Development of a Multi-gene-based Immune Prognostic Signature in Ovarian Cancer

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Research

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Abstract

**Background:** Chemotherapeutic resistance is responsible for treatment failure. Immunotherapy is important in ovarian cancer (OC). Systematic exploration of immunogenic landscape and reliable immune gene-based prognostic biomarkers or signature is necessary to be identified. This study aims to identify the immune gene-based prognostic biomarkers and regulatory factors, further to develop an individualized prediction signature.

**Methods:** This study systematically explored the gene expression profiles from RNA-seq data set for The Cancer Genome Atlas (TCGA) ovarian cancer. Differentially expressed and survival-associated immune genes and transcription factors (TFs) were identified using immune genes from ImmPort dataset and TFs from Cistoma database. We developed the prognostic signature based on survival associated immune genes with LASSO (Least absolute shrinkage and selection operator) Cox regression analysis. Further, Network analysis was performed to uncover the potential molecular mechanisms of immune-related genes with the help of computational biology.

**Results:** The prognostic signature, a weighted combination of the 21 immune-related genes, performed moderately in survival prediction with AUC was 0.746, 0.735, and 0.749 for 1, 3, and 5 year overall survival, respectively. Network analysis uncovered the regulatory role of TFs in immune genes. Intriguingly, the prognostic signature reflected infiltration of some immune cell subtypes.

**Conclusions:** We first constructed a signature with 21 immune genes of clinical significance, which showed promising predictive value in the surveillance, prognosis, even immunotherapy response of OC patients.

Introduction

Ovarian cancer (OC) causes the most deaths among gynecological cancers, with more than 22000 new cases and 14000 deaths each year in the United States[1]. It is challenging that the incidence of OC, even recurrence rate and chemotherapy-resistant cases will be greatly increased despite the development of aggressive frontline treatment.

Evidence has shown that OC is immunogenic [2]. Intra-tumoural CD8+ T cells had a clear correlation with improved clinical outcome. Hamanishi et al. reported that the overall response rate for nivolumab treatment was 15% and the disease control rate is 45% [3]. 2015 ASCO annual meeting presented the pembrolizumab (anti-PD-1) and avelumab (anti-PD-L1) as the immune checkpoint targets. Furthermore, clinical trials, NCT02718417 (Javelin Ovarian 100), ENGOT-ov29-GCIG (ATALANTE), NCT02580058 (Javelin Ovarian 200), and NRG-GY009 as indicated, are ongoing or planned for the testing of potential efficacy. However, ovarian cancer is featured with high clonal heterogeneity and specific dissemination patterns. Single chemotherapy or immunotherapy was not so efficiency, while combinatorial therapy may increase the risk of adverse effects[4]. Therefore neo-antigens and effective biomarkers should be investigated, and the prognostic signature based on immune genes should be addressed.
Furthermore, recent genome-wide studies addressed the impact of diverse gene regulatory mechanisms in immune homeostasis. Emerging evidence showed that transcriptional networks drive functional changes during immune activation and subsequent immune resolution. Thus, identification of transcriptional regulators in the immune system and their gene regulatory networks is critical.

This current study aimed to investigate the correlation between immune genes and survival of OC cases, and to develop an immune gene-based signature to evaluate disease progression and therapy choice. Bioinformatics analysis was conducted based on the transcriptomes and immune gene expression profiling data from 376 ovarian cancer patients in TCGA and 88 normal ovarian tissues in GTEx (Genotype-Tissue Expression) dataset. A signature based on 21 immune genes was identified, and it is closely related with aggressive clinical outcomes of OC. Moreover, network analysis showed the close association between transcription factors and immune genes. This study could provide critical theory and clues for the prognostic prediction and therapeutic selection for OC cases.

Materials And Methods

Data collection and preprocessing

The workflow is shown in FigS1. RNA-Seq data as well as clinical information was downloaded from TCGA dataset including 379 serous ovarian cancer cases, and three overlapping samples (TCGA.13.1489.02A, TCGA. 29.2414.02A, TCGA.61.2008.02A) were removed. RNA-Seq data and clinical information for 88 normal ovarian samples were obtained from GTEx in xena (https://xenabrowser.net/). GEO (Gene Expression Omnibus) datasets, with accession number GSE26712 and GSE63885 based on GPL96 [HG-U133A] Affymetrix Human Genome U133A Array were downloaded for validation. Information of cases included was summarized in Appendix Table1. Gene expression level was defined as the average value for multiple probes. All statistics were under R condition, and NormalizeBetweenArrays was used to normalize expression distribution.

