Identification of a gene set correlated with immune status in ovarian cancer by transcriptome-wide data mining

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Abstract

Background : Ovarian cancer (OC) is a serious tumor disease in gynecology. Many papers have reported that high tumor mutational burden (TMB) can generate many neoantigens to result in a higher degree of tumor immune infiltration, so our study aims to predict the key molecules in OC immunotherapy by combined TMB with immunoactivity-related gene.

Method: We divided OC cases into two groups: the low & high TMB group hinged on the somatic mutation data from the Cancer Genome Atlas (TCGA). We also used single-sample gene set enrichment analysis (ssGSEA) scores of immune cell types to conduct unsupervised clustering of OC patients in the TCGA cohort and some of them were defined as the low & high immunity group. Besides, to further understand the function of these genes, we conducted Gene Ontology, Kyoto Encyclopedia of Genes and Genomes pathway, protein-protein interaction network, survival prognosis analysis and immune infiltration analysis. Finally, the effects on prognosis and immunotherapy in OC patients were explored by the Group on Earth Observations verification the patients' responses to immunotherapy.

Results: We found that the higher the TMB was associated with the higher OC grades. Moreover, both high TMB and high immunity were significantly correlated with a good prognosis of OC. Then, 14 up-regulated differential expression genes (Up-DEGs) that were closely related to the prognosis of OC patients were screened according to the high TMB group and the high immunity group. Next, pathway analysis revealed that Up-DGEs were mainly involved in immune response and T cell proliferation. Finally, four genes had a good prognosis and were validated in the GEO dataset which included CXCL13, FCRLA, PLA2G2D, and MS4A1. We also identified that four genes had a good prognosis in melanoma patients treated with anti-PD-L1 and anti-CTLA-4 in the TIDE database.

Conclusion: High TMB can promote immune cell infiltration and increases immune activity. And our analysis also demonstrated that the higher the TMB, the higher the immune activity, the better the prognosis of OC. Altogether, we found that CXCL13, FCRLA, PLA2G2D, and MS4A1 may be biomarkers for OC immunotherapy. Keywords: ovarian cancer, TMB, immune cells infiltration, survival prognosis.

Background

OC is one of the three primary gynecological malignancies and the most fatal gynecological tumor around the world. Despite having various treatments, the survival rate has been low and has not improved significantly [1]. The mortality rate was the highest in gynecological tumors, and the five-year survival rate of the patients was no more than 35% [2]. Due to the lack of specific symptoms or effective screening strategies [3], and more than 70% of patients are in the middle and late stages at the time of diagnosis, which resulting in a higher risk of recurrence and poor prognosis [4]. Besides, up to 70% of patients with OC still recurrence after 12 to 18 months when they were given traditional therapies, hence, targeted therapy has become the most promising treatment [2]. Therefore, it’s urgent and vital to identify new survival biomarkers for OC treatment.

It has made a breakthrough in the field of cancer immunotherapy through targeting immune checkpoints to enhance the anti-tumor immune response, such as advanced squamous non-small cell lung cancer (NSCLC) and metastatic melanoma [5]. However, this immunotherapy can merely contribute to a small number of patients and the vast majority of patients have limited or no response to this treatment [6]. At present, there is little or no clinical immunotherapy for OC. Thus, it's critical to identify molecular biomarkers that predict cancer immunotherapy responders combined with cancer genomics analysis. Like several markers, such as PD-L1 and CTLA-4 have been evaluated in specific tumor types [7–9].

On the one hand, an emerging biomarker of the immunotherapeutic response is the total number of mutations present in tumor specimens. This is termed TMB. Lots of studies have considered that TMB as a predictor of tumor immune response [10–12] and explored the relationship between TMB and immunotherapy response [13, 14]. TMB was defined as the total number of replacement and insert/deletion mutations per megabase in the exon coding region of the genome examined in tumor samples [15]. Just as its name implies, highly mutated tumors may produce more neoantigens, and making them more susceptible targets for T cells [15], which in turn activates the immune function of the body and leading to an anti-tumor immune response [16]. Alternatively, some researches have been reported that the higher the TMB, the better the clinical efficacy of immune checkpoint inhibitors and also have better survival prognosis [17].

On the other hand, tumor microenvironment (TME) has also been considered to have an essential impact on tumor immunity [18–21]. TME is the cellular environment in which the tumor is located and it's the site between tumor cells and adjacent normal tissues. And TME is composed of cancer cells and a variety of stromal cells, cytokines, chemokines, etc. Stromal cells include immune cells, fibroblasts, endothelial cells, etc. Cytokines include TNF, VEGF, IL-1, etc. Chemokines include CCL27, CCL21, CXCL12, etc [22, 23]. However, prior to this report, the roles of the TMB combined with immunoactivity-related gene sets in predicting the biomarkers for prognosis of OC was unclear. Thus considering the correlation between the TMB, the immune cell types and the efficacy of cancer treatment, so the TMB and the immunoactivity-related gene sets were used to predict the biomarkers associated with the prognosis of OC was the focus of our studies. Therefore our findings will undoubtedly accelerate the validation and the realization of TMB and TME as the significant predictive markers for OC's clinical and prognosis.

In this study, we sought to identify prognostic biomarkers for OC by an integrated TMB and TME analysis, and we downloaded and analyzed the OC expression profile data and mutation annotation files from the TCGA database. To investigate the relationship of TMB and TME with OC, the OC samples from the TCGA database would be classed into the high TMB group and the low TMB group, and then according to the ssGSEA score, the immunoactivity-related genes sets were divided into three clusters, some of which were defined as the high immunity group and the low immunity group. Next, we gained lots of essential genes of the immune microenvironment for predicting prognosis in OC patients. Further researches are required to explore the potential of these critical OC-related genes in diagnosis, prognosis, and targeted therapy. In addition, to further learn more about the function of these genes, Gene Ontology (GO), Kyoto

Encyclopedia of Genes and Genomes pathway (KEGG), protein-protein interaction (PPI) networks, survival prognosis analysis, immune infiltration analysis and the analysis of patient response to immunotherapy were performed successively. Finally, we preferred four essential genes with a good prognosis for OC, and our studies can provide new prognostic biomarkers for the immunotherapy of OC (Fig. 1).

Methods

Database

RNAseq and mutation annotation files for OC patients were gained from the TCGA database portal (https://portal.gdc.cancer.gov/). Clinical data such as staging type, survival status and time were also downloaded from the TCGA database. The ESTIMATE algorithm was used to calculate the immune scores.

Implementation of ssGSEA

We obtained the marker gene sets for immune cell types from the TCGA database. Then we used the ssGSEA program to derive the enrichment scores of each immune-related term. The ssGSEA applies gene signatures expressed by immune cell populations to individual cancer samples. The computational approach used in our study included immune cell types that are involved in innate immunity and adaptive immunity. Tumors with qualitatively different immune cell infiltration patterns were grouped using hierarchical clustering.

Heatmaps and clustering analysis

Heatmaps and clustering were generated using the R package.

Identification of DEGs

R package limma was used for data analysis. Fold change > 2, $p < 0.05$ were taken as the cutoffs to screen the DEGs.

Functional annotation and pathway analysis of DEGs

The online tool, Database for Annotation, Visualization and Integrated Discovery (DAVID, https://david.ncifcrf.gov/) provided full-scale information for a list of genes by GO and KEGG pathway analysis. ClusterProfiler and enrich plot packages to predict the function of DEGs. GO and KEGG pathway analysis with the standard of $p < 0.05$, the results were downloaded in text format. Gene set variation analysis (GSVA) is an open-source software package for R and can be downloaded on the website, then condensing information from Up-DEGs and Down-DEGs expression profiles respectively into KEGG pathway. Compared with single-gene analysis, the advantages of this method include noise and dimension reduction [24]. Moreover, Unsupervised hierarchical clustering of these top KEGG pathways was able to distinguish between normal ovaries group (GTEx datasets) and OC (TCGA datasets).

