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5 signature genes revealed by single-cell profiling identified unique immune subtypes affecting the prognosis of ovarian cancer

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Abstract. – OBJECTIVE: Ovarian cancer (OC) ranks among the most prevalent gynecological malignancies, with surgery, chemotherapy, and immunotherapy constituting primary treatment modalities. However, despite advancements, immunotherapy, particularly immune checkpoint inhibitors, has yielded suboptimal outcomes. The pressing need to identify biomarkers predictive of clinical prognosis underscores our objective. We aim to discern gene signatures and establish prognostic subgroups, specifically in the context of immunotherapy and chemotherapy, guiding clinical decision-making.

MATERIALS AND METHODS: We used the Tumor Immunotherapy Gene Expression Resource (TIGER) and The Cancer Genome Atlas (TCGA) databases to extract signature genes of prognostic significance. Unsupervised consensus clustering was employed to classify patients based on these signature genes. The Tumor Immune Estimation Resource (TIMER) database, along with the R packages "maftools" and "ESTIMATE" facilitated immune infiltration estimation. Gene set variation analysis (GSVA) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis were implemented to probe immune-related cell signaling pathways among distinct subtypes. The Tumor Immune Dysfunction and Exclusion (TIDE) database was used to assess immunotherapy effects, while the R package "OncoPredict" evaluated drug sensitivity differences among subtypes.

RESULTS: We identified five prognostically influential genes in ovarian cancer: ***IGFBP7, JCHAIN***, ***CCDC80***, ***VSIG4***, and ***MS4A1***. Utilizing these signature genes, we categorized TCGA-OV patients into five clusters, each associated with varying clinical prognoses. Notably, 2 clusters exhibited superior prognoses, accompanied by enhanced immune cell infiltration. KEGG enrichment analysis revealed their heightened enrichment in cellular immunity and immune cell interaction pathways. Given the elevated expression levels of multiple immune checkpoint molecules, these clusters may substantially benefit from immune checkpoint inhibitor therapy. Additionally, chemotherapy sensitivity analysis indicated their favorable responses to first or second-line chemotherapy regimens.

CONCLUSIONS: We subclustered ovarian cancer patients by 5 signature genes obtained from the Single-cell RNA sequencing (scRNA-seq) dataset, which demonstrated a good typing effect. Patients in the two molecular subtypes showed better survival, higher immune cell infiltration, and higher drug sensitivity. This meticulous typing may help clinicians to quickly assess the prognosis of patients and the response to immunotherapy and chemotherapy.

Key Words:
Ovary Cancer, scRNA-seq, Gene signature, Immune subtypes.

Introduction

Ovarian cancer (OC) represents one of the most challenging gynecological malignancies, characterized by the worst prognosis among such tumors, with approximately 22,000 new diagnoses annually. Early clinical-stage diagnosis remains elusive, often resulting in detection at an advanced stage[1]. The recurrence rate of ovarian cancer is extremely high after the standard treatment protocol of comprehensive staging surgery supplemented with platinum or paclitaxel chemotherapy, and patients with recurrence are prone to platinum drug resistance after multiple chemotherapy sessions, and the treatment effect becomes increasingly low. Bevacizumab or Poly (ADP-ribose) polymerase (PARP) inhibitors have been proven to be used in maintenance therapy,

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showing favorable effects in prolonging progression-free survival (PFS), while little effect on overall survival (OS) was observed[2,3], suggesting the need for more effective maintenance therapy, including immunotherapy.

Immunotherapy enhances the anti-cancer immune response by multiple pathways, including immunostimulatory cytokines, application of tumor-specific/associated antigen vaccines, and monoclonal antibodies targeting immunosuppressive receptors expressed by immune cells or ligands expressed by tumor cells[4]. The blockade of inhibitory receptors/ligands is often called immune checkpoint inhibitions (ICIs), which target the common immune checkpoint such as programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1), cytotoxic T-lymphocyte antigen-4 (CTLA-4), Lymphocyte activation gene 3 (LAG-3), T-cell immunoglobulin mucin 3 (TIM3), Nuclear Receptor Subfamily 2 Group F Member 6 (NR2F6), T cell immunoreceptor with Ig and ITIM domains (TIGIT), B and T lymphocyte attenuator (BTLA), V-domain Ig suppressor of T cell activation (VISTA)[5]. Blockade of PD-1 and CTLA-4 is currently the most promising ICI approach so that the immune suppression can be reversed. They have exhibited remarkable clinical efficacy, especially in melanoma, non-small cell lung cancer (NSCLC), colon cancer, and renal cell carcinoma[6-8]. However, only 20-30% of patients treated with these ICIs achieve[9,10] long-term survival time, making it an urgent issue to identify biomarkers for prediction of clinical outcome.

In this study, we identified five gene markers with prognostic significance in OC patients and further identified five molecular subtypes using a dataset of OC single-cell sequencing. In the meanwhile, we clarified the differences in immune cell infiltration, drug sensitivity, and prognosis among patients in these different molecular subtypes. Ultimately, our findings shed light on novel tumor molecular subtypes capable of accurately characterizing OC patients with distinct immune profiles, offering potential guidance for personalized clinical decision-making.