Differentially expressed immune gene (DEIGs)

1811 immune related genes were obtained from ImmPort database (https://immport.niaid.nih.gov) including cytokine-related genes, T-cell signaling genes, B-cell pathway genes, NK (natural killer) cells signaling genes etc. TCGA and GTEx RNA-Seq data were used to identify the DEIGs between serous ovarian cancers and normal cases. The P-value thresholds were established by Bonferroni-correction method, which set the significance level to be 0.05 divided by number of tests. Consequently, DEIGs were selected by p-value <2.76×10^{-5} (0.05/1811) and absolute fold change >2. The R package “Limma” was used to find out differentially expressed immune genes.

Immune-gene based Prognostic signature construction
First, we performed univariate Cox regression analysis to evaluate the overall survival (OS) of patients with differentially expressed immune genes to identify the survival-associated immune genes (SAIGs). LASSO (Least absolute shrinkage and selection operator) Cox regression analysis, by constructing a penalty function, was used to identify the predictive genes and construct the multi-gene-based prognostic model. Based on the expression level of each gene and the regression coefficient, we conducted a risk score (RS) \( RS = \sum w_i x_i \) (\( x_i \) represents the expression level of each gene involved in the model and represents the corresponding regression coefficient). In details, patients were divided into high-risk and low-risk groups based on the median of RS. Then we used Kaplan-Meier (K-M) survival analysis to compare the survival between groups, and performed ROC (receiver operating characteristic) curve to evaluate the prediction value of this prognostic model with area under curve (AUC). Additionally, multivariable Cox regression analysis was used to define the independent prediction value of risk score from other prognostic factors including age, grade, stage, and debulking status. GSE26712 and GSE63885 datasets were used to prove the prediction value of the prognostic model.

**Transcription factors - Immune genes regulatory network**

318 transcription factors were obtained from Cistoma database (http://cistrome.org/). Firstly, differentially expressed transcription genes (DETGs) were identified with \( p < 1.57 \times 10^{-4} \) (0.05/318, Bonferroni-correction method) and absolute fold change >2. We analyzed the correlation between 318 DETGs and 71 SAIGs, and constructed the regulatory network with the following condition: P-value < 2.21x10^{-6} (0.05/(318*71), Bonferroni-correction method) and Pearson correlation coefficient > 0.3. Cytoscape software was used to visualize the network.

**Correlation between immune genes and clinical features**

To evaluate the correlation between immune genes and clinical features including age, grade and stage, we compared the risk score of patients with age < 60 or age ≥ 60, early or advanced stage in high grade serous ovarian cancer. Student’s t-test (for binary clinical variables) and ruskal Wallis test (for multiple clinical variables) were used.

**Correlation between immune gene and immune cells**

CIBERSORT algorithm, namely gene expression deconvolution Algorithm, was used to evaluate the relative abundance of 22 kinds of immune cell with normalized gene expression data. The 22 cell types inferred by CIBERSORT encompass T cells, B cells, natural killer cells, macrophages, dendritic cells, and neutrophils, amongst others. We uploaded TCGA RNA-Seq data to the CIBERSORT web portal (http://cibersort.stanford.edu/), and set the default signature matrix as 1,000 permutations. We select samples with CIBERSORT-P value < 0.05 for further analysis. Correlation analysis was conducted to estimate the relationship between risk score and immune cells.
Results

Immune gene-based prognostic signature is an independent prognostic factor in OC patients.

Firstly we examined the DEIGs between the 376 ovarian cancer cases in TCGA dataset and 88 normal ovarian tissues in GTEx dataset. As shown in Fig 1A-1B, a total of 495 differentially expressed genes were identified with p-value <2.76×10^{-5} and absolute fold change >2, containing 188 downregulated and 307 upregulated genes (table S1).