PPI Networks Construction and Module Analysis

In order to analyze the links between these proteins, DEGs were uploaded to the Search Tool for the Retrieval of Interacting Genes (STRING, https://string-db.org/). Only single networks with more than ten nodes were included for further analysis.

Survival Analysis of Hub Genes.

Download the datum matrix containing all ovarian patients' prognosis information from the GEO database. The data was processed by GraphPad Prism 8 and then exported the results. Kaplan-Meier plots were drawn to demonstrate the relationship between patients' overall survival and gene expression levels of DEGs. The relationship was tested by the log-rank test.

Statistical and computational analyses

SPSS 20.0 software was used for statistical analysis. $p < 0.05$ was considered statistically significant in all statistical tests. All the statistical and computational analyses were performed using the R package.

Results

The TMB scores and the immunoactivity-related gene sets are conjoined with OC's overall survival.

Datasets comprising RNAseq from 379 TCGA tumors and mutation annotation files from 436 TCGA tumors (272 which overlapped with RNAseq data) were obtained from the TCGA Data Portal in March 2019 (https://portal.gdc.cancer.gov/). We evaluated all OC cases with complete gene expression data and clinical information in TCGA. And from mutation annotation files, we presented the Word Cloud of gene symbols identified in OC which the mutation sites > 5 based on the WordArt (https://wordart.com). We found that TP53 is the most significantly mutated gene (**Figure S1A**). We then selected the top 25 genes to execute some higher-level studies that we employed the DISCOVER (Discrete Independence Statistic Controlling for Observations with Varying Event Rates) [25] algorithm to examine the association between co-occurrence and mutual exclusion among these genes and we found that the pairwise combinations that the highest significance of mutual exclusivity was TTN and LRP2 ($p < 0.001$) (**Figure S1B**). In order to better visualized the mutually exclusive genes pairs, we altered 420 (96.33%) of 436 samples and in a waterfall plot shown that the most frequently mutated genes were TP53 and TTN ($p < 0.05$, FDR < 0.01) (**Figure S1C**). Based on their somatic mutation data, TMB was distributed between 0.0263 to 6.5260 and the rank order of TMB across OC cases from highest to lowest is Grade 3 & Grade 4 > Grade 1 & Grade 2, that is, the higher the OC grade, the higher the TMB.

Based on their somatic mutation data, TMB was distributed between 0.0263 to 6.5260 and the rank order of TMB across OC cases from highest to lowest is Grade 3 & Grade 4 > Grade 1 & Grade 2, that is, the higher the OC grade, the higher the TMB (**Figure 2A**, n=417, except for the blank values, p=0.048). And then based on the ssGSEA scores of immune cell types, we divided the immunoactivity-related gene sets

into three immune infiltration clusters by unsupervised clustering, and some of which were defined as high immunity clusters (n=193) and low immunity clusters (n=149) (**Figure 2B**). Besides, the significant differences in tumor purity were found in the microenvironment between the high immunity clusters and the low immunity clusters. In addition, we used tumor purity in the microenvironment to validate the grouping and found that the tumor purity in the high immunity group was significantly lower than that in the low immunity group. This result proves the reliability of our classification (**Figure S1D**). Moreover, from this trend, it was found that there was a significant correlation between the high immunity clusters and the expression of PD-L1 (**Figure 2B**). To figure out the potential interrelationship between the overall survival and TMB groups and/or immunity groups, we assessed the prognosis of OC samples. In **Figure 2C,** Kaplan-Meier survival curves showed that the overall survival time of patients with the high TMB group was longer than the low TMB group and this was statistically significant. (2.58 years vs. 1.92 years, p=0.0295 in log-rank test). In **Figure 2D**, the result showed that the overall survival time of the high immunity group was higher than that of the low immunity group and this was statistically significant. (2.50 years vs. 2.45 years, p=0.00082 in log-rank test). These results indicated that the higher the TMB, the better the prognosis and the higher the OC grade. Besides, the high immunity cluster was also associated with a good prognosis of OC.

KEGG pathway and GO term analysis of DEGs associated with OC.

First, we compared the relationship between the immunity group and the immune cell infiltration in OC. Then we compared the infiltration degree of 22 different immune cells subpopulation [26] between the low immunity group and the high immunity group of cancers. We found that in the correlation between immunoactivity-related gene sets and immune infiltration, most of these immune cells have a higher infiltration degree in the high immunity group than in the low immunity group of OC (**Figure S2**). Next, as to reveal the correlation between the TMB, the immunoactivity-related gene sets, and global gene expression profiles, we crossed the high TMB gene expression signature with the high immune gene expression signature by Venn diagrams and finally obtained 14 DEGs (**Figure 3A**). Heatmaps of 14 DEGs in the up-regulated group were shown in **Figure 3B**. In the next work, we conducted unsupervised hierarchical clustering of KEGG pathways related to Up-DEGs (**Figure 3C**) and it's shown that these KEGG pathways were able to distinguish OC from normal controls with high specificity and sensitivity. And most of these KEGG pathways are immune-related such as "Cytokine cytokine receptor interaction", "Chemokine signaling pathway", as well as "Primary immunodeficiency". Moreover, in order to summarize the potential function of the DEGs, we performed functional enrichment analysis of the 14 up-regulated genes between high TMB and high immunity group. GO terms and KEGG pathway analysis identified including immune and inflammatory response, cytokine activities and T cell proliferation in the Up-DEGs group (**Figure 3D and 3E**). These results indicated that the gene sets at the intersection of Higher TMB gene expression signature and higher immune gene expression signature have active immune functions.

Protein-protein interactions among genes associated with prognosis in OC

In order to better make out the interactions between the identified DEGs, we acquired PPI networks using the STRING tool. The network was consists of Up-DEGs module (**Figure 4**), in the Up-DEGs module, we uploaded 14 DEGs to STRING to construct PPI networks and screened nine hub genes, it indicated that 9 nodes and 10 edges when minimum required interaction score was set 1.5 and disconnected nodes were hidden in the network in STRING website (**Figure 4A**). We showed a connection number with other members of the module for further analysis (**Figure 4**). In the Up-DEGs module (**Figure 4B**), CXCL13 involving 6 nodes, which has the most key link with other members of the module in the network. For the Up-DEGs module (**Figure 4B**), several key genes associated with T cell proliferation, inflammation and immune response were located at the center of the module, including PLA2G2D, CXCL13, and FCRLA. These results indicated that PLA2G2D, MS4A1, ADAMDEC1, FCRLA, CXCL13 interact more with other molecules, suggesting that these genes are more likely to be key genes.

Correlation of expression of individual DEGs in overall survival

For the sake of probe the potential effects of individual DEGs in overall survival time, Kaplan-Meier survival curves were generated from the TCGA database. Among a total of 14 Up-DEGs, four genes were validated to be significantly relevant to a good survival and prognosis outcomes, including PLA2G2D, CXCL13, FCRLA, and MS4A1 (log-rank test, $p < 0.05$) (**Figure 5A, 5B, 5C, 5D**). To valid the prognostic of these genes, we downloaded and analyzed gene expression data from the Group on Earth Observations (GEO). Meta-analysis depicting forest plots of DEGs further indicated that OC showed a significant survival benefit with high TMB gene expression signature of the four genes and were all independent predictors of OC (**Figure 5E, 5F, 5G, 5H**). These results indicated that CXCL13, FCRLA, PLA2G2D, and MS4A1 were validated as prognostic biomarkers for the immunotherapy of OC.