Materials and Methods

Data Sources of Ovarian Cancer Microenvironmental Signature Genes

A single-cell RNA sequencing dataset of OC was obtained through the TIGER database (available at: http://tiger.canceromics.org/)[11]. Signature genes from endothelial cells, epithelial cells, fibroblasts, myeloid cells, B cells, total T cells, CD4+ T cells, CD8+ T cells and natural killer (NK) cells (logFC>1) were retrieved, resulting in 303 signature genes.

Identifying the Prognostic Relevance of Signature Genes

We obtained the ovarian serous cystadenocarcinoma patients' dataset (TCGA-OV) from the Cancer Genome Atlas (TCGA) database (available at: https://portal.gdc.cancer.gov/), which included transcriptome sequencing data, clinicopathological feature data, and follow-up information of 378 patients. Univariate and multivariate COX regression analysis identified marker genes with prognostic value (p-value <0.05).

Identification of Molecular Subtypes

"ConsensusClusterPlus" R package (available at: https://bioconductor.org/packages/release/bioc/html/ConsensusClusterPlus.html) was used to classify and identify patients by unsupervised consensus clustering. The clustered patients were identified by PCA analysis.

Immune Cell Infiltration Analysis

The infiltration level of 22 immune cells in TCGA-OV patients was obtained from the Tumor Immune Estimation Resource (TIMER) database (available at: http://timer.cistrome.org/), and the infiltration level of macrophages, neutrophils, myelogenous cells such as T cell and B cell in different subtypes of patients was evaluated. The immune cell score and microenvironment score of patients with different subtypes were evaluated with "Estimation of STromal and Immune cells in MAlignant Tumor tissues using Expression data" (ESTIMATE) R package (available at: https://bioinformatics.mdanderson.org/estimate/rpackage.html).

Determination of Tumor Mutation Burden

The evaluation of copy number variation and tumor mutation burden (TMB) in patients with different subtypes was conducted by the "maftools" R package (available at: https://www.bioconductor.org/packages/release/bioc/html/maftools.html).

TIDE Score

TIDE database (available at: http://tide.dfci.harvard.edu/) was used to evaluate the sensitivity

of different subtypes of patients to ICI treatment and the difference in immune resistance score.

Identification of Drug Sensitivity

The R package "OncoPredict" (available at: https://cran.r-project.org/web/packages/oncoPredict/index.html) was used to evaluate the difference in drug sensitivity of different subtypes of patients to first-line or second-line chemotherapy drugs for ovarian cancer.

Results

Five Prognostic Specific Tumor Microenvironment Marker Genes for Ovarian Cancer Were Identified

We obtained the OC single-cell database set through the TIGER database and screened 303 characteristic genes of tumor microenvironment cells (logFC>1 and logFC of tumor cells<1). Then, after univariate and multivariate COX regression analysis of the TCGA-OV, five unique genes that affect the prognosis of OC in microenvironment cells (including B cell, endothelial, epithelial cell, erythrocyte, fibroblast, malignant, myeloid, plasma and T cell) were identified, namely *IGFBP7*, *JCHAIN*, *CCDC80*, *VSIG4*, and *MS4A1*. Table I shows the results of the COX analysis, and Figure 1A shows the distribution characteristics of these five genes in the tumor microenvironment. The survival analysis results of 5 genes are displayed in Figure 1C-G.

The Patients Were Divided into 5 Subtypes with Consensus Clustering

In order to determine whether these five specific marker genes can be used for fine classification of patients, we conducted cluster analysis on patients through unsupervised consensus clustering. The clustering results in Figure 2A-D show that patients can be successfully divided into 5 subtypes - the best clustering result can be obtained when the K value is 5. The heat map in Figure 2E shows the expression characteristics of 5 genes in different subtypes of patients, including tumor grade, stage, age, and survival status in different clusters. Survival analysis showed that the survival rates of different patients were significantly different. The prognosis of patients in cluster 3 and cluster 5 was better, while that of cluster 1, cluster 2, and cluster 4 was poor (Figure 2F).

Analysis of Immune Characteristics of Different Subtypes

PCA showed the difference in transcriptome expression in patients with different subtypes

Table I. Univariate and multivariate COX regression analysis of tumor microenvironment marker genes for TCGA-OV patients.

Gene	Univariate analysis			Multivariate analysis		
	HR	95% CI	*p*-value	HR	95% CI	*p*-value
DNPH1	0.554	0.554 (0.335-0.915)	0.021	-	-	-
MZB1	0.918	0.918 (0.843-0.999)	0.046	-	-	-
IFT27	0.7	0.700 (0.506-0.968)	0.031	-	-	-
CTSD	2.388	2.388 (1.073-5.314)	0.033	-	-	-
VWF	1.383	1.383 (1.029-1.858)	0.031	-	-	-
FKBP11	0.612	0.612 (0.397-0.944)	0.026	-	-	-
IGFBP7	0.455	0.455 (0.212-0.975)	0.043	0.352	0.352 (0.150-0.826)	0.016
DUSP1	1.607	1.607 (1.086-2.380)	0.018	-	-	-
IGLC2	0.889	0.889 (0.805-0.982)	0.021	-	-	-
HMGN3	0.414	0.414 (0.199-0.860)	0.018	-	-	-
CD79A	0.908	0.908 (0.833-0.991)	0.03	-	-	-
JCHAIN	0.903	0.903 (0.828-0.986)	0.023	0.795	0.795 (0.659-0.958)	0.016
CCDC80	1.203	1.203(1.007-1.437)	0.041	1.397	1.397 (1.113-1.753)	0.004
FOS	1.487	1.487 (1.051-2.106)	0.025	-	-	-
HSBP1	0.47	0.470 (0.261-0.847)	0.012	-	-	-
TRBC1	0.895	0.895 (0.808-0.992)	0.034	-	-	-
CETN2	0.46	0.460 (0.235-0.899)	0.023	-	-	-
VSIG4	1.303	1.303 (1.047-1.621)	0.018	1.605	1.605 (1.185-2.176)	0.002
CXCR4	0.644	0.644 (0.415-0.998)	0.049	-	-	-
MS4A1	0.858	0.858 (0.772-0.953)	0.004	0.868	0.868 (0.741-1.017)	0.081
IGHGP	0.933	0.933 (0.877-0.992)	0.028	-	-	-