Further, we addressed 71 survival-associated immune genes with p-value <0.05 by univariate COX analysis with 495 DEIGs (FigS2). Then we selected the top 40 survival-associated immune genes by ranking p-value from low to high from this set of genes for further analysis. LASSO cox regression was performed (FigS3) and 21 hub genes were screened for prognostic model construction, including IL27RA, GAL, RBP1, ANGPT4, EBI3, C5AR1, MSR1, HCK, SYK, CYBB, PI3, CD86, FABP4, CX3CR1, ITGB2, PENK, PRLR, RARG, ESM1, BCL10, and OBP2A. The 21 hub genes and the coefficient were shown in Table S2, and the risk score of 376 patients in TCGA dataset were defined. Samples were divided into high-risk and low-risk groups by the median value of risk score. As expected, the proposed model could successfully separate OC samples with high OS from those with low OS (Fig2A, P=2.92e−14), while higher risk score was related with poor prognostic survival and clinical outcome (Fig2B & 2C). Moreover, the prognostic signature had good prediction value for clinical outcome of ovarian cancer patients, with the AUC 0.746, 0.715, and 0.749 for 1 year, 3 year, and 5 year overall survival, respectively (Fig2D). Fig2E showed the expression profiles of these 21 hub genes in the final model.

Furthermore, elder age, advanced stage, sub-optimal debulking status and higher risk score were associated with poor overall survival by using univariate Cox regression analysis (Fig3A), while the risk score of prognostic signature remained as an independent prognostic factor when applying the multivariate Cox regression analysis after adjusting for clinical and pathologic factors such as age, debulking status, and stage(HR=1.483, 95%CI:1.355-1.622, p<0.001, Fig3B).

The immune gene-based signature has great prediction value in other two independent datasets.

To further validate the prediction value of the model, another two independent groups (GSE26712 and GSE63885 datasets) were downloaded as the validation groups. The corresponding risk score of each patients were calculated with the constructed model above and the patients were divided into high-risk and low-risk sub-groups by the medium of risk score. Kaplan-Meier curves were carried out in these two independent cohorts, showing that patients in high-risk subgroup was correlated with poor prognosis than patients in low-risk group(Fig4A & 4B, P=0.032 and P=0.022, respectively).
Immune gene-based prognostic signature is closely correlated with clinicopathological factors.

The results for clinicopathological factors-based stratification analyses showed that the risk score is positively related with patients’ age and stage (FigS4). Comparing to the patients with age ≥60, elder patients had a higher risk score indicating poor clinical outcome (P=0.019, FigS4A). In addition, the risk score is positively correlated with stage (P=0.048, FigS4B), and patients with advanced stage showed higher risk score in ovarian cancer (table S3).

Immune gene-based prognostic signature is related with infiltration of specific immune cell subtypes.

To understand the correlation between gene signature and specific immune cell, we performed CIBERSORT algorithm to determine the proportions of 22 immune cells infiltrates. High density of Macrophages M0 (Cor=−0.263, p=2.235e−04, FigS5A), NK cells resting (Cor=−0.155, p=0.032, FigS5B), and T cells follicular helper (Cor=−0.163, p=0.024, FigS5C) infiltration was negatively correlated with higher risk score, while Macrophages M2 (Cor=0.262, p=2.356e−04, FigS5D), Neutrophils (Cor=0.176, p=0.014, FigS5E), and T cells CD8 (Cor=0.171, p=0.018, FigS5F) were positively correlated with higher risk score.

Immune gene-based prognostic signature is related with and regulated by transcription factors.

The immune response is strictly controlled and regulated for the production of inflammatory cytokine. Transcription factors showed critical role in regulating gene expression, allowing immune response to occur in a controlled effective manner [5]. Besides, transcription factor can act as an immuno-metabolism regulator and control immune cell metabolism, playing an important role in the regulation of immune, malignant, and metabolic diseases [6]. Hence, to identify the interaction networks between transcription factors and immune genes is imperative in ovarian cancer. We identified 130 differentially expressed TFs among 318 genes in ovarian cancer cases compared with normal tissues (Table S4). We built a regulatory network with 71 survival-associated immune genes and 130 TFs. As shown in Fig5, most of the survival-associated immune genes in the network are positively correlated with higher risk score which indicated poor clinical survival. Specially, we identified four TFs, whose expression levels were significantly correlated with survival-associated immune genes, including CIITA, BATF, VDR, CBX2. Survival-associated immune genes positively or negatively correlated with transcription factors, which predicted good or poor clinical survival in serous ovarian cancer. The TF-immune genes regulatory network acutely demonstrated the regulatory mechanism among these immune-related genes.


