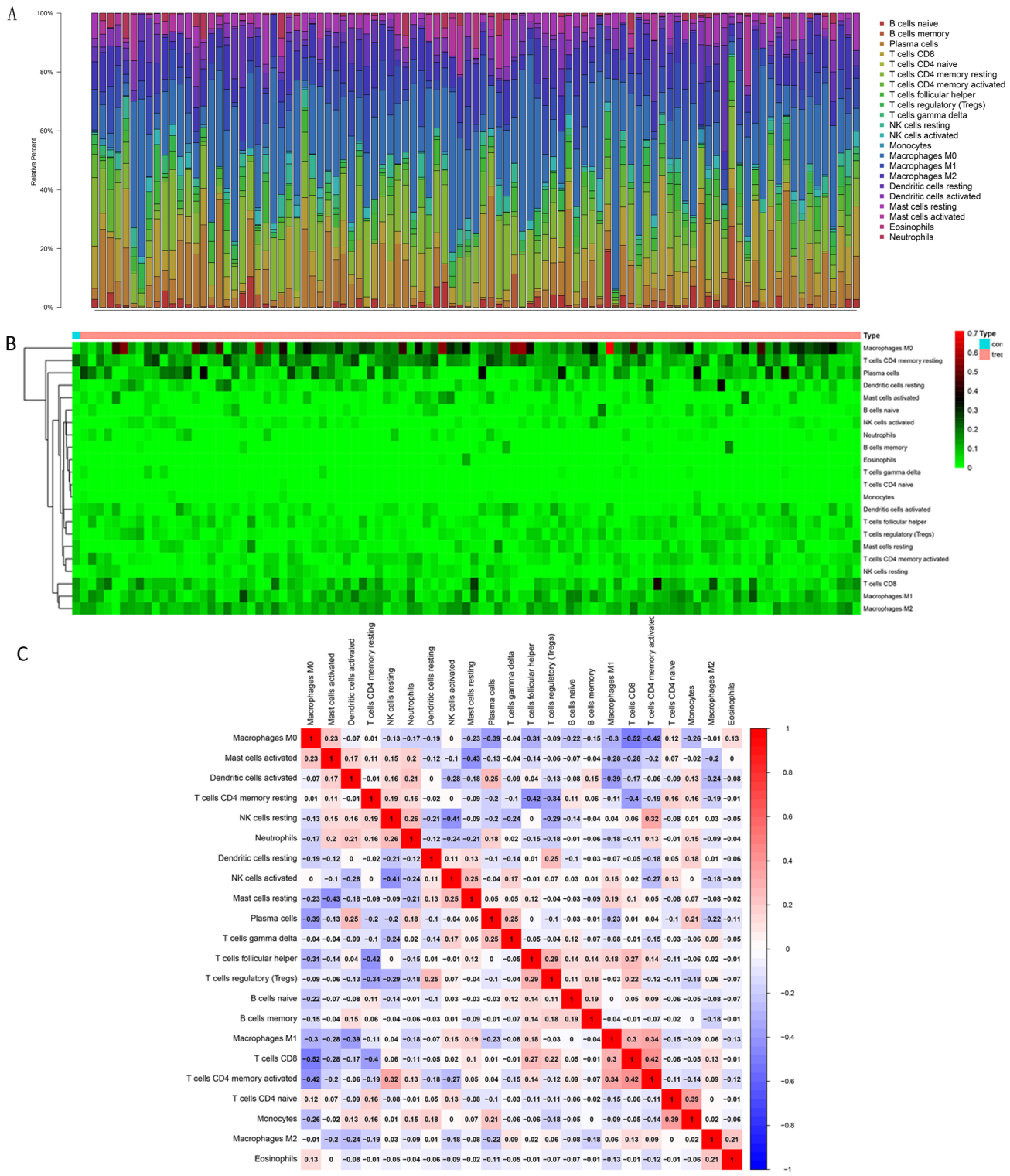

Figure 2

Boxplot, heatmap and corHeatmap visualization showed 22 TIICs subsets of immune response based on TCGA.

