Integrative network analysis identifies an immune-based prognostic signature as the determinant for the mesenchymal subtype in epithelial ovarian cancer

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Abstract

Background: Epithelial ovarian cancer (EOC) has been classified into four molecular subtypes, of which the mesenchymal subtype has the poorest survival. Our goal is to develop an immune-based prognostic signature by incorporating molecular subtypes for EOC patients.

Methods: The gene expression profiles of EOC samples were collected from seven public datasets as well as an internal retrospective validation cohort, containing 1192 EOC patients. Network analysis was applied to integrate the mesenchymal modalities and immune signature to establish an immune-based prognostic signature for EOC (IPSEOC). The signature was trained and validated in eight independent datasets.

Results: Seven immune genes were identified as key regulators of the mesenchymal subtype and were used to construct the IPSEOC. The IPSEOC significantly divided patients into high- and low-risk groups in discovery (OS: P < .0001), 6 independent public validation sets (OS: P = .04 to P = .002), and an internal retrospective validation cohort (OS: P = .0025). Furthermore, pathway analysis revealed that differences between risk groups were mainly activation of mesenchymal-related signalling. Moreover, a significant correlation existed between the IPSEOC values versus clinical phenotypes including late tumor stages, drug resistance.

Conclusion: We propose an immune-based signature, which is a promising prognostic biomarker in ovarian cancer. Prospective studies are needed to further validate its analytical accuracy and test the clinical utility.

Abbreviations: EMT = epithelial-mesenchymal transition, EOC = epithelial ovarian cancer, GEO = Gene Expression Omnibus, GO = gene ontology, GSEA = gene set enrichment analysis, IRGs = immune genes, MRA = master regulator analysis, OS = overall survival, PFS = progression-free survival, RMA = robust multiarray analysis.

Keywords: epithelial ovarian cancer, immune signature, mesenchymal, prognosis

1. Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic cancer, characterized by pathological and molecular heterogeneity.[1] Despite new screening and treatment strategies, EOC patients still have a poor prognosis. Death rates from ovarian cancer in developed countries have declined only slightly over the past 30 years, largely because of a lack of obvious symptoms, with nearly 60% of cases diagnosed as late stages.[2] Standard treatment for EOC is debulking surgery followed by chemotherapy. Because of the diffuse nature of EOC, a significant proportion of patients who initially had a complete pathological response experienced relapse and died of chemoresistance.[3] Therefore, researchers and clinicians need to establish robust prognostic pipelines for accurately identifying EOC patients, who might benefit from targeted therapies.

Large-scale public cohorts with tumor gene expression data provide a broader opportunity to search for reliable prognostic markers for ovarian cancer. Several studies have developed markers based on gene expression for EOC prognosis prediction.[4,5] However, due to the heterogeneity of EOC, most of the markers have low prognostic efficacy and cannot be directly used in clinical practice. Recently, four ovarian cancer subtypes with different molecular and clinical characteristics were found,[6] among which the mesenchymal subtype had the poorest...
prognostic value in cancers including. There is growing evidence that the immune system has an important role in the development and progression of cancer. Many previous studies have shown that ovarian cancer is an immunogenic tumor and that immunotherapy targeting the immune checkpoint is strongly pursued. In addition, previous studies have tentatively shown that the immune system has prognostic value in cancers including ovarian cancer. Considering the highly heterogeneous nature of EOC and the complex immune microenvironment, we applied network analysis to integrate mesenchymal modalities and immune signature underlying the mesenchymal subtype to develop an immune gene-based prognostic signature for EOC (IPSEOC). The master regulator analysis showed that seven immune genes were the key factors of the mesenchymal subtype. With sufficient validation of 6 independent public datasets and an internal cohort, we proved the model stability and reliability. Although immune prognostic markers for ovarian cancer have been reported, no research has been done for prognosis prediction of EOC patients who might benefit from more rigorous treatments.

2. Materials and methods

2.1. Ethical approval

The researchers were authorized to conduct the study by the Ethics Committee of the Beilun People’s Hospital, Ningbo, China. All procedures were implemented in accordance with the Declaration of Helsinki and relevant policies in China.