**Discussion**

Immune related genes play a significant role in tumor progression and immunotherapy. An integrative, genome-wide profiling study to establish the immune gene-based signature to predict the clinical prognosis is urgently needed, and the molecular regulatory mechanisms of immune related genes have not been identified. In the present study, we conducted comprehensive analysis and identified 21 survival associated immune gene-based signature with the TCGA dataset as the training set and two independent GEO datasets as the validation set. The large number of ovarian cancer cases for this study facilitated robust and general results. We identified several immune related genes that significantly correlated with the progression of ovarian cancer, and they may be the valuable clinical biomarkers. Moreover, we proposed the prognostic signature based on selected, differentially expressed, and survival-associated immune related genes to predict clinical outcome. The signature was the independent prognostic factor in overall survival. Furthermore, the network analysis showed that immune genes were closely related with transcription factors, and this has been a critical regulatory mechanism for immune response.

Patients with ovarian cancer are at substantial risk for recurrence and chemotherapy resistant. Immunotherapeutic approaches such as personalized antigen-specific immunotherapy have been recognized as curative potential targets[7]. Currently, the immune-based interventions have gained approval in many solid tumors and hematologic malignancies. However, ovarian cancer has the features of extensive malignant and immunologic heterogeneity. New tumor antigens and prediction signature are critically needed to select cases that are at high risk for recurrence and chemotherapy resistant. Previous studies demonstrated that next-generation sequencing or large-scale sequencing analysis is now available to identify the tumor neo-antigens for personalizing cancer immunotherapies[8], but they had the limitation of small sample size and intra-study heterogeneity [9]. Bioinformatics systematic analysis will enable a more in-depth exploration. In this study, we combined gene expression profiling from TCGA dataset, which had relative large samples with 376 cases, and GEO datasets. The 21 immune genes were identified as reliable biomarkers of ovarian cancer prognoses. Besides, exploration of immune gene patterns and survival-associated immune genes with computational biology that are specifically designed to perform analysis across different platforms can minimize the technical or samples bias, providing further and general insights into biomarkers identification. As such, this immune-gene based prognostic signature may serve as a generalized, individualized estimate of survival of ovarian cancer.

Several clinical trials have demonstrated the potential use of cancer immunotherapy in ovarian cancer, but the immunotherapeutic responses are variable in different patients with high cost for treatment. Therefore, predictive biomarkers that can identify treatment response are urgently needed. Significant research on immune relevant prognostic signature proposed by Wen Jiang et al has led to studies to find biomarkers predicting prognoses and immunotherapeutic responses in bladder cancer, but differences exist between Wen’s article and the present study. Firstly for the study cases included, we used the cases including ovarian cancer patients and normal ones, while only patients were included in Wen’s article. Secondly for the identification of immune associated genes, we identified the differentially expressed genes between cancer and normal cases, while Wen was focused on the differentially expressed genes.
associated with immune infiltration. Thirdly for the application, Wen constructed the tumor immune infiltration–associated gene (TIM) signature that can predict the immunotherapeutic response and reflect the immune cells infiltration, while the gene signature construction based on survival-associated immune genes in this article stratified ovarian cancer patients into two distinct subgroups related with survival outcomes. Therefore, this proposed signature combined the molecular and clinical characteristics, and identified biomarkers to provide a more accurate estimation of overall survival in ovarian cancer. Combinatorial prognostic immune gene-based signature can illuminate how specific genomic aberration types associated with clinical outcome[10]. The correlation between the gene signature and prognostic factors provoked perspectives on the good predictive value of gene signature on distinct grade or stage disease and further on overall survival in OC.