Correlation of four genes expression with immune infiltration level in OC and pathway analysis.

Another vital aspect of this research is the correlation between DEG expression and different levels of immune infiltration in OC. We used TIMER (Tumor Immune Estimation Resource, https://cistrome.shinyapps.io/timer/), an online database, to detect the infiltration of immune cells in tumor tissues using the RNA-seq. Our results demonstrated that CXCL13, MS4A1, and PLA2G2D have a negative correlation with tumor purity in OC, especially CXCL13, MS4A1, PLA2G2D (**Figure 6A, 6C, 6D**). CXCL13 expression showed a very weak relationship with B cell infiltration level in OC (**Figures 6A**) while FCRLA, MS4A1, PLA2G2D have no significant correlations with B cell infiltration level in OC (**Figure 6B, 6C, 6D**). Moreover, There were moderate to strong positive relationships between the expression levels of PLA2G2D, FCRLA, MS4A1, CXCL13, and infiltration level of $CD4^+$ T cells, and also have a prominent positive correlation between expression level and infiltration level of $CD8^+$ T cells in OC, especially CXCL13, MS4A1, PLA2G2D, FCRLA (**Figures 6A, 6B, 6C, 6D**). More information can be got from **Table S1**.

In the next work, we conducted gene set enrichment analysis (GSEA) to analyze the four genes and found that all of four genes were related to immunity, Both CXCL13, FCRLA, MS4A1, and PLA2G2D were associated with B cell receptor, chemokine, cytokine-cytokine receptor interaction, natural killer cell-

mediated cytotoxicity and T cell receptor signaling pathway (**Figure 6E, 6F, 6G, 6H**). Besides these, both CXCL13, FCRLA, PLA2G2D were associated with primary immunodeficiency (**Figure 6E, 6F, 6H**). MS4A1 was associated with autoimmune thyroid disease (**Figure 6G**). All of these findings revealed that CXCL13, FCRLA, MS4A1 and PLA2G2D's expression was closely related to immunity were enriched for hallmarks of OC. More information can be got from **Table S2**. These results indicated that CXCL13, FCRLA, PLA2G2D, and MS4A1 were validated as prognostic biomarkers for the immunotherapy of OC. These results indicate that CXCL13, FCRLA, PLA2G2D, and MS4A1 are positively correlated with immune cell infiltration, and these genes are enriched to be associated with immune pathways by GSEA enrichment method.

Prognostic evaluation of four genes in melanoma patients treated with immunocheckpoint inhibitors.

In addition, we used an online database, TIDE(Tumor Immune Dysfunction and Exclusion, http://tide.dfci.harvard.edu/query/), to predict patient response rates to immune checkpoint inhibitors. As shown in **Figure 7**, our results showed that among the melanoma patients treated with anti-PD-L1 or anti-CTLA4, The CXCL13, MS4A1, FCRLA, and PLA2G2D were positively correlated with the number of CTL infiltration, the two-sided t-test *p* values for correlations in CXCL13, MS4A1, FCRLA, PLA2G2D and mean are1.43×10^{-10}, 8.12×10^{-2}, 6.6×10^{-10} and 2.1×10^{-4}, respectively. And the high expression of these genes was associated with a better prognosis of patients.

The last but not least, we researched the protein expression of CXCL13, FCRLA, and MS4A1 in OC and normal ovarian tissues based on the IHC (immunohistochemistry) in The Human Protein Atlas (https://www.proteinatlas.org). We found that the staining intensity of CXCL13 and MS4A1 was slightly different in normal ovarian tissues and OC tissues, the protein expression levels in cancer were slightly lower than in adjacent cancers, but the difference was not significant. Contrary to the mRNA level of FCRLA, the level of FCRLA protein was up-regulated in OC tissues compared to the normal tissues (**Figure S3**). PLA2G2D could not find the relevant protein map in the database for the time being, which may indicate that the gene is a molecule worth digging and exploring in the future, and it is expected to play a greater role as a prognostic marker.

Discussion

OC is the most deadly malignancy among all gynecological tumors in the world and it is also one of the three most common malignancies in gynecological tumors, so it's always been a puzzle that people want to solve [27, 28]. Hence, at present, it's imperative to comprehend the possible molecular mechanisms of OC progress and so as to find the new targets for the treatment of OC patients. At present, targeting PD-1, and PD-L1's [29] immunotherapies have been allowed to be used to treated NSCLC [30], and prostate, urothelial malignancies, renal cell carcinoma [31], but currently there was little or even no clinical immunotherapy for OC. However, neoantigen produced by somatic cell mutation in the tumor is an important means to promote immune recognition of cancer [32]. Many studies have confirmed that TMB was associated with immunotherapy [33, 34]. As we all know, the main hypothesis in the field of

immunotherapy is that tumors with increased TMB produce more neoantigens, hence there are more immunogenic [35]. And new research suggests high TMB and neoantigen load in tumors have been associated with an enhanced response to immune checkpoint blockade therapy [36]. And some researches have also shown that high TMB was inclined to confer a survival benefit in diverse cancers with more non-recurrent mutations, and higher TMB also has been reported to have higher immune infiltration in tumors [37]. Such as melanoma [38] and NSCLC [39], with high TMB have shown better response and treatment results in immune checkpoint blockade treatment. Because we know that genes can regulate the protein expression, the more mutations in the genes, the more abnormal proteins are likely to be produced, and in a large number of malignancies with non-recurrent mutations, high TMB means the increase of neoantigens, finally rendering tumor cells lose their ability of growth advantage or invasiveness [40]. So TMB may be good targets for immuno-oncology treatment development.

On the other hand, TME has also been reported to have a substantial effect on the gene expression of tumor tissues, thus affecting clinical outcomes [41–43]. The role of the immunoactivity-related gene in tumors have been reported as a prognostic indicator [44]. This marker has two advantages: on the one hand, it's the most powerful prognostic factor for disease-free and overall survival especially in early-stage cancers; on the other hand, it can provide new treatments for immunotherapy [45]. And we know, tumor cells are constantly evolving to reduce the production of neoantigen generation and to escape the mutational burden associated with anti-tumor response in the TME [46]. So we combined TMB with the immunoactivity-related gene sets as prognostic indicators for OC. Therefore comprehending whether a highly immunogenic tumor is directly associated with a mutational burden could provide a mechanistic explanation and treatment direction for the survival and prognosis of clinical research.

In our current work, we tried to identify some immune critical genes associated with TMB and TME in the TCGA database that contribute to OC's overall survival. We then grouped TMB scores and immunoactivity-related gene sets into the high and the low group in OC, and in this study, we used TMB scores based on independent cancer types rather than all types of cancer to distinguish the high TMB and the low TMB in OC [15]. First, we assessed the relationship between OC grade and TMB, Strikingly, we found that high tumor stages were correlated with the TMB scores in OC. Moreover, we also found that there was a positive correlation between high immunity group and the expression level of PD-L1, which was statistically significant, indicated that such this immunological grouping was appropriate and effective to a certain extent, and provided a theoretical basis for the immunotherapy of OC (Fig. 2). When we then compared the genes expression levels in the high TMB group and with those in the low TMB group, we found that the high TMB tends to higher overall survival. Similar to the TMB group, we also found that the survival prognosis of OC was significantly correlated with the high immunity group (Fig. 2). Altogether, our studies indicated that the high TMB and the high immunity showed a trend towards survival benefit in OC. Our previous results found that there was a significant correlation with immune infiltration in the high immunity group, and the degree of infiltration was significantly higher in the high immunity group.