2.2. Patient series

We retrospectively collected and comprehensively analyzed the gene expression profiles (GEPs) from 7 independent datasets, containing 1151 cases. The complete lists of all GEPs are shown in Supplemental Table 1, http://links.lww.com/MD/E946. These datasets involved patients from the GSE26712 (n = 182), the GSE9891 (n = 285), the GSE26193 (n = 107), the GSE18520 (n = 63), the GSE35963 (n = 153), the GSE49997 (n = 204), and the GSE13876 (n = 157). The expression data of all cohorts together with the corresponding clinical parameters were downloaded from Gene Expression Omnibus (GEO). The molecular subtyping information for GSE26712 and GSE9891 was retrieved from Verhaak’s study. The BL-OV cohort contains frozen and paraffin-embedded tissues from 41 ovarian cancer cases that were collected from Beilun People’s Hospital (Ningbo, China). The detailed clinical characteristics of the 8 datasets were described in Table 1. The design and workflow of this study were illustrated in Figure 1A.

2.3. Expression data preprocessing

GEPs were downloaded from GEO by “GEOquery” (R package, version 1.0.7) and normalized with the robust multiarray analysis (RMA). For each cohort, the GEPs were collapsed from probe IDs to genes symbols, if multiple probe IDs correspond to the same genes symbol, the one with the highest mean value was kept as the representative of the corresponding gene. For the BL-OV cohort, whole transcriptomes were sequenced on the Illumina HiSeq platform (Novogen, China). Reads were first trimmed to remove linker sequences and low-quality bases using Cutadapt (version 1.2.1) and then mapped to the hg38, followed by calculation of gene counts using SATR (version 2.5).

2.4. Integrated network analysis

Immune genes (IRGs) were downloaded from the ImmPort database. IRGs measured by all cohorts were kept. Network analysis was applied to integrate mesenchymal modalities and immune genes underlying the mesenchymal subtype. Together, we used the GSE26712 dataset as the training cohort. Fifty-two immune genes (log2FC > 0.75, FDR < 0.01) and 1131 target genes (log2FC > 0.25, FDR < 0.01) differentially expressed by comparing the mesenchymal subtype with the other three

### Table 1

Demographic and clinic characteristic descriptions for ovarian cancer patients in different datasets.

|                | Training cohort | Validation cohorts |
|----------------|-----------------|-------------------|
|                | GSE26712 (n = 182) | GSE9891 (n = 285) | GSE26193 (n = 107) | GSE18520 (n = 63) | GSE35963 (n = 153) | GSE49997 (n = 204) | GSE13876 (n = 157) | BL-OV (n = 41) |
| **Age (years)** |                 |                   |                   |                   |                   |                   |                   |                   |
| I              | 61 (26–84)      | 59 (22–80)       | 63 (24–89)       | 57 (26–85)       | 57 (21–84)       | 55 (36–69)       |
| II             | 18 (6%)         | 20 (10%)         | 11 (10%)         | 2 (3%)           | 9 (4%)           | 11 (27%)         |
| III            | 144 (79%)       | 218 (76%)        | 59 (55%)         | 53 (84%)         | 109 (71%)        | 154 (71%)        | 25 (61)          |
| IV             | 36 (20%)        | 22 (8%)          | 17 (16%)         | 10 (16%)         | 40 (26%)         | 31 (15%)         | 2 (5%)           |
| Unknown        | 2 (1%)          | 3 (1%)           | 10 (5%)          |                   |                   |                   |                   |                   |
| **Grade**      |                 |                   |                   |                   |                   |                   |                   |                   |
| 1              | 19 (7%)         | 7 (7%)           |                   |                   |                   |                   |                   | 14 (9%)          |
| 2              | 29 (16%)        | 97 (34%)         | 33 (31%)         |                   |                   |                   |                   | 45 (29%)         |
| 3              | 108 (59%)       | 163 (67%)        | 67 (63%)         | 53 (84%)         | 150 (98%)        | 143 (70%)        | 85 (54%)         |
| Unknown        | 45 (25%)        | 6 (2%)           | 10 (16%)         |                   |                   |                   |                   |                   |
| **Debulking**  |                 |                   |                   |                   |                   |                   |                   |                   |
| optimal        | 66 (36%)        | 160 (66%)        | 28 (44%)         | 103 (67%)        | 137 (67%)        |                   |                   |                   |
| suboptimal     | 71 (39%)        | 88 (31%)         | 11 (17%)         | 47 (31%)         | 57 (28%)         |                   |                   |                   |
| Unknown        | 45 (25%)        | 37 (13%)         | 24 (38%)         | 3 (2%)           | 10 (5%)          |                   |                   |                   |
subtypes (Immunoreactive, Differentiated and Proliferative). Integrated network analysis was performed by the “RTN” (R package, version 2.10.0).[31] Master regulator analysis (MRA) was done to examine significantly overrepresented epithelial-mesenchymal transition (EMT) genes[32] within each immune gene’s regulon. Seven immune genes of top significance were kept as the key factors of the mesenchymal subtype.