However, immunotherapy can be prevented by tumor immunological function disruption, and the off-target activity of immune-stimulatory factors may result in severe toxicity. Moreover, individual immunotherapy is not efficiency for strong anti-tumor potential, while combinatorial immunotherapy may increase the risk and severity of adverse effects [4]. Thus, finding tumor-mediated immunosuppression or immunostimulation targets is still challenging[11]. Immunomodulatory gene circuit platform is potential for tumor-specific immune-stimulation by de novo cancer-specific promoter synthesis, with RNA-based design and transcription factors encoding. Differentiated TFs were identified and multiple binding motifs for cancer-specific TFs were encoded to generate synthetic OC-specific promoters, resulting in compact and tumor-specific promoters[12]. It is promising that we can identify the specific TFs for promoters encoding. In the present study, we have demonstrated the interaction between transcription factors and immune genes, showing that the majority of poor survival-associated immune genes were positively correlated with the expression of TFs in serous OC. The most critical TFs were CIITA, BATF, VDR, and CBX2. Among them, CIITA has been shown to drive MHC Class II expressing tumor cells as professional antigen presenting cell (APC) performers, thus activating the immune cells and constructing the specific optimal anti-tumor vaccine[13]. BATF can induce the T cell exhaustion during chronic infection, which is characterized by expression of inhibitory receptors and protect cells from excessive immunopathology[14, 15]. Besides, BATF inhibition can ameliorate the pathophysiologic responses in allergic asthma acting as the important transcription factor by regulating T and B-cell differentiation[16]. Vitamin D and the vitamin D receptor (VDR) is important in immunological regulation in disease such as inflammatory bowel diseases (IBD) and human immunodeficiency virus infection by modulating the function of monocytes/macrophages during infection[17, 18]. Furthermore, polycomb chromobox (CBX) proteins, especially CBX2 were down-regulated in macrophage upon viral infection. Cbx2 knockdown or silencing inhibited IFN-β production and played a critical role in antiviral innate immunity[19]. On the basis of the aforementioned findings, the specific TFs for promoters encoding may be readily translated to clinical practice.

Immune cells infiltration is the important features in tumor microenvironment (TME) of ovarian cancer. Early immune response is always presented with multiple types of immune cell infiltration and cell-mediated immunity-associated gene expression alteration. In ovarian cancer, macrophages and T cells are the main infiltrated type, along with neutrophil and NK cells. Previous studies showed that
alternatively activated macrophages (M2) and neutrophils possess the pro-tumor roles and T follicular helper cells (Tfh) play an important role in immune cell recruitment. In our study, we showed that the genes of patients with high risk scores were enriched in pro-tumor or anti-inflammatory pathways relating with M2 macrophages and neutrophils, while the genes of patients with low risk scores were enriched in inflammatory pathways relating with M0 macrophages, Tfh cells and NK cells. The association reflected the landscape of immune infiltration in TME of ovarian cancer. The gene signature would be highly related with the TME especially tumor immune microenvironment and could stratify patients into high-infiltrating TME subgroups and low-infiltrating TME subgroups, and it would be an attractive target for prediction of immune infiltration and therapy intervention.

Our study has some advantages. Firstly, we performed analysis with TCGA dataset which showed larger sample size, thus our gene signature was reliable and general. Secondly, the current study was based on the immune-related genes which showed a strong biological background, thus our study has the advantage on other models which screened from RNA-seq or the whole genome profiling, providing the novel biomarkers and targets for early diagnosis and molecule-targeted therapy exploration. Thirdly, our prognostic model had a promising survival prediction ability which was shown in ROC curves, and our signature simplified the complicated effects of immune genes in clinical outcomes and immunotherapy responses, making it easier for prognosis and therapy response prediction.

But our study also showed some limitation. First, we used the datasets from both GEO and TCGA to get more sufficient validation, but it will show some statistic cohort bias and heterogeneity for the difference of platforms and differences in clinical care, clinical setting, and treatment. Second, only overall survival was remained to estimate the association between immune gene signature and clinical outcome to decrease the missing rate. This approach increased statistical power and data integrality, but it is also a limitation insofar as some patients’ information will be lost, and the signature will be more accurate if other survival parameters are included. Third, this study is developed with genes in ImmPort database, and further biological experiments and validation are warranted in ovarian cancer in the future. At the same time, the gene signature was validated in other two independent GEO datasets, but it will be more reliable with prospective cohort study in the future.