So next, by comparing the expression level of each gene in the high TMB group and the high immunity group, we screened out 14 Up-DEGs. Then, we analyzed the intersection of DEGs in the high TMB group (top 200 genes) and the high immunity group (427 genes) and found that many of DEGs were involved in many immune regulatory pathways, as shown by GO and KEGG terms analysis (Fig. 3). This is consistent with previous reports that the functions of immune cells have something to do with TME in OC [47]. In the current work, we focused on the genes characteristic in the TMB and TME of OC patients, which in turn influenced the development of OC and thereby contributed to patients' overall survival.

Moreover, we constructed the PPI network and in Up-DEGs module's genes were identified by using STRING software. Therefore we built up the PPI modules (Fig. 4), and all of these participated in immune response/infiltrates. Besides, we performed an overall survival analysis of among the 14 DEGs up-regulated between the high TMB group and the high immunity group, it identified that there were four genes were associated with prognosis in OC patients. All of the overall survival was good, including PLA2G2D, CXCL13, FCRLA, MS4A1. Surprisingly, the increased expression of these immune-related genes was connected with better survival prognosis in OC patients, and perhaps these gene products promoted anti-cancer immune responses and inhibited tumor metastasis. And we were interested in CXCL13, FCRLA, MS4A1, and PLA2G2D as they exhibited a significant predictive value for OC prognosis and these genes were identified as key genes by confirmation and survival analysis in OC (Fig. 5). We also validated the four genes related to the prognosis of OC patients from the GEO database (Fig. 5). And in the PPI network, CXCL13, FCRLA, MS4A1, and PLA2G2D are also highly interconnected nodes. And the four genes could serve as potential biomarkers for OC.

CXCL13 was the B cell chemoattractant, has recently been linked with TFH cell infiltration and improved survival, interestingly, functions of CXCL13 have been shown to be associated with risk of breast cancer [48]. It can promote the migration of B lymphocytes. FCRLA encodes a protein similar to the gamma immunoglobulin (IgG) Fc fragment receptor. And its encoded proteins are selectively expressed in B cells and maybe participated in the development and progression of B cells. The FCRLA is shown to be a potential target Ag in immunotherapy for B-cell lymphoma [49]. MS4A1's official full name is membrane-spanning 4-domains, subfamily A, member 1 and it's a member of the membrane-spanning 4A gene family. In addition, it can encode B-lymphocyte surface molecules that play an important role in the development and differentiation of B cells, which plays a role in the development and differentiation of B-cells into plasma cells. And it has been reported that MS4A1 plays a vital role in the apoptosis of B-cell lymphoma Ramos cells [50]. PLA2G2D involved in inflammation and immune response, and some studies indicate that PLA2G2D can improve inflammation by mobilizing lipid-promoting mediators and suggests that the enzyme may be used to treat inflammatory diseases. And PLA2G2D is preferentially expressed in dendritic cells and macrophages and has pro-differentiation functions [51]. Therefore, in summary, these genes were all related to immunotherapy to some extent and were associated with the good prognosis of OC, which can be further used as evidence for clinical immunotherapy of OC. We also evaluated the relationship of 4 genes with immune infiltration level in OC (Fig. 6), and these four genes were found to be associated with immune response and immune infiltration. Finally, because the TIDE score is the best

predictor of anti-PD1 and anti-CTLA4 therapy[52], therefore, the authors investigated the correlation between the expression of CXCL13, MS4A1, FCRLA, PLA2G2D and the response rate of immunocheckpoint inhibitors (Fig. 7). It was found that in melanoma patients treated with anti-PD-L1 or anti-CTLA4, these four genes were positively correlated with the number of CTL infiltration, and their high expression was associated with a better prognosis of patients. These key genes were showed here to correlate with the immune response to melanoma, but there was currently no data on anti-PD-L1 or anti-CTLA4 immunotherapy for OC, which could serve as predictor markers for the future immune response to OC. Our results may provide additional data in decoding the complex interaction of tumor and tumor environment in GBM. In conclusion, our results may provide more additional data for the interaction between the TMB and TME in OC.

Some studies have shown and described TMB across more than 100,000 clinical cancer samples from advanced disease, these data can help to guide more clinical trials of immunotherapy [15]. The interaction between OC and the TME seriously affected the evolution of tumors, which in turn affected tumor recurrence and the overall prognosis of patients. Hence, understanding the relationship between the combination of TMB and immune microenvironment and the survival prognosis and clinical outcomes of certain malignancies was very favorable and promising for future immunotherapy. And our study, combined with the overall analysis of TMB and TME, we found some independent survival biomarkers for the treatment of OC. Next, our future work will focus on the analysis of the immune response mechanism.

Conclusions

Our researches provided a new perspective on the relationship between TMB and tumor immune response in OCs. Higher TMB can promote immune cell infiltration and increases immune activity. And our analysis also demonstrated that the higher the TMB, the higher the immune activity, the better the prognosis of OC. In summary, We found that CXCL13, FCRLA, PLA2G2D, and MS4A1 may be biomarkers for OC immunotherapy. Finally, through further study of these genes that may shed new light on the potential relationship between tumor microenvironment and OC prognosis.

Abbreviations

TMB: Tumor Mutational Burden; Tumor Microenvironment: TME; OC: Ovarian Cancer; TCGA: the Cancer Genome Atlas; ssGSEA: Single-sample Gene Set Enrichment Analysis; Up-DEGs: Up-regulated Differential Expression Genes; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes pathway; PPI: Protein-Protein Interaction; DAVID: Database for Annotation, Visualization and Integrated Discovery; GSVA: Gene Set Variation Analysis; STRING: Search Tool for the Retrieval of Interacting Genes; DISCOVER: Discrete Independence Statistic Controlling for Observations with Varying Event Rates; GEO: Group on Earth Observations; OS: Overall Survival; TIMER: Tumor Immune Estimation Resource; GSEA: Gene Set Enrichment Analysis; TIDE: Tumor Immune Dysfunction and Exclusion; IHC: immunohistochemistry.

Declarations

Ethics approval and consent to participate

Not Applicable

Consent for publication

We have obtained consent to publish this paper from all the participants of this research.

Availability of data and materials

All data generated or analyzed during the study period were included in this published article and its supplementary information.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Lili Fan conceived and designed this study, also crafted figures and tables, and was responsible for the critical reading of the manuscript. Also, she contributed to the data collection and analysis and the critical reading of the manuscript. Han Lei, Ying Lin, and Tianxiang Zhang were responsible for the writing and helped with the figures and tables. Han Lei, Zhengwei Zhou, Zhipeng Yan, Guang Shu, and Gang Yin supervised and contributed to the critical reading of the manuscript.