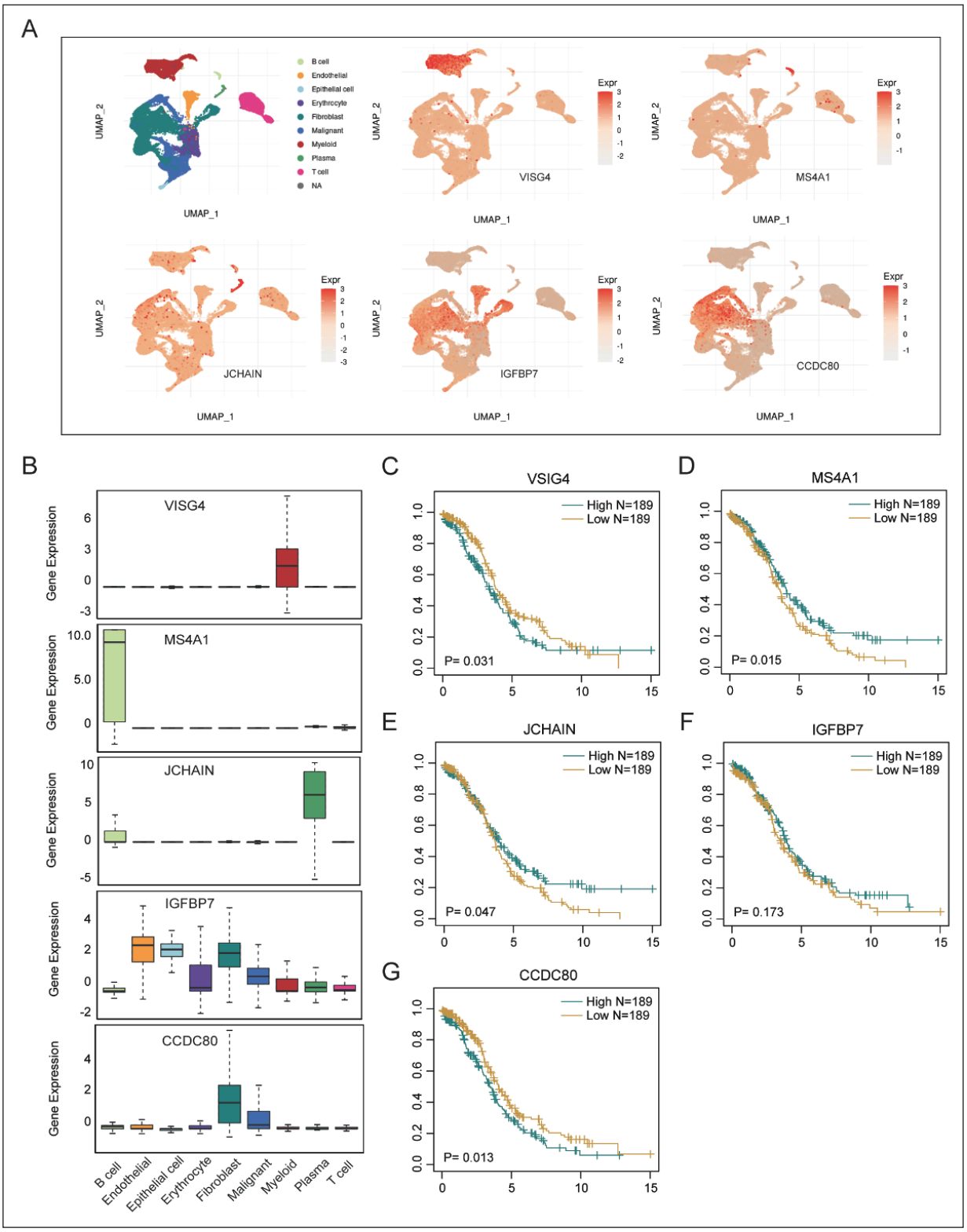

Figure 1. 5 prognostic-specific tumor microenvironment marker genes for ovarian cancer were identified. **A-B**, The distribution and gene expression characteristics of these five genes in the tumor microenvironment reviewed by scRNA-seq. **C-G**, The survival analysis results of 5 genes by KM plot.

