Original Research

Identification of two molecular subtypes of dysregulated immune lncRNAs in ovarian cancer

Xiaojun Liu*, Jinghai Gao*, Jing Wang, Jing Chu, Jiahao You and Zhijun Jin

Department of Gynaecology and Obstetrics, Changzheng Hospital, Naval Medical University, Shanghai 200003, China
Corresponding author: Jinghai Gao. Email: gaojinghai@smmu.edu.cn
*These authors contributed equally to this work.

Impact statement

Long non-coding RNA (lncRNA) has increasingly been identified as a key regulator in pathologies such as cancer. However, expressed lncRNA and its role in mapping the ovarian cancer subpopulation are still largely unknown. Based on lncRNA, two molecular subtypes of ovarian cancer were identified and had significant prognostic differences and immunological characteristics.

Abstract

Long non-coding RNA (lncRNA) has increasingly been identified as a key regulator in pathologies such as cancer. Multiple platforms were used for comprehensive analysis of ovarian cancer to identify molecular subgroups. However, lncRNA and its role in mapping the ovarian cancer subpopulation are still largely unknown. RNA-sequencing and clinical characteristics of ovarian cancer were acquired from The Cancer Genome Atlas database (TCGA). A total of 52 lncRNAs were identified as aberrant immune lncRNAs specific to ovarian cancer. We redefined two different molecular subtypes, C1(188) and C2(184 samples), in "iClusterPlus" R package, among which C2 grouped ovarian cancer samples have higher survival probability and longer median survival time ($P < 0.05$) with activated IFN-gamma response, Wound Healing and Cytotoxic lymphocytes signal; 456 differentially expressed genes were acquired in C1 and C2 subtypes using limma (3.40.6) package, among which 419 were up-regulated and 37 were down-regulated, in TCGA dataset. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis revealed that these genes were actively involved in ECM-receptor interaction, PI3K-Akt signaling pathway interaction KEGG pathway. Compared with the existing immune subtype, the Cluster2 sample showed a substantial increase in the proportion of the existing C2 immune subtype, accounting for 81.37%, which was associated with good prognosis. Our C1 subtype contains only 56.49% of the existing immune C1 and C4, which also explains the poor prognosis of C1. Furthermore, 52 immune-related lncRNAs were used to divide the TCGA-endometrial cancer and cervical cancer samples into two categories, and C2 had a good prognosis. The differentially expressed genes were highly correlated with immune-cell-related pathways. Based on lncRNA, two molecular subtypes of ovarian cancer were identified and had significant prognostic differences and immunological characteristics.

Keywords: lncRNA, ovarian cancer, The Cancer Genome Atlas database, bioinformatics, molecular subtypes

Experimental Biology and Medicine **2021; 246: 547–559. DOI: 10.1177/1535370220972024**

Introduction

Ovarian cancer is a prevalent malignant tumor of the feminine reproductive system, and its mortality rate is the highest of all gynecological malignant tumors[1]; 70% of sufferers are diagnosed at an advanced stage due to hidden disease, no obvious symptoms at early stage and effective early diagnosis method.[2] Although some progress has been made in surgical techniques, molecular targeted drugs, and chemotherapy drugs, the efficacy of ovarian cancer is still not satisfactory, with a five-year survival probability of not exceeding 35%.[3] Due to the large population base in China, the situation is more severe. A study in 2015 found that there were about 52,100 women diagnosed with ovarian cancer, among whom about 22,500 died of ovarian cancer.[4] Last few years, although many new treatment methods have been discovered, the prognosis of ovarian cancer cells has not been appreciably advanced. Therefore, a thorough of the molecular behavior of malignancy of ovarian cancer cells and its possible system has an important theoretical basis.

Long non-coding RNA(LncRNA) is a term used to refer to non-coding RNA whose nucleotide length is greater than 200.[5] Currently, investigation of lncRNA is still in the primary stage. With the progress of more and more researches

on lncRNA, LncRNA plays an essential function in epigenetic regulation, dose-compensation effect, cell differentiation regulation, and cell cycle regulation, and has become a research hotspot in genetics. More and more work suggest that lncRNA can not only regulate innate immune response, but also regulate more complex adaptive immune response.[6,7] In addition, lncRNA may be a key regulator of tumor microenvironment (TME),[8,9] forming a complex and heterogeneous environment composed of stromal cells and infiltrating immune cells.[10] In ovarian cancer, elevated expression of IR155HG is tied to higher infiltration of immune cell subsets.[11] LncRNA HOTTIP promotes the expression of PD-L1 in immune escape and neutrophils, while suppresses T cell proliferation and tumor immunotherapy.[12] The engagement of lncRNA in immune regulation is sophisticated, and plenty of pivotal immunomodulatory lncRNAs as yet unidentified. Therefore, there is an urgent need to find out novel immune-associated lncRNAs and to characterize their role in ovarian cancer.

In this work, we first produced a co-expression network of immune-associated mRNA and lncRNA to acquire 52 immune-associated prognostic lncRNAs. We then built two immune-associated molecular subtypes of lncRNA in ovarian cancer samples. In addition, we performed an analysis of the characteristics of immune microenvironment and functional enrichment. Finally, we examined whether 52 immune-associated lncRNAs could contribute to predicting the prognosis of patients with endometrial cancer.