2.5. Development of the immune-based prognostic signature for EOC (IPSEOC)

Seven immune genes are differentially up-expressed in the poorest survival subtype and are the master regulatory factors of the mesenchymal subtype-specific genes (including EMT genes). The Cox proportional-hazards model was applied to test their association with overall survival. Based on these seven immune genes, we develop a cox-model named the immune-based prognostic signature for EOC (IPSEOC) as follows: risk score = (0.0201 × FOS) + (0.0212 × PLAU) + (0.0612 × COLEC12) + (0.0174 × INHBA) + (0.3910 × EDNRA) + (0.0836 × TGFB1) + (0.0853 × CTGF). The upper quartile risk value was set as the cut-off to separate patients into high- and low-risk groups across all datasets.

2.6. Validation of the IPSEOC

The IPSEOC score was further evaluated in the 6 independent public validation cohorts as well as an internal retrospective validation cohort in terms of OS and PFS by the log-rank test, respectively. The IPSEOC then was evaluated with other clinical parameters in the uni- and multivariate cox analysis. In the multivariable Cox regression, debulking status and tumor stage were included as covariates.

2.7. Profiling of immune cells infiltration

To analyze the immunobiological characteristics of high- and low-risk groups, we used CIBERSORT,[33] to characterize immune cells’ abundance of tumor tissue GEPs. Based on a set of reference immune cell GEPs, CIBERSORT used support vector regression[33] to deconvolute tumor tissue gene expression profile with each type of immune cell enrichment. More specifically, standardized gene expression profiles were submitted to the CIBERSORT Web portal (http://cibersort.stanford.edu/) with 1000 permutations. For each sample, CIBERSORT quantified the relative proportions of 22 infiltrated immune cell types.

2.8. Gene ontology (GO) analysis and gene set enrichment analysis (GSEA)

GO analysis was conducted for the significantly up-regulated genes in the high-risk group using gProfiler.[34] GSEA[35] was conducted using “fgsea” (Bioconductor package, version 1.12.0) with 1000 permutations. Gene sets were retrieved from the Molecular Signature Database (MSigDB hallmark and kegg, version 7).[35] A P-value below .05 was used to choose significant gene sets.

2.9. Statistical analysis

Continuous variables were compared using the Wilcoxon signed-rank test or Kruskal-Wallis rank-sum test. Kaplan-Meier analysis was performed using the log-rank test using “survival” (R package, version 2.41.3). Uni- and multivariable analyses were conducted by the Cox proportional hazards model. For all tests, a P-value below .05 was used to choose significant gene sets. Statistical significance is presented as following *P < .05, **P < .01, ***P < .001. All the statistical tests were conducted using R (version 3.6.1).
3. Results

3.1. Integrative analysis reveals seven immune genes as master regulators for the mesenchymal subtype of EOC

EOC is a molecularly heterogeneous disease. In recent studies, four molecular subtypes have been identified, among which the mesenchymal subtype has the worst prognosis (Supplemental Fig. 1, http://links.lww.com/MD/E940). To investigate the immune system role underlying the mesenchymal subtype, a total of 1192 patients with EOC from 7 independent public cohorts as well as an internal retrospective validation cohort were included (Table 1). We applied network analysis to integrate mesenchymal modalities and immune genes in the GSE26712 cohort (Fig. 1A). The networks consist of immune genes that are significantly up-regulated in the mesenchymal subtype compared with the other three subtypes and were found to regulate most of the mesenchymal specific target genes (Fig. 1B). Master regulator analysis (MRA) identified seven immune genes (CTGF, FOS, PLAU, COLEC12, INHBA, TGFBI1, and EDNRA) as the key factors of the mesenchymal subtype (Supplemental Table 2, http://links.lww.com/MD/E947). These seven immune genes are significantly up-regulated in the mesenchymal subtype (Table 2). Collectively, these results indicate that the risk score model is robust in evaluating the survival of EOC patients.