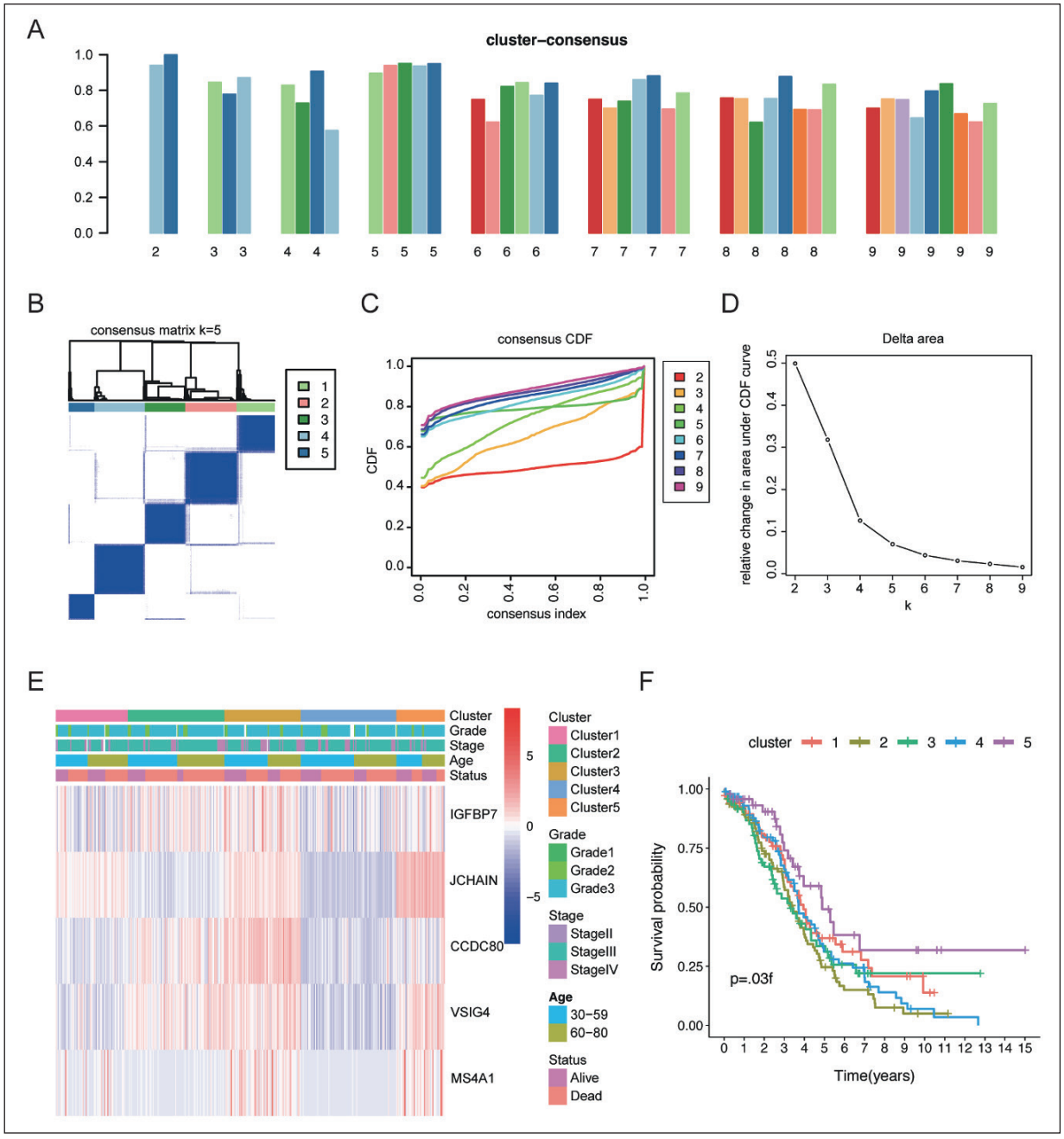

Figure 2. Consistency clustering divides patients into 5 subgroups. Unsupervised consensus clustering divides patients into 5 subgroups when K=5 can achieve the best clustering results (**A-D**). **E**, Heatmap displays the clinical status of patients in different clusters. **F**, 5 cluster patients show different survival status.

(Figure 3A). To determine whether the subtypes identified above can accurately reflect different immune characteristics, we evaluated the amount of immune cell infiltration in TIMER. It can be seen that there are significant differences in the contents of B cells, T cells, neutrophils, macrophages, and DC in different subtypes of patients (Figure 3D) under the TIMER algorithm. Among them, the contents of immune cells in C3 and C5 patients are higher, while those in C4 patients are lower, indicating that C3 and C5 patients are relatively active in immunity, while C4 belongs to the "immune desert type". Further, through the Cibersort algorithm, we found that C5 patients showed the highest content of CD8+ T cells and Th cells, while C3 patients had a higher content