In summary, the current study constructed prognostic signature with the immune-related genes, providing a good ability for prognostic prediction. Network analysis revealed the regulatory relationship and the interaction between immune genes and transcription factors, providing the biomarkers for immunomodulators. Prospective and validation studies are necessary for further establishment of prediction accuracy with this gene signature. The network analysis is warranted to be validated to identify the critical role of transcription factors in survival outcome.

Declarations

Ethics approval and consent to participate
Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and materials**

The datasets generated and analysed during the current study are available in the TCGA and GEO datasets that provide free online tools and resources.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

CT designed the article and conducted the analysis. SH write the article and check the results.

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**Abbreviations**

OC: Ovarian cancer

TCGA: The Cancer Genome Atlas

TFs: Transcription factors

CTLA-4: Cytotoxic T lymphocyte-associated protein4

PD-1: Programmed cell death 1

NK: Natural killer
DEIGs: Differentially expressed immune gene
OS: Overall survival
LASSO: Least absolute shrinkage and selection operator
RS: Risk score
K-M: Kaplan-Meier
ROC: Receiver operating characteristic
AUC: Area under the curve
DETG: Differentially expressed transcription genes
DEGs: Differential expression genes
APC: Antigen presenting cell
VDR: Vitamin D receptor
IBD: Inflammatory bowel diseases
TME: Tumor microenvironment

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Figures
Figure 1

DEIGs between ovarian cancer and normal cases. (A) Heat map of the DEIGs. The above horizontal axis shows the information of samples including normal cases (N=88) and ovarian cancer cases (N=376), respectively. The left longitudinal axis shows the clustering results. The color change from red to green represents the expression of immune genes changed from high to low. (B) Volcano Plot of the DEIGs. The
red and blue points in the figure show the DEIGs with statistical significant (p-value <2.76×10^{-5} and absolute fold change >2). DEIG, differential expression immune genes.

Figure 2

Prognostic signature Construction. (A) Kaplan-Meier curve of prognostic predictors for ovarian cancer. (B&C) Patients with higher risk score in this model predicted poor prognostic survival and clinical outcome. (D) ROC curves of prognostic predictors for ovarian cancer with 1 year, 3 year, and 5 year overall
survival. (E) The expression distribution of the 21 hub genes in the prognostic model. LASSO, Least absolute shrinkage and selection operator; ROC, Receiver operating characteristic.

**A**

| pvalue  | Hazard ratio          |
|---------|-----------------------|
| GRADE   | 0.232 1.290(0.850–1.958) |
| AGE     | 0.003 1.020(1.007–1.033) |
| STAGE   | 0.037 1.372(1.019–1.847) |
| Debulking | 0.009 0.673(0.500–0.905) |
| Riskscore | <0.001 1.495(1.370–1.633) |

**B**

| pvalue  | Hazard ratio          |
|---------|-----------------------|
| AGE     | 0.021 1.016(1.002–1.030) |
| STAGE   | 0.028 1.419(1.039–1.938) |
| Debulking | 0.108 0.779(0.575–1.056) |
| Riskscore | <0.001 1.483(1.355–1.622) |

**Figure 3**

Univariate and Multivariable Cox regression survival analysis of prognostic signature adjusted for clinical factors in serous ovarian carcinoma. (A) Univariate Cox regression analysis, and (B) Multivariable Cox
regression analysis, showed that risk score was associated with poor overall survival and is the independent factor for clinical survival.

Figure 4

The prediction value of this prognostic signature with other two independent datasets. Validation showed that high-risk was correlated with poor prognosis in GSE26712 (A), and GSE63885 (B).
Figure 5

The correlation network analysis between survival-associated transcription factors and immune genes in serous ovarian cancer patients constructed by Cytoscape. Survival-associated immune genes (circle) positively (red lines) or negatively (green lines) correlated with transcription factors (triangle), which predicted good (green circle) or poor (red circle) clinical survival in serous ovarian cancer patients.

Supplementary Files

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