3.2. Development of the immune-based prognostic signature for EOC (IPSEOC)

Using the GSE26712 cohort as the training set, we defined the IPSEOC using Lasso Cox proportional hazards regression of these seven immune genes: risk score = (0.0201 \times \text{CTGF}) + (0.0212 \times \text{PLAU}) + (0.0612 \times \text{COLEC12}) + (0.0174 \times \text{INHBA}) + (0.3910 \times \text{EDNRA}) + (-0.0836 \times \text{TGFBI1}) + (-0.0853 \times \text{CTGF}). Risk scores were calculated in all the training and validation cohorts (Supplemental Table 3, http://links.lww.com/MD/E948 and Supplemental Table 4, http://links.lww.com/MD/E949). The risk-score plots clearly demonstrate the difference between alive and dead patients (Supplemental Fig. 3, http://links.lww.com/MD/E942). For detecting the mesenchymal subtype, the IPSEOC achieved an AUC of 0.84 in the training dataset (Fig. 4A and Supplemental Fig. 4, http://links.lww.com/MD/E943) and an AUC of 0.93 in one validation dataset (Fig. 4B and Supplemental Fig. 4, http://links.lww.com/MD/E943). The upper quartile risk value was set as the cut-off to separate patients into high- and low-risk groups across all datasets. The predictive value of the IPSEOC was first evaluated in the GSE26712 cohort. The IPSEOC significantly stratified patients into high- and low-risk groups in terms of OS (HR: 2.36, 95% CI: 1.62 – 3.44; P = 4.24 \times 10^{-6}). Patients with significantly high risk scores had a more poor prognosis (Fig. 5A and Supplemental Table 3, http://links.lww.com/MD/E948).

3.3. Validation of the IPSEOC

To verify the prognostic power of the IPSEOC, we calculated the survival difference within the two risk groups in 6 validation cohorts. As expected, the IPSEOC significantly stratified patients into high- and low-risk groups in terms of OS (HR range: 1.61 [95% CI: 1.07–2.42; P = 2.34 \times 10^{-2}] to 3.39 [95% CI: 1.53–7.53; P = 2.06 \times 10^{-2}]) (Fig. 5B–G and Supplemental Table 3, http://links.lww.com/MD/E948) and PFS (HR range: 1.75 [95% CI: 1.20–2.40; P = 5.50 \times 10^{-2} to 1.64 [95% CI: 1.32–3.54; P = 1.73 \times 10^{-3}]) (Fig. 5A, C, D, and Supplemental Table 3, http://links.lww.com/MD/E948) in the 6 validation cohorts. Furthermore, the IPSEOC also significantly stratified patients in the internal retrospective validation cohort (Fig. 5H, OS: HR: 2.26, 95% CI: 1.08–4.73; P = 2.54 \times 10^{-2}). In the meta-analysis for all validation datasets, the prognostic effects of the IPSEOC are more obvious in terms of OS (HR: 1.84, 95% CI: 1.54 – 2.21; P = 1.90 \times 10^{-11}) (Fig. 5I) and PFS (HR: 1.70, 95% CI: 1.36 – 2.11; P = 1.89 \times 10^{-6}) (Fig. 6C). Furthermore, it remains an independent predictor of prognosis in the uni- and multivariate Cox model, after adjusting for stage and debulking status (Table 2). Collectively, these results indicate that the risk score model is robust in evaluating the survival of EOC patients.
Figure 3. Prognostic associations of the seven immune signature genes in training cohort (A) and validation datasets GSE9891 (B) and GSE26193 (C). P-values are based on uni-cox analysis.

Figure 4. ROC curves for detection efficiency of the mesenchymal subtype in the training cohort GSE26712 (A) and one validation cohort GSE9891 (B).
3.4. In silico functional assessment of the IPSEOC

To gain insight into the biological differences between risk groups, we performed immune cell infiltration, GO and GSEA analysis. Immune cell types, such as Macrophages M2 and T cells CD8 were enriched in training and validation cohorts (Fig. 7A and Supplemental Fig. 5, http://links.lww.com/MD/E945). We observed a significantly higher proportion of Macrophages M2 in the high-risk group and a significantly higher enrichment of monocytes in the low-risk group (Fig. 7B). GO analysis showed that the 700 differentially expressed genes between risk groups were mostly involved in immune responses and tumor metastasis, such as MAPK signaling, cell migration, and focal adhesion (Fig. 8A). Enrichment analysis between high- and low-risk groups identified that many mesenchymal phenotype-related pathways, including the TGF-beta signaling, epithelial-mesenchymal transition, angiogenesis, and focal adhesion, were positively enriched in the high-risk group ($P < .01$) (Fig. 8B and Supplemental Table 5, http://links.lww.com/MD/E951).
3.5. The IPSEOC is a strong clinical indicator of ovarian cancer patients

We observed that the risk score levels were significantly increased with tumor stages, of which stage IV patients have the highest risk score (Fig. 9A). Besides, in the suboptimal group, the risk scores were significantly higher than the optimal group (Fig. 9B). In addition, the risk scores were significantly higher in chemo-resistant EOC than in chemosensitive EOC (Fig. 9C). Moreover, patients with high-grade also showed higher risk scores than patients with low-grade (Fig. 9D).