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Figures

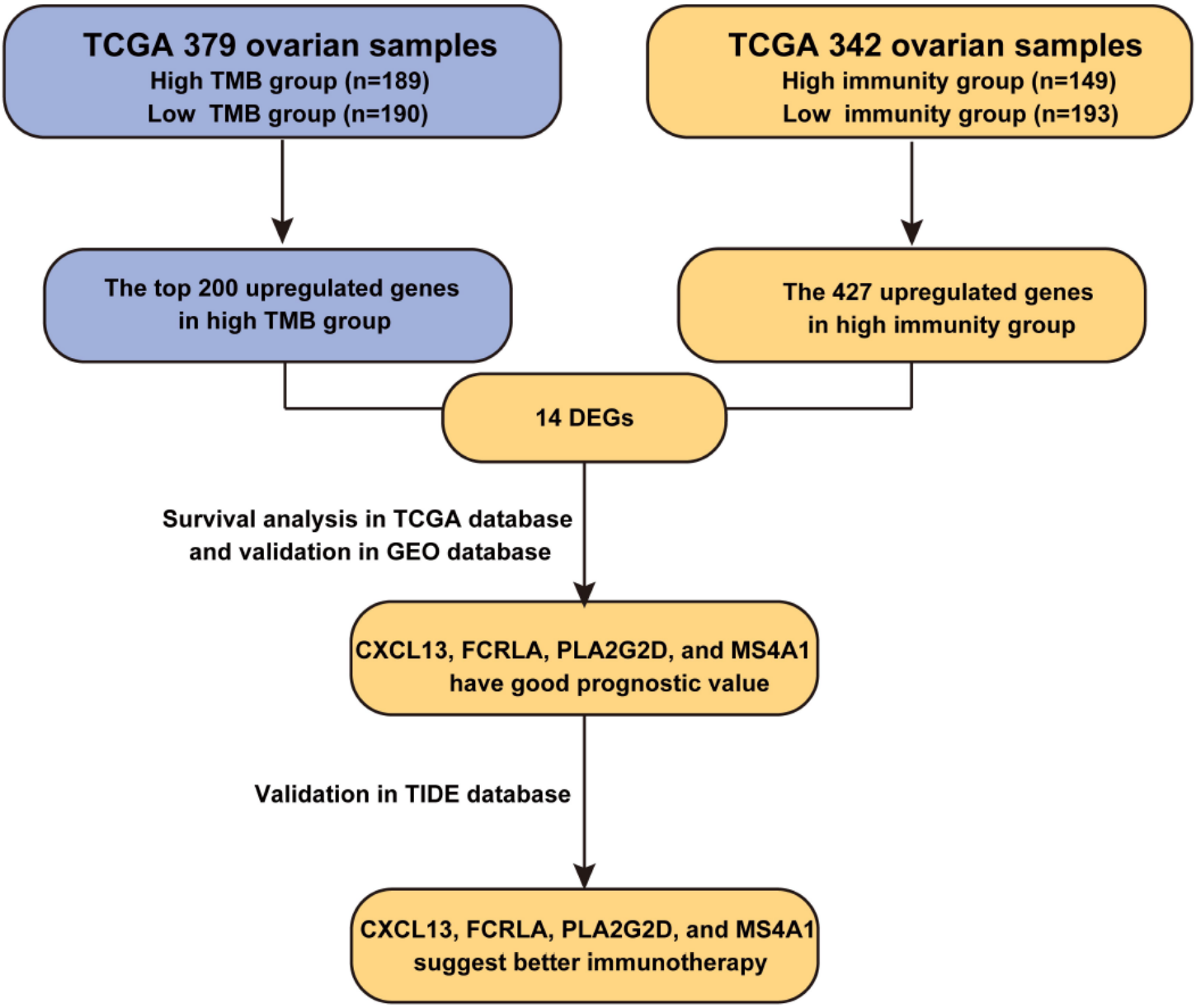

Figure 1

The workflow of the current work.

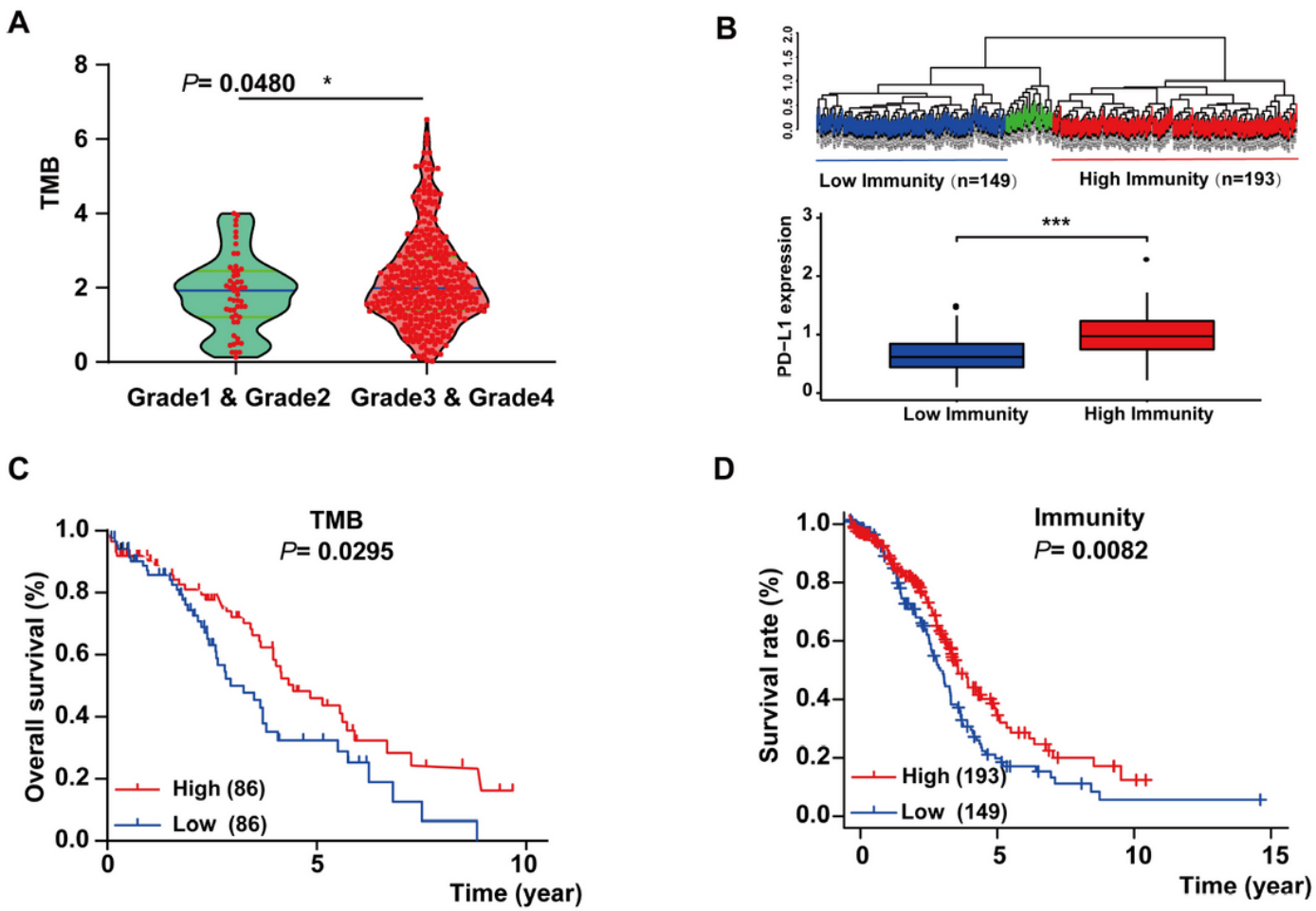

Figure 2

The TMB scores and the immunoactivity-related gene sets are conjoined with OC's overall survival. (A) The violin plots showed the interrelationship between TMB and OC grades. It exhibited that there was a powerful relationship between OC grades and the level of TMB scores (n=417, except for the blank values, p=0.0480). (B) Up: Unsupervised clustering of OC patients from the TCGA cohort using ssGSEA scores from immune cell types. Hierarchical clustering was performed with Euclidean distance and Ward linkage. Three distinct immune infiltration clusters, here we define some of them as low immunity and high immunity. Down: the correlation analysis between the expression level of PD-L1 and the high & low immunity clusters obtained by ssGSEA score based on immune cell type (As displayed by Mann-Whitney test, $p < 0.0001$). (C) OC patients were classified into two groups based on their TMB scores: the samples with high TMB scores in the top 20% and the low TMB samples with TMB scores in the bottom 20% of all OC samples. According to the Kaplan-Meier survival curve, the total survival time of the high TMB group is longer than the low TMB group (2.58 years vs. 1.92 years, n=430, except for the blank values, as displayed by the long-rank test, p=0.0295). (D) OC patients were classified into two groups based on their immunity: those with a high immunity group in 193 patients and those with a low immunity group in 149 patients. The total survival time of the high immunity group is longer than the low immunity group (2.77 years vs. 2.67 years, n=364, except for the blank values, as displayed by the log-rank test, p=0.0082).