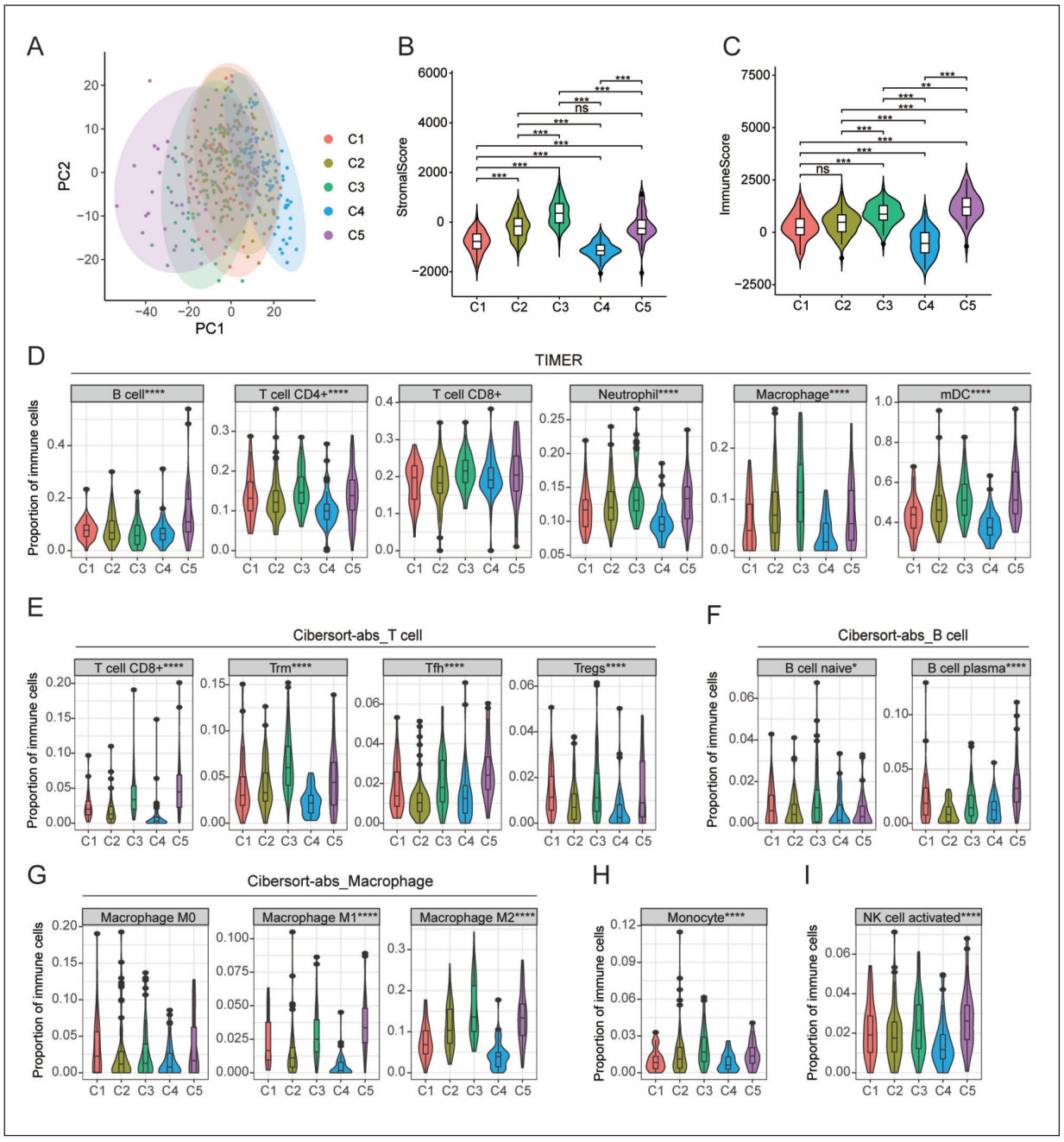

Figure 3. Immune infiltration analysis of the 5 clusters. **A**, PCA analysis of the 5 clusters. **B-C**, Estimate immunoassay showed different immune score and stromal score among 5 clusters. **D-I**, TIMER and Cibersort display different immune infiltration compositions among 5 clusters. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

of CD4+ memory-resting cells than C5 patients (Figure 3E). The content of immature B cells was higher in C3 patients, while the content of plasma cells was higher in C5 patients (Figure 3F). In addition, M1 macrophages were higher in C5 patients, while M2 macrophages were higher in C3 patients, which further proves this difference in these clusters (Figure 3G). Similarly, this difference between the two clusters can be found in monocytes and NK cells (Figure 3H-I). The above differences indicate that the immune activation status of C5 patients is better than that of C3 patients. In addition, the microenvironment score further shows this difference. C4 patients

have the worst stromal cell score and immune cell score, while C3 patients have the highest stromal cell score, and C5 patients have the highest immune cell score (Figure 3B-C).

Analysis of Molecular Characteristics of Subtypes

In order to explore the difference in pathways between different subtypes, we used "GSVA" to evaluate the variation of KEGG-related pathways in different clusters. It showed the difference in pathway score between different subtypes, and there were many significant differences between C5 and C4, C3 and C4, including chemokine signaling, cytokine/cytokine receptor interaction, T cell/B cell receptor signaling, NK cell-mediated cytotoxicity, Janus kinase-signal transducer and transcriptional activator (JAKSTAT) signaling and primary immunodeficiency pathway (Figure 4A-B), which are closely related to cellular immunity and the interaction between immune cells. In addition, we used maftool to evaluate the difference in tumor mutation load in patients with different subtypes. It can be seen that the most common mutation type among the two clusters is a missense mutation, while *TP53* is the gene most prone to mutation. The mutation load of C5 is also significantly higher than that of C4 patients, which indicates that C5 patients may have better immune responses (Figure 4C-D).