Materials and methods

Data acquisition and processing

The latest expression data and clinical follow-up information of ovarian cancer patient and endometrial cancer and cervical cancer patient tissues were downloaded from the TCGA GDC API[13] on 20 April 2020. For TCGA data set, the expression spectrum was divided into lncRNA and mRNA by gene annotation of GTF (V32 version) file in GENECODE, and the Ensembl ID of these genes was converted into Symbol form. For the TCGA-ovarian cancer data set, in order to retain the gene set with biological significance, we deleted the genes whose expression values were all 0 in the samples, and obtained the expression profiles of 19,498 protein-coding genes and 13,630 lncRNAs. The RNA-seq data of TCGA were processed in the following steps:

1. Only tumor samples of solid tumors are retained.
2. Samples that do not express spectrum were removed.
3. Samples without clinical follow-up information were removed.
4. Samples without survival data were removed.
5. Samples without survival status were removed.

The work flow chart is shown in Figure 1.

Immune function-related pathways

In this study, the Immport database[14] contains a large number of immune-related genes, which are widely used in immune-related research. A total of 17 immune function-related pathways were obtained, including antigen processing and presentation, antimicrobials, BCR signaling, chemokines and chemokine receptors, cytokines and cytokine receptors, interferons, interferon receptors, interleukins, interleukins, natural killer cell cytotoxicity, TCR signaling pathway, TGFb family members, TGFb family members, TNF family members, and TNF family members. All include 1811 related protein-coding genes.

Co-expression analysis

In order to explore the co-expression relationship of genes in ovarian cancer samples, we used the Pearson correlation method to calculate the correlation between mRNA and lncRNA expression in the samples. First, we deleted all genes whose TPM expression value was 0 in tumor samples. At the same time, to ensure that the expression value is close to the normal distribution, log2 conversion is performed for the expression spectrum. Next, Pearson correlation coefficients and significance P values of all mRNA and lncRNA in the samples were calculated in R language. Moreover, at the threshold of $|R|>0.5$ and $P<0.05$, significant co-expression relationships between all mRNA and lncRNA were obtained in ovarian cancer samples.

Recognition of immune function lncRNA regulators

To explore the relationship between lncRNA and immune function, Gene Set Enrichment Analysis (GSEA)[15]method was used to calculate the enrichment relationship between lncRNA and 17 immune function-related pathways. The expression correlation coefficient R value and P value of mRNA and lncRNA in ovarian cancer and normal samples were integrated to obtain the Score value representing the correlation between the two genes. The formula is as follows: $\text{Score} = -\log10P \times \text{sign}(R)$.

For each lncRNA, all mRNAs were sequenced from small to large based on correlation scores, and the enrichment significance and lncRES scores of each lncRNA and each immune-functionally related pathway were calculated using GSEA method.[15] Under the condition of $FDR<0.05$ and $|\text{lncRES}|>0.995$, lncRNA regulators of immune function in ovarian cancer samples were obtained.

Enrichment analysis of candidate dysregulated immune lncRNA and immune cells

To verify the role of dysfunctional immune lncRNA in ovarian cancer, 24 immune cells (CD4_naive, CD8_naive, Cytotoxic, Exhausted, Tr1, nTreg, iTreg, Th1, Th2, Th17, Tfh, Central_memory, Effector_memory, NKT, MAIT, DC, Bcell, monocyte, macrophage, NK, neutrophil, Gamma_delta, CD4_T, CD8_T) marker gene collection were obtained from ImmuCellAI. Next, dysregulated immune lncRNAs with significant correlation with marker genes of 24 immune cells were extracted, and the hypergeometric enrichment analysis method was used to

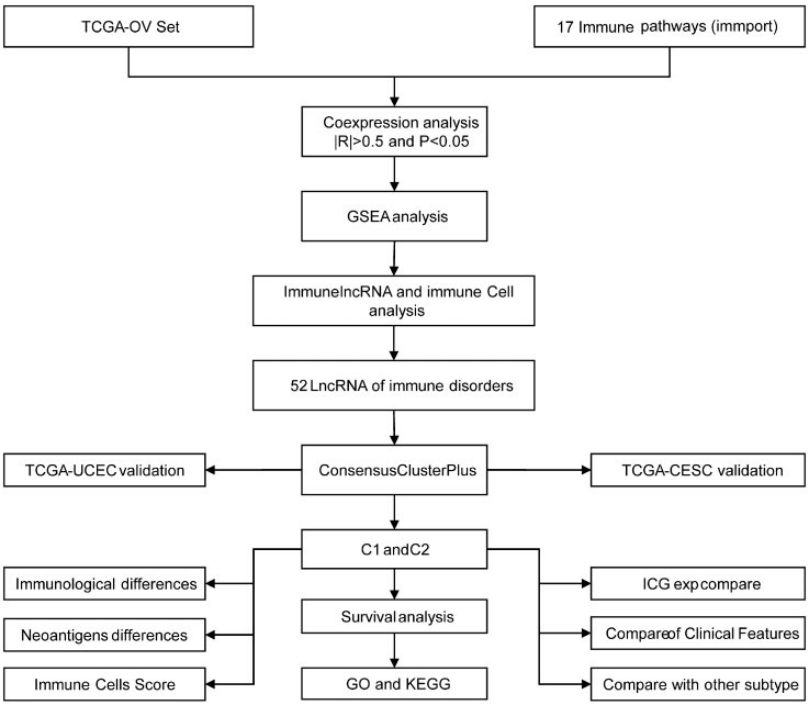

Figure 1. Workflow.