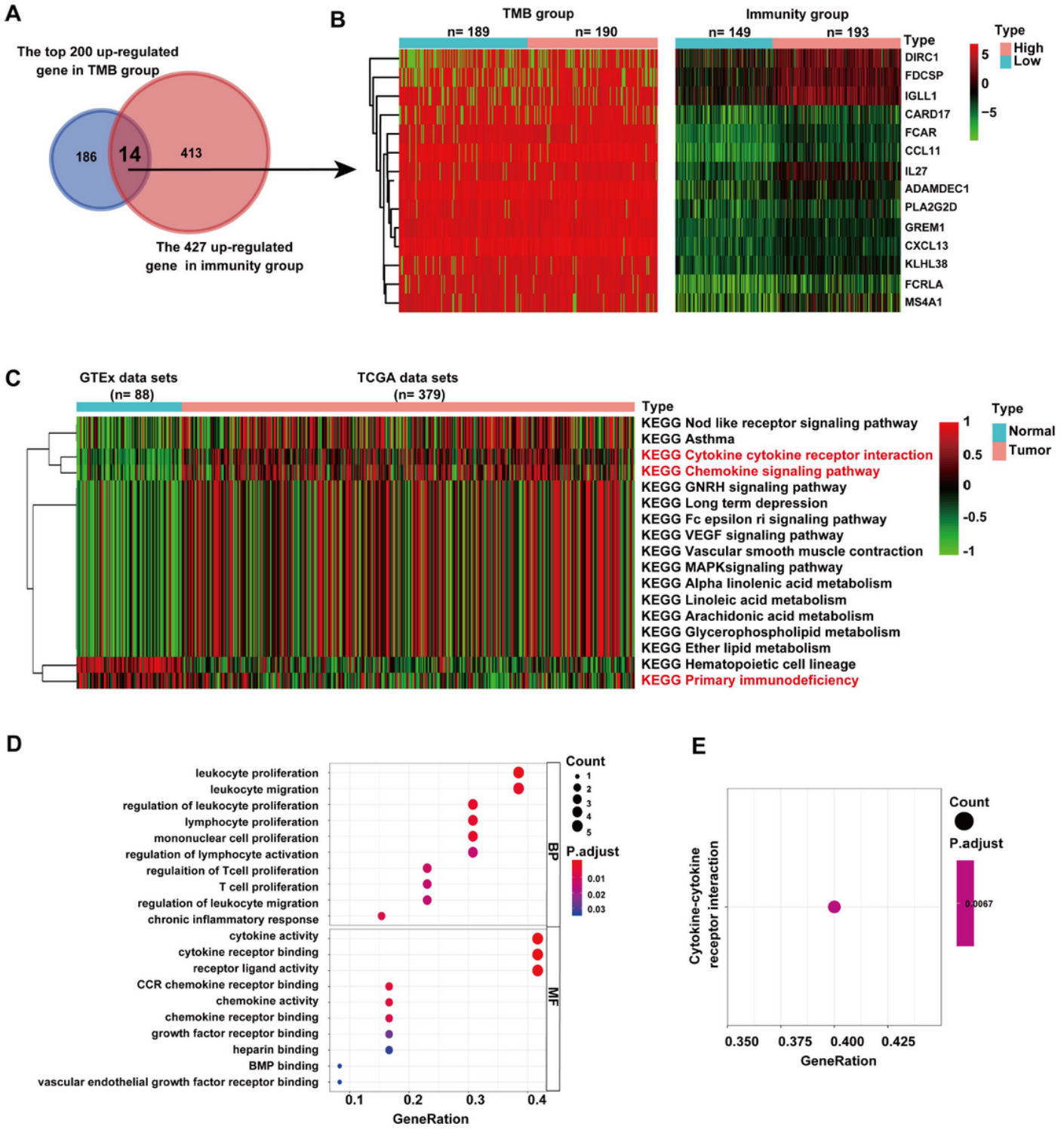

Figure 3

KEGG pathway and GO term analysis of DEGs associated with OC. (A) Venn diagrams displayed the amount of conjointly up-regulated DEGs in both the TMB group and the high immunoactivity-related gene sets group. (B) Heatmaps of 14 DEGs in the up-regulated group. (C) Unsupervised hierarchical clustering of KEGG pathways associated with Up-DEGs. Blue represents normal samples, data from GTEx (n=88), red represents treat samples, data from TCGA (n=379). The average linkage method and the Pearson distance measurement method were used to depict the Heatmaps. High expression genes are shown in

red, low expression genes in green, and genes with the same level of expression are shown in black. False discovery rate (FDR) < 0.05, fold change > 1. (D, E) A remarkably rich overview of the GO and KEGG terms. The X-axis serves as the proportion of relevant genes, and the y-axis serves as the GO and KEGG terms. Each bubble means a term. The size of the bubble implies the account of relevant genes. Lighter colors suggest the smaller P values. D: Enriched GO terms. E: Enriched KEGG terms. FDR of GO and KEGG pathway analysis was acquired from the DAVID functional annotation tool. p < 0.05.

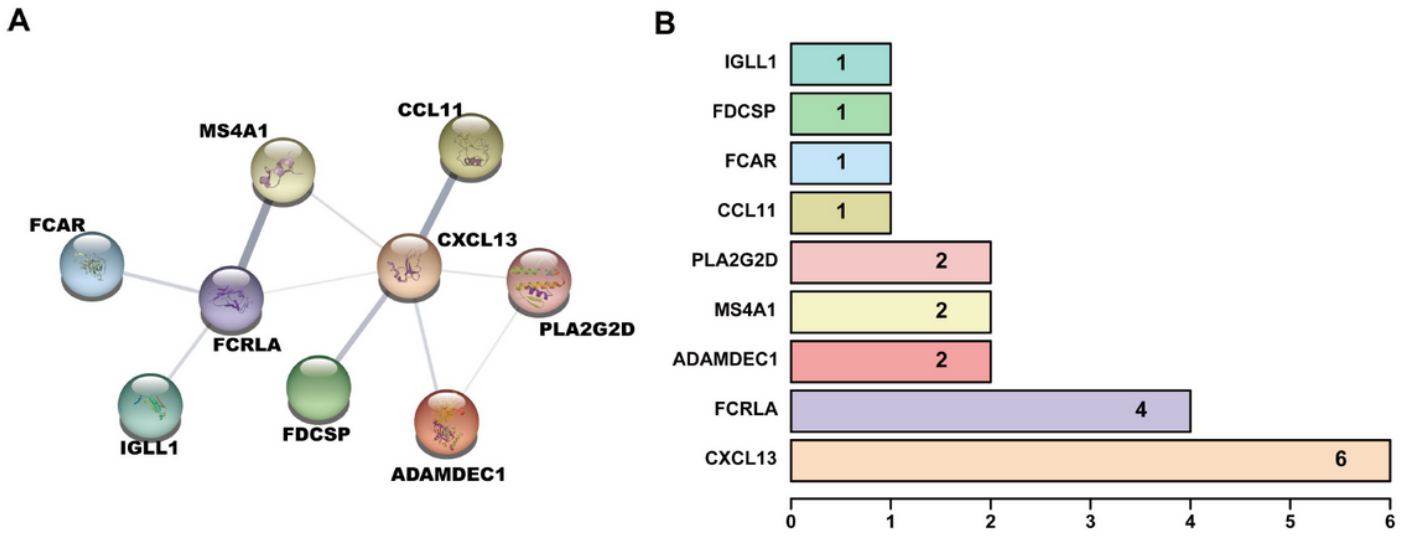

Figure 4

PPI networks of Up-DEGs Modules. (A) PPI network established by the STRING database for the Up-DEGs module. Minimum required interaction score >0.04, Disconnected nodes were hidden in the network. (B) Quantification diagram of the Up-DEGs module.