Prognosis and Treatment Response of Different Subtypes

Firstly, we evaluated the correlation between these five marker genes and immune checkpoints, as well as the difference in the expression level of immune checkpoints among these 5 clusters. It can be found that Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), Hepatitis A virus cellular receptor 2 (HAVCR2), Lymphocyte-activation gene 3 (*LAG3*), Programmed cell death protein 1 (PDCD1) and so forth were in varied good degrees of correlation with these marker genes, and the correlation ratio of *VSIG4* and HAVCR2 can reach 0.8 (Figure 5A). Similarly, the expression of TIGIT, CD274, CD226, *LAG3*, HAVCR2, PDCD1, V-set immunoregulatory receptor (VSIR) in C3 and C5 was higher than that in other clusters (Figure 5B-C). By the use of the TIDE database, we found that C3 and C5 patients also had higher TIDE scores, immune rejection scores, and dysfunction scores, indicating that these subtypes of patients may be more sensitive to ICI treatment (Figure 5D-F). We further evaluated the sensitivity difference of different subtypes of patients in terms of chemical treatment. Among the four first-line or second-line drugs (docetaxel, gemcitabine, cisplatin, and paclitaxel), C3 and C5 patients had higher sensitivity (Figure 5G-J).

Discussion

Over the past few decades, tumor immunotherapy has flourished, providing new opportunities for the treatment and long-term management of solid tumors. However, the results of immunotherapy for ovarian cancer are still unsatisfactory. The improved prediction of immunotherapeutic response can be achieved by evaluating the target therapy sensitive or resistant subsets according to specific tumor biomarkers. Here, we identified five prognosis-related marker genes from a single-cell sequence cohort of immunotherapy and further identified clusters with good immunotherapy response and chemotherapy sensitivity. With just a few gene detections, ovary cancer patients' response to treatment was well judged. It is helpful to explore the intrinsic molecular mechanism behind the different immunotherapeutic responses of ovary cancer patients.

In our study, 5 out of 356 genes revealed by single-cell profiling of ovarian cancer were identified by univariate and multifactorial COX regression analysis, which led to successful clustering of the TCGA-OV patients. The genes *IGFBP7*, *JCHAIN*, *CCDC80*, *VSIG4* and *MS4A1* were screened for molecular subtyping. Our results showed that the patients in cluster 3 and cluster 5 might get a better prognosis and should be more sensitive to immunotherapy, including PD-1, *LAG3*, TIGIT and CTLA4 therapy options. Immune infiltration analysis showed that the patients in cluster 3 and cluster 5 get a better infiltration of CD8+ T cells and Th cells, immature B cells. Further, TIDE scores provide more evidence of the immune activation status of cluster 3 and cluster 5.

Five characteristic genes were identified, including *IGFBP7*, *JCHAIN*, *CCDC80*, *VSIG4* and *MS4A1*. *IGFBP7* shows tumor suppressive activity in specific tumors by regulating cell proliferation, apoptosis, epithelial-mesenchymal transition (EMT), angiogenesis, and increased immune infiltration[12-14]. *JCHAIN* gene encodes immunoglobulin J-chains, and it enables IgA/IgM binding activity and protein homodimerization activity. It is reported that *JCHAIN* is related