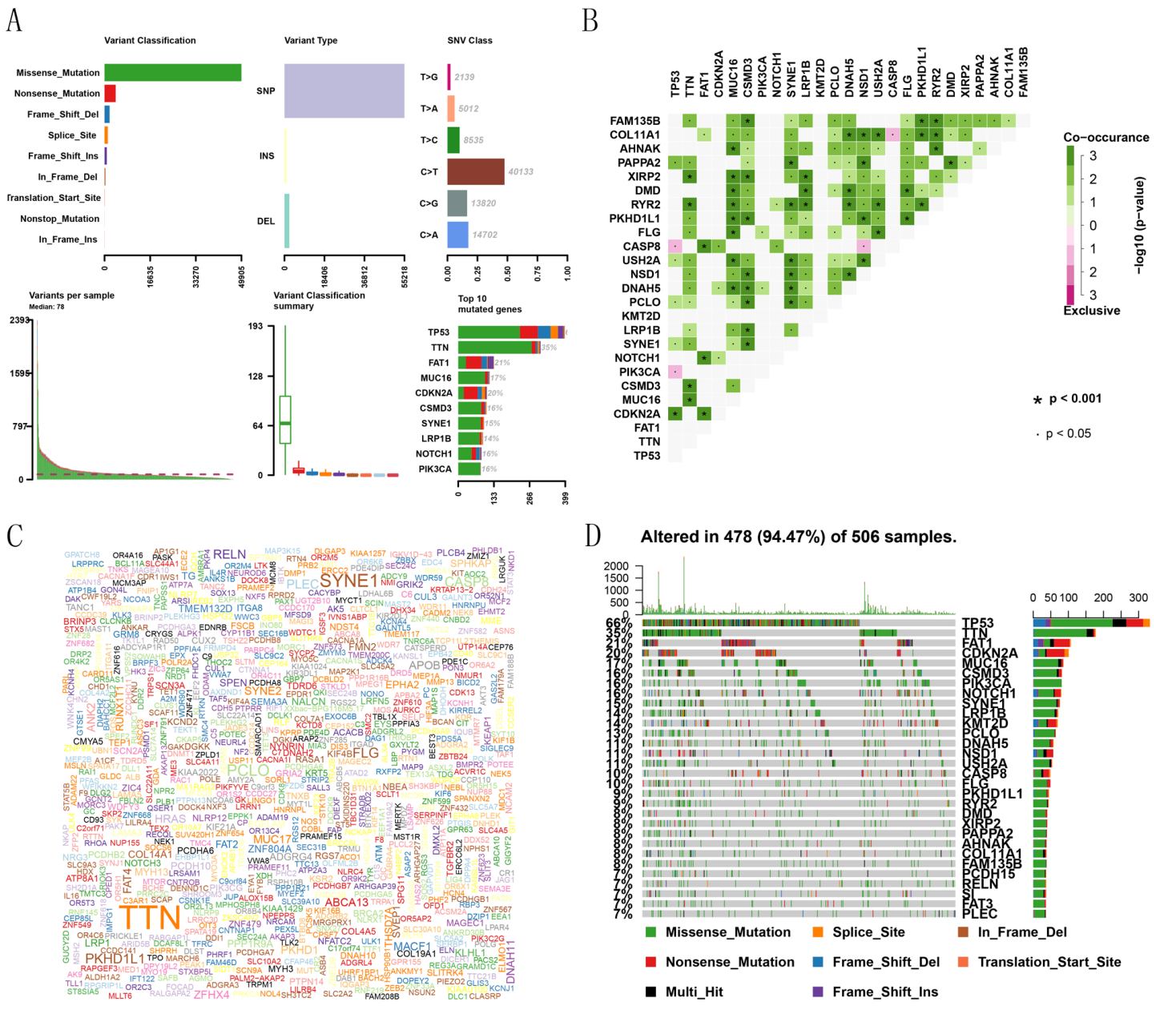

Figure 3

The result of tumor mutational burden in Head and Neck Cancer (cBioPortal). (A) The most common somatic mutations variant classification was missense mutation, the most common DNA sequence polymorphism type was SNP, the most common single nucleotide variants (SNV) class was C>T, the variants per sample median was 78 in HNSCC patients. Top 10 mutated genes that related to TMB was TP53, TTN, FAT1, MUC16, CDKN2A, CSMD3, SYNE1, LRP1B, NOTCH1 and PIK3CA. (B) CorHeatmap showed interaction between different mutated genes and the results showed that there are co-expression relationships among many mutated genes. (C-D) The gene cloudmap demonstrated that the most remarkable mutated genes were TTN and the waterfall plot tell us that the gene altered in 478 (94.47%) of 506 samples as well as the relative percent, variant classification of every single mutated genes.