4. Discussion

Epithelial ovarian cancer is the most lethal gynecological cancer with pathological and molecular heterogeneity characteristics.[1] Clinical characteristics such as histopathological typing, tumor staging, and debulking status are common indicators for...
Figure 8. (A) Gene ontology (GO) of the up-expressed genes between the high- vs low-immune risk groups. (B) Gene Set Enrichment Analysis (GSEA) between the high- vs low-immune risk groups.

Figure 9. (A) The risk score across tumor stages in different datasets. (B) The risk score between debulking status in different datasets. (C) The risk score for drug response in GSE26712. (D) The risk score across different tumor grades.
assessing patient risk.\textsuperscript{37} At present, various multi-gene prognostic markers\textsuperscript{4,5,38} have been developed, but their prediction efficiencies were still uncertain. First, the sample size of the involved cohorts was small for sufficient validation to evaluate the stability of the prognostic signature. Second, most of them fail to consider the effect of disease heterogeneity on predictive performance, which easily leads to overfitting. Therefore, a new signature that can accurately recognize patients with poor EOC survival is urgently needed to give more rigorous treatments.

The EOC transcriptome was unsupervised classified into four molecular subtypes with different molecular and clinical characteristics.\textsuperscript{6–8} Prognostic signature screened based on molecular portraits specific to the worst prognosis subtype may be used for risk stratification of EOC patients.\textsuperscript{39,40} In this study, we established an immune gene-based prognostic signature for EOC (IPSEOC) by integrating mesenchymal modalities and the immune system underlying the mesenchymal subtype and validated it in 6 independent validation cohorts as well as an internal retrospective validation cohort. The large sample size provided sufficient validation for the IPSEOC and made it more robust. Shen et al have reported an immune signature by single sample gene set enrichment (ssGSEA) analysis, however, their research did not take into account ovarian cancer heterogeneity, while all validation datasets were public datasets and were not validated in internal clinical samples. To the best of our knowledge, no research has been done for risk stratification by integrating the immune system and the characteristics of the mesenchymal subtype in EOC. The IPSEOC was constructed by seven immune genes as the key factors of the mesenchymal subtype and could stratify patients into different risk groups. Within these seven immune genes, over-expression of EDNRA was found to be associated with tumor metastasis and poor outcome in advanced bladder cancer.\textsuperscript{41} Upregulated INHBA expression is associated with poor survival in gastric cancer.\textsuperscript{42} High expression of CTGF had a significant correlation with tumor progression and survival with Gliomas.\textsuperscript{43} The defined high-risk group showed a worse OS and PFS than the low-risk group and displayed chemoresistant, inferring that more rigorous therapies would benefit them. The IPSEOC remained an independent prognostic predictor in multivariate Cox proportional hazards analysis after adjusting for other clinical factors. Most genes within the differentially expressed genes between risk groups were mostly involved in immune responses and tumor metastasis. Previous studies have described Macrophages M2 contributing to poor prognosis,\textsuperscript{44} while Monocytes indicate a better prognosis.\textsuperscript{45} We observed a significantly higher proportion of Macrophages M2 in the high-risk group and a significantly higher enrichment of monocytes in the low-risk group. Further, the pathway enrichment analysis adds the evidence for its association with cancer and the clinical application potential. Mesenchymal phenotype-related pathways, such as EMT, angiogenesis, and focal adhesion, were positively enriched in the high-risk group. Our findings inferred the important role of IPSEOC in tumor invasion and therefore, could serve as a robust prognostic signature in EOC.

This study still has some limitations. First of all, the prognostic signature was screened from gene expression profiles generated from microarray platforms, which are expensive, difficult to operate and involve professional bioinformatics expertise, so it is difficult to be popularized in daily clinical application. Second, the training and validation data sets were all from retrospective studies in the study, including fresh frozen samples. Therefore, the efficiency and stability of FFPE samples are still in doubt. In the following improvement process, more datasets containing more clinical characteristics need to be included for more extensive screening and validation.

Taken together, our network analysis established an immune gene-based signature, which could effectively predict EOC patients’ survival. Our study is the first attempt to integrate tumor heterogeneity and the immune system to develop the prognostic signature for EOC.

**Author contributions**

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