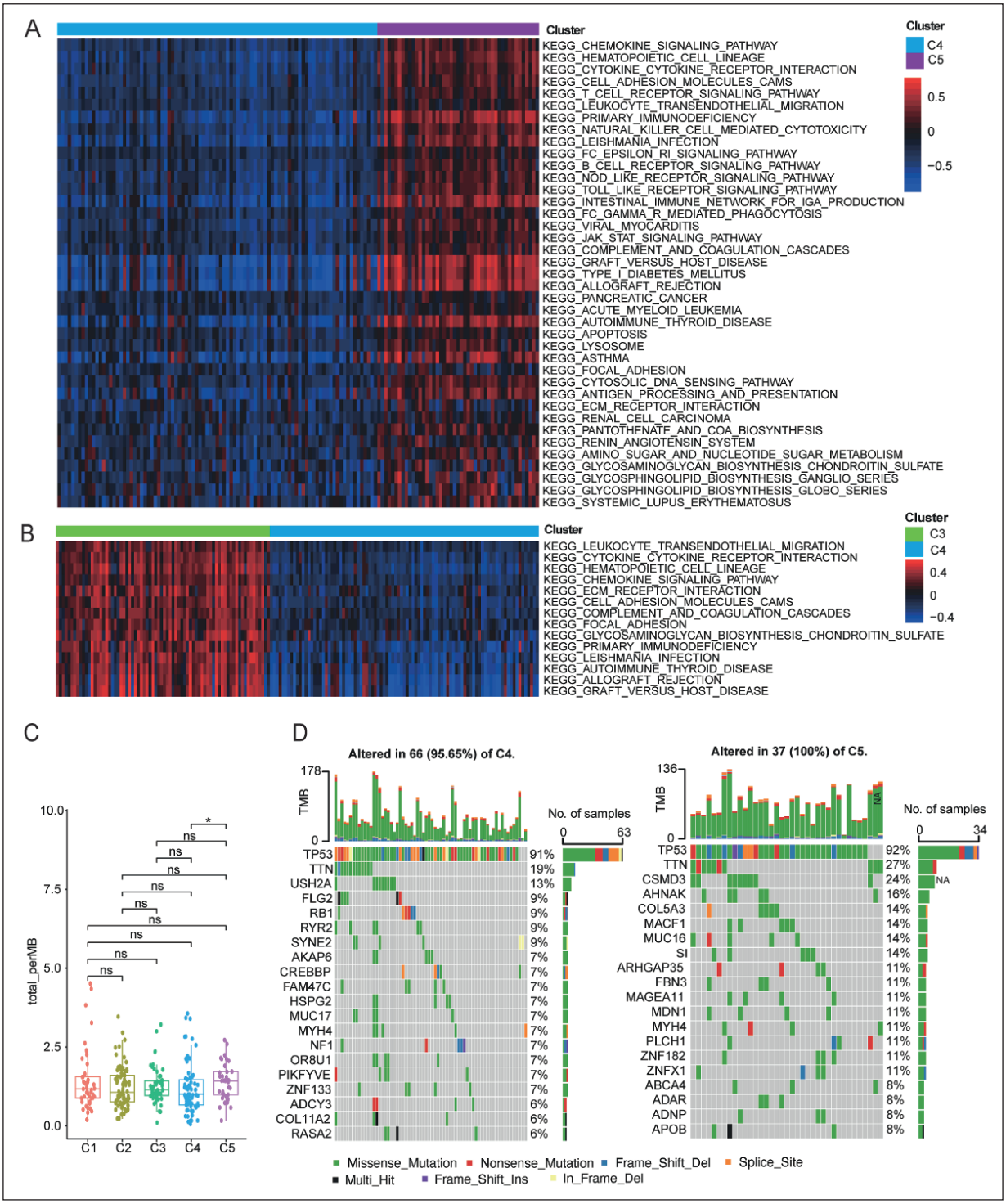

Figure 4. Analysis of molecular characteristics of the 5 subtypes. **A-B**, GSVA analysis of KEGG in 5 subtypes. **C-D**, Tumor mutation burden (TMB) analysis among the 5 subtypes. *$p<0.05$.

to the clinical prognosis of patients with acute lymphoblastic leukemia[15]. In addition, it was also found[16] to be associated with the survival of head and neck squamous cell carcinoma, together with *CHAC2*, *CLEC9A* and 8 other genes. Besides, *JCHAIN* may have a key impact on drug resistance and tumorigenesis of Marek's disease[17]. Previous studies[18,19] have found that *CCDC80*