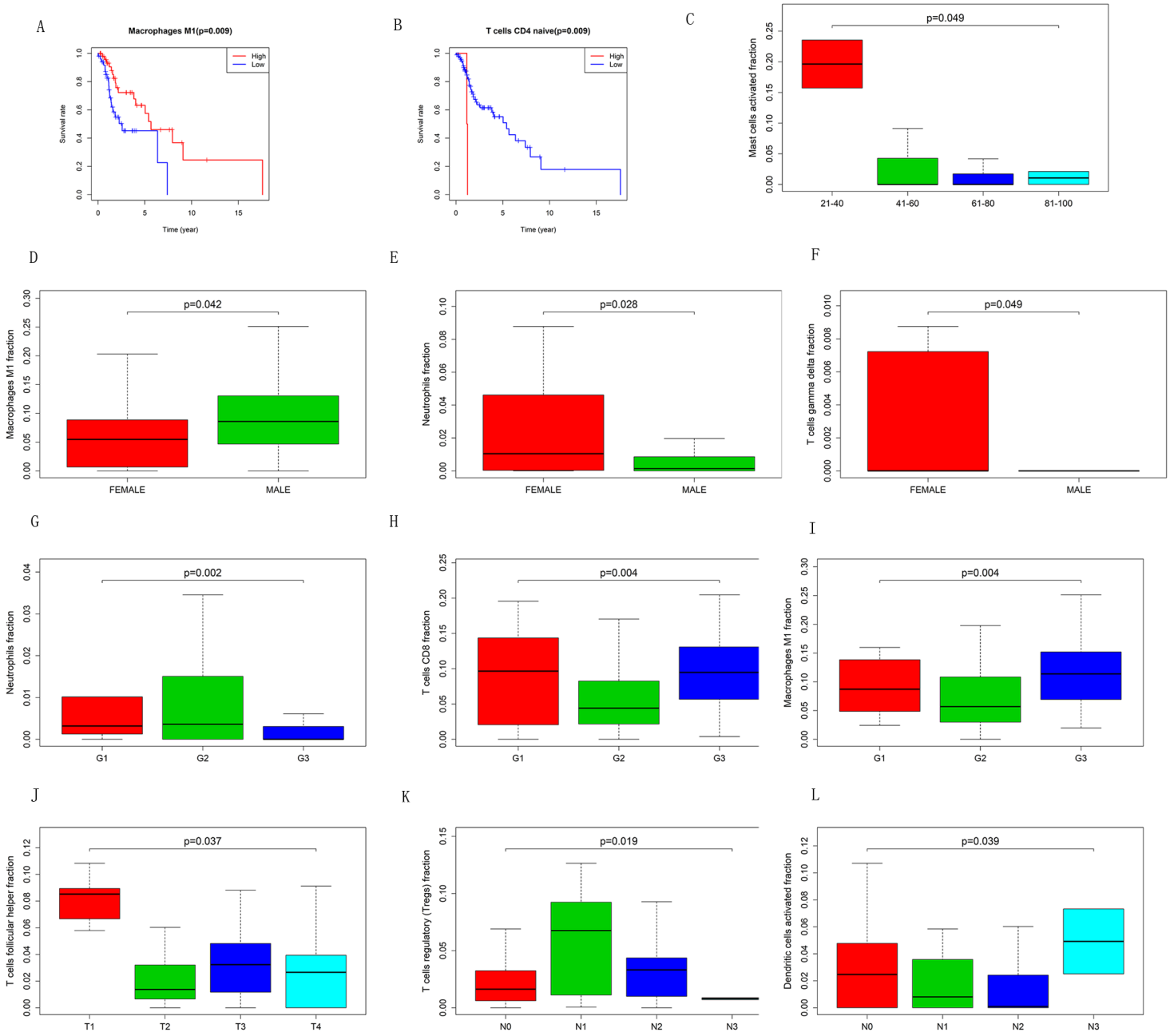

Figure 4

Survival rate between TMB and the relationship between tumor-infiltrating immune cells and patients' clinical factors. (A-B) The survival rate between TMB and macrophages M1 (P=0.009) and T cells CD4 naïve. (C-L) The relationship between tumor-infiltrating immune cells and patients' clinical factors.

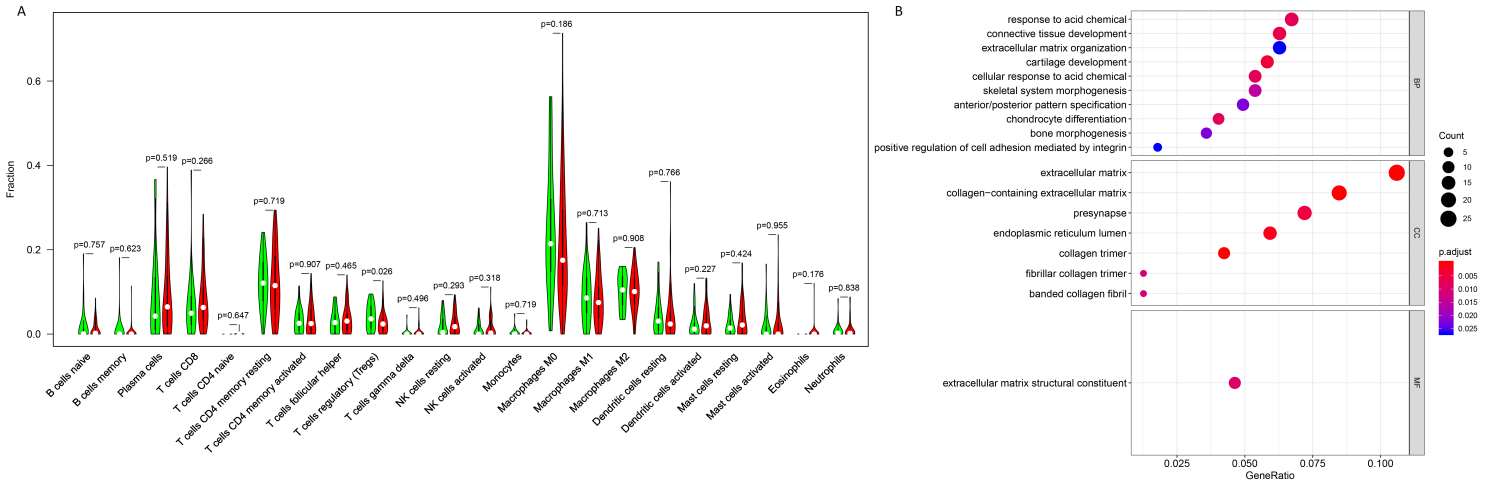

Figure 5

TMB and differentially expressed genes. (A) The clustering results. (B) GO analysis results. (C) The relationship between TMB and patients' gender. (D) The expression of T cells regulatory (Tregs) was significant difference and may plays an important role in regulate tumor cells (P=0.026). (E) The relationship between TMB and patients' grade (P=0.064).