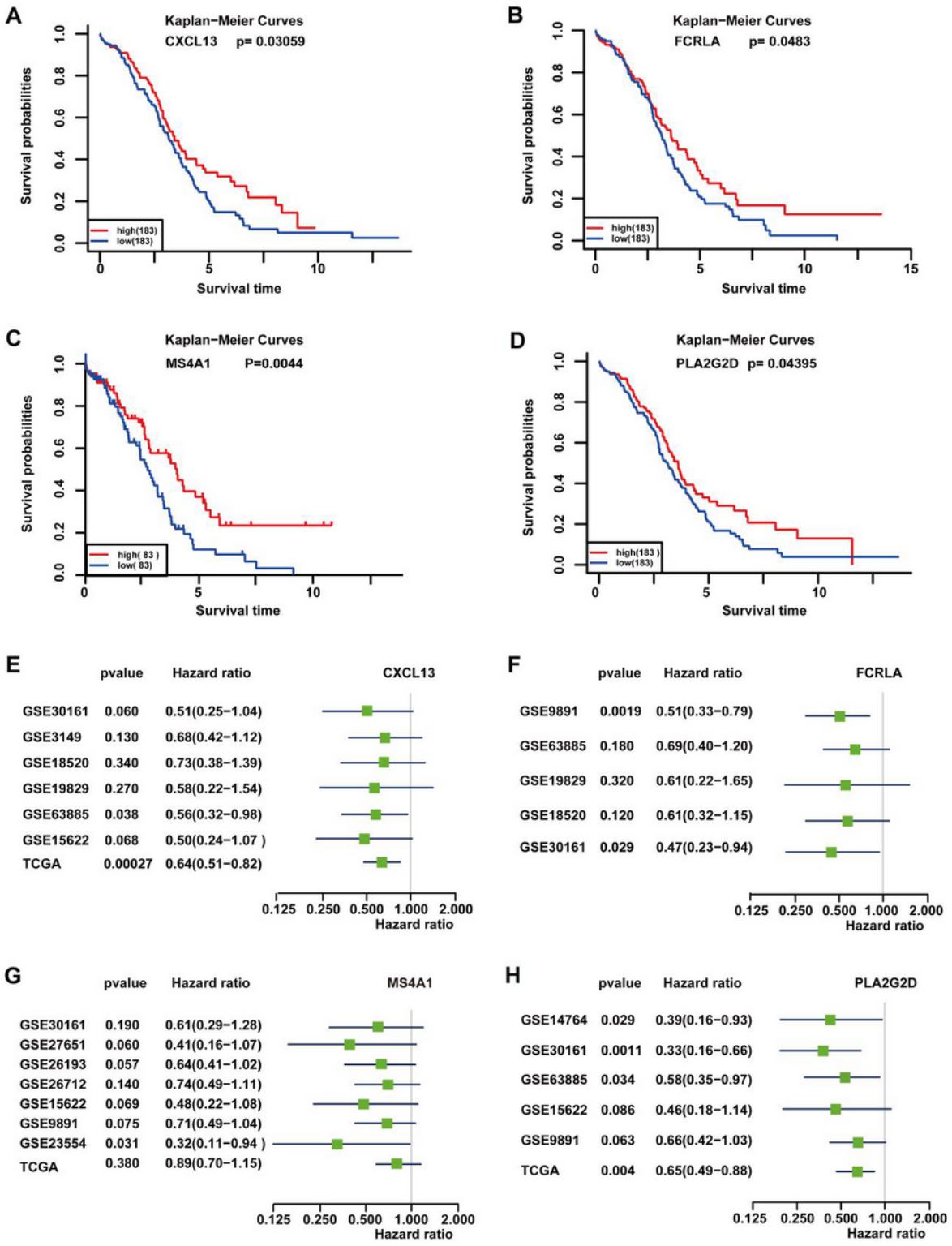

Figure 5

Correlation of expression of individual Up-DEGs in overall survival. (A-D) Kaplan-Meier survival curves were produced for selected DEGs extracted from the comparison of groups of high (red line) and low (blue line) gene expression. The elevated expression of CXCL13 (A), FCRLA (B), MS4A1 (C) and PLA2G2D (D) were related to better overall survival (OS) in OC. $p < 0.05$ in the Log-rank test. (E-H) Verification of the correlation between DEGs extracted from the TCGA database and overall survival time in the GEO cohort.

Forest plots showed the association with the clinical benefits of the four genes in the survival prognosis study and can as a univariate predictor of OC.