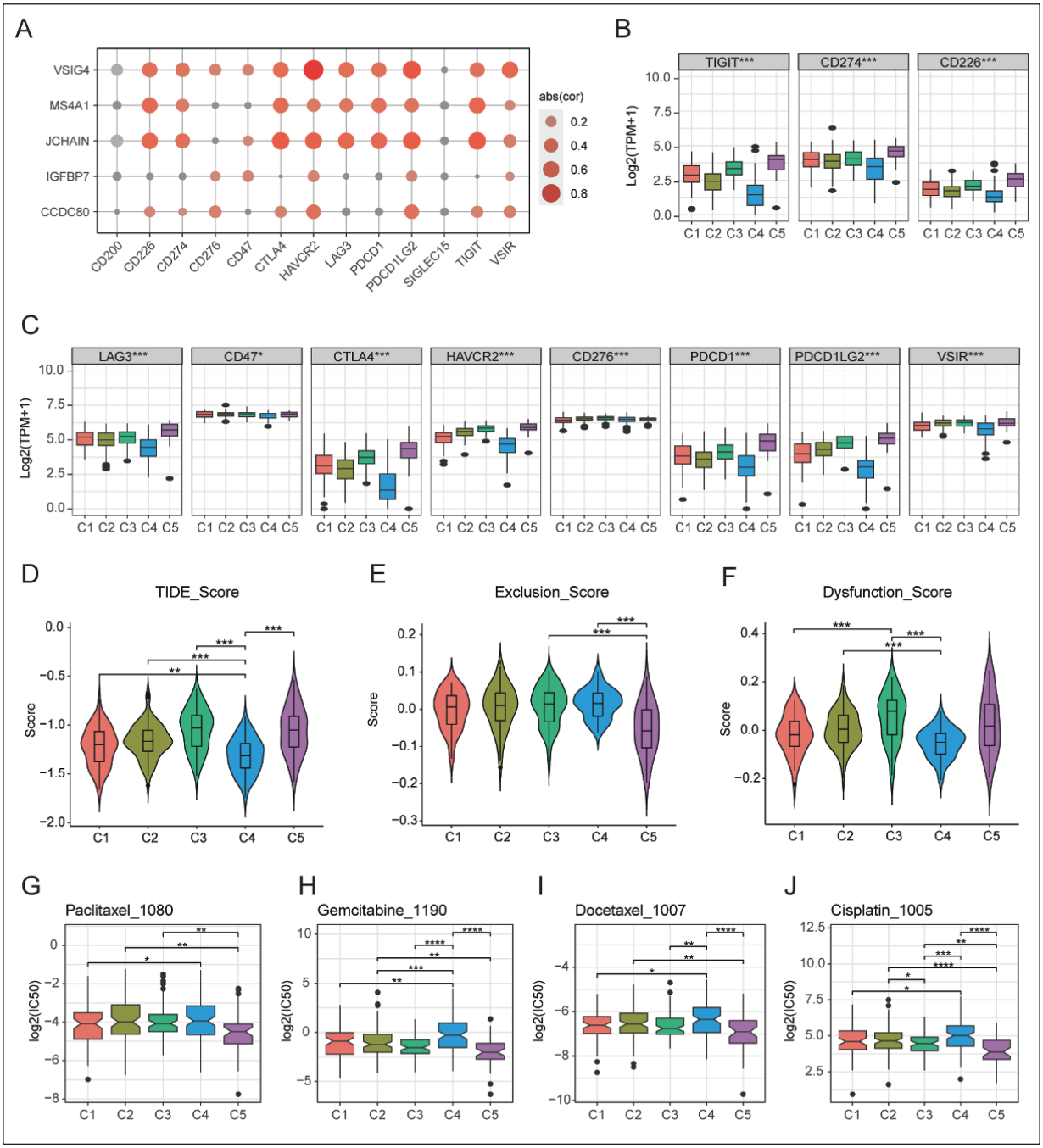

Figure 5. Sensitivity of immunotherapy and chemotherapy in the 5 clusters. **A-C**, Correlation between these five marker genes and immune checkpoints. **D-F**, Differences in TIDE scores among the 5 clusters. **G-J**, Analysis of drug sensitivity in the 5 clusters. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

may play an inhibitory role in tumorigenesis of thyroid cancer, pancreatic cancer, and colon cancer. Liang et al[20] demonstrated that *CCDC80* gets a downward trend in both mRNA and protein levels in ovary cancer and may act as a tumor suppressor by influencing TME and metabolism. VSIG4, VSIG3, and TIGIT belong to the B7 family-related proteins of the VSIG family and are co-inhibitory receptors in the process of T cell activation. Among them, VSIG4 is a type I transmembrane receptor that is only expressed in the tissue-resident macrophage subpopulation, which

has potential therapeutic effects on immune-mediated inflammatory diseases and is also considered a new target for immunosuppression of immune checkpoints in cancer treatment[21,22]. *MS4A1* is the gene encoding the B-cell surface marker CD40, which has a close influence on B cell proliferation, differentiation, and activation[23,24]. *MS4A1* expression was found to be positively correlated with patient survival in colorectal cancer, and *MS4A1* expression can be used as a diagnostic marker[25]. Similarly, the expression level of CD20 in the tumor-infiltrating lymphocytes of ovarian cancer patients is positively correlated with the clinical prognosis[26], which is consistent with our findings. It can be seen that these characteristic genes that we found are mostly related to immune molecules, and immune cells demonstrated prognostic relevance in a wide range of different tumors.

The correlation analysis with CTLA4, HAVCR2, PD1, TIGHT and other immune checkpoint molecules showed that these five signature genes were positively correlated with T cell exhaustion in varying degrees. In the presence of chronic infection and tumors, T cells are continuously exposed to inflammatory factors and tumor antigens such that they become dysfunctional and then enter a state called exhaustion, as evidenced by the expression of multiple inhibitory receptors (immune checkpoint molecules)[27]. The application of immune checkpoint inhibitors can effectively rescue T cell exhaustion and restore the tumor immune function of T cells, while chemotherapeutic drugs such as paclitaxel and cisplatin can activate T-cell function and increase immune cell infiltration to some extent[28]. As a result, cluster 3 and cluster 5, with higher expression of immune checkpoint molecules, exhibited higher TIDE scores and exclusion scores, which mean a higher likelihood of immunosurveillance escape and lower immunotherapy success rates. Combination therapy of chemotherapy and immunotherapy appears to be a promising approach in treating ovarian cancer, as evidenced by the improved chemotherapy prediction scores of cluster 3 and cluster 5.

Limitations

With regard to the methodological aspects of our study, it is important to acknowledge certain limitations. First, reliance on retrospective datasets such as TCGA has inherent limitations, and prospective studies would be beneficial to validate our findings. Furthermore, the heterogeneity within ovarian cancer presents a challenge, and future investigations should consider larger sample sizes to capture the full spectrum of this heterogeneity. In addition, the dynamic nature of the tumor microenvironment warrants longitudinal studies to unravel temporal changes and their impact on patient outcomes.

Thus, the above findings suggest that we identified five prognosis-related signature genes obtained from single-cell sequencing and subsequently identified clusters with better immune infiltration and chemotherapy effect based on consistent clustering of TCGA-OV.

Conclusions

Our investigation pinpointed five prognostically significant marker genes through the analysis of a single-cell sequencing cohort, facilitating the effective categorization of TCGA-OV patients into unique molecular subtypes. The discernible differences observed in immune infiltration, drug responsiveness, and overall prognosis among these subtypes offer crucial insights with implications for forthcoming clinical endeavors. In summary, our study provides a comprehensive analysis of the ovarian cancer microenvironment, uncovering molecular subtypes and their associated clinical implications. The limitations highlighted underscore the need for continued research to refine our understanding of the intricate interplay between the tumor and its microenvironment, ultimately paving the way for personalized therapeutic strategies in ovarian cancer.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Approval

All datasets in the study were downloaded from public databases, which allowed researchers to download and analyze public datasets for scientific purposes.

Informed Consent

Not applicable.

Availability of Data and Materials

Access to all the data is mentioned in the article and can also be obtained by contacting the authors.

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Authors' Contributions

Zhu Yang and Qiang Yi conceived and designed the study, and Siyang Xiang and Qinke Li made the data analysis and wrote the manuscript. All of the authors read and approved the final manuscript and agreed to publish this paper in this journal.

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