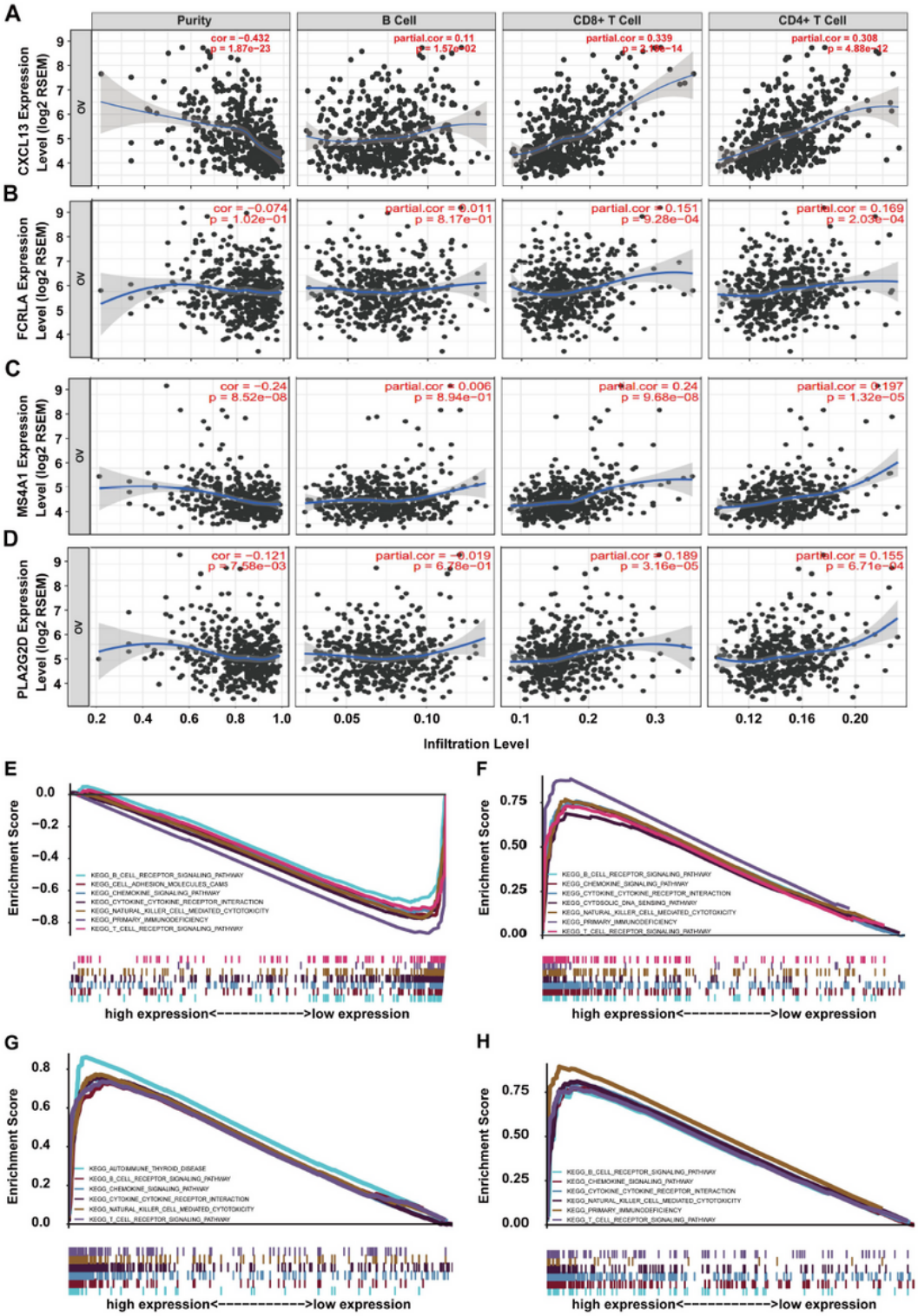

Figure 6

Correlation of four genes expression with immune infiltration level in OC and pathway analysis. (A-D) Scatter diagram of the correlation between the expression levels of CXCL13, FCRLA, MS4A1, PLA2G2D and immune infiltration level in OC. (E-H) An evaluated enrichment degree within the four genes-

expressing. KEGG pathway analysis of CXCL13, FCRLA, MS4A1, and PLA2G2D. The vertical bars on the x-axis of the GSEA diagram represent the positions of genes in a given set in a sorted list. Positive GSEA curve means positive enrichment and the negative GSEA curve means anti-enrichment.

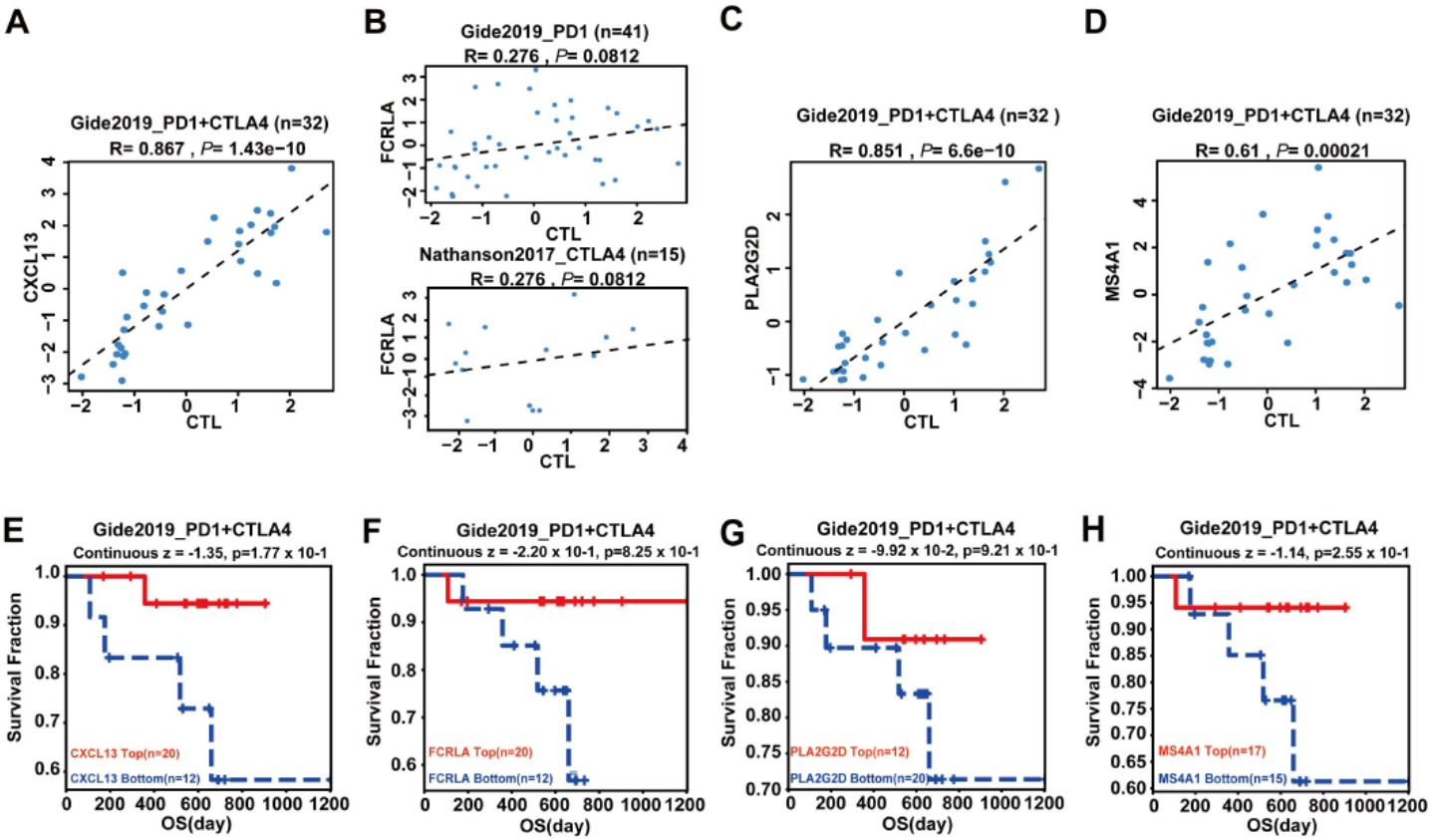

Figure 7

Prognostic evaluation of four genes in melanoma patients treated with immunocheckpoint inhibitors. (A-D) Scatter diagram of the correlation between the expression levels of CXCL13 (A), FCRLA (B), PLA2G2D (C) and MS4A1 (D) the response rate of immune checkpoint inhibitors in melanoma. The expression correlations of top (> 0) and bottom (< 0) were obtained in patients with melanoma treated with anti-PD1 and anti-CTLA4. The x-axis shows the CTL level for each tumor (average expression level of CD8A, CD8B, GZMA, GZMB, and PRF1). The Pearson correlation (R) between the plotted values is shown in the upper of each plot. (E-F) Kaplan–Meier plots of overall survival (OS) for melanoma patients with top half and bottom half CXCL13 (E), FCRLA (F), PLA2G2D (G) and MS4A1 (H) expression levels, using the data from an anti-PD1 and anti-CTLA4 study. The p-value was calculated by testing the association between high/low expression and overall survival with the two-sided Wald test in a Cox-PH regression. The z-score of each gene is calculated by dividing the interaction coefficient by its standard error. The elevated expression of CXCL13 (E), FCRLA (F), PLA2G2D (G) and MS4A1 (H) were related to better OS in melanoma. $p < 0.05$ in the Log-rank test.

Supplementary Files

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

- TableS1.xls
- TableS2.xls
- SupplementaryMaterial.pdf