identify the significant enrichment relationship between candidate dysregulated immune lncRNAs and immune cells. The hypergeometric calculation formula is as follows

$$\mathrm{P}ij = \frac{C_M^k C_{N-M}^{n-k}}{C_N^n}$$

P*ij* represents the marker gene enrichment significance of lncRNA *i* and immune cell *J*, *N* represents the number of significant correlation mRNAs of lncRNA *i* in ovarian cancer samples, *M* represents the number of marker genes contained in immune cell *j*, *k* represents the number of significant correlation genes of lncRNA *i* and immune cell *j*, and *N* represents the number of all mRNAs, namely 19,498. In addition, when K is less than 3, the test results are considered to have no significance. Based on this method, we calculated the expression correlation and enrichment significance between each candidate lncRNA and 24 immune cells, and identified the significant enrichment relationship between all candidate dysregulated immune lncRNA regulators and immune cells at the threshold of $P < 0.05$. Finally, we screened out ovarian cancer-specific dysregulated immune lncRNAs that were significantly enriched in at least 12 immune cells.

Identification of molecular subclasses

According to the expression values of dysregulated immune lncRNAs in cancer samples, consensus clustering was used to classify ovarian cancer samples and endometrial cancer, that is, to use R-packets to unsupervised clustering the samples and determine the number of clustering.

Survival analysis of molecular subclasses

Survival analysis was conducted for the samples according to the survival time (OS, DSS) and survival status of different sample categories. Kaplan–Meier method was used to estimate the survival rate and median survival time, and the difference between different sample subclasses was obtained by comparing each group based on the log-rank test.

Differential genes and functional enrichment analysis of molecular subclasses

The differential genes among the molecular subtypes were analyzed using the limma package,[16] $\mathrm{FDR} < 0.05$ and $|\mathrm{FC}|, > 1.5$ were used as thresholds for filtering, the genes that met the conditions were used as the differential genes for further analysis. For differential genes, we used the R software package WebGestaltR (0.4.3)[17] for functional enrichment analysis of GO and KEGG, with $\mathrm{FDR} < 0.05$ as the threshold of significant enrichment.

Analysis of immunological properties of molecular subclasses

In order to analyze the differences in biological characteristics among different samples, various immunological features of TCGA ovarian cancer samples were obtained from published article,[18] including important immunological molecular features such as wound healing, IFN-gamma response, and TGF-beta response. Meanwhile, the software MCPcounter[19] was conducted to calculate the immune scores of 10 cells in the tumor samples. Wilcox rank sum test was used to compare the biological characteristics and characterization of subclasses of different samples.

Validation of dysregulated immune lncRNA on endometrial cancer dataset

In order to verify whether these 52 lncRNAs have immune-related functions in different cancer species, TCGA-endometrial cancer data set was used for verification. The immune lncRNA gene was used to classify the TCGA-endometrial cancer data, and then the differentially expressed genes among different groups were compared and analyzed by functional annotation of the differentially expressed genes.

Results

Identification of ovarian cancer-specific dysregulated immune lncRNAs

The correlation analysis of mRNA and lncRNA expression showed that a total of 7135 lncRNAs were strongly correlated with mRNA in ovarian cancer samples, and each lncRNA was significantly correlated with 43 mRNA on average. Based on GSEA method and enrichment results of 17 immune function pathways, 10,792 significant lncRNA-immune function pathway pairs were identified in ovarian cancer samples, including 3084 lncRNA regulators enriched in different immune function sets.

In order to further ensure the immunomodulating role of candidate lncRNAs in ovarian cancer, marker gene sets of 24 immune cells were collected from the literature. Under the condition of $P < 0.05$, a total of 6718 significant lncRNA-immune cell pairs were obtained, and we further screened 52 lncRNAs with significant enrichment relationships with at least 12 immune cells as the dysregulated immune lncRNAs specific to ovarian cancer (Figure 2).

Identification of molecular subtypes of ovarian cancer

Considering that the classification of molecular subclasses of cancer samples is of great significance for personalized treatment of ovarian cancer, 52 expression values of immune lncRNAs specific to ovarian cancer were used to classify the samples. Based on the method in ConsensusClusterPlus of R package, ovarian cancer samples in TCGA are better divided into two categories, C1 and C2 contain 188 and 184 samples, respectively (Figure 3(a)). Further, survival analysis of TCGA ovarian cancer samples showed significant differences in OS time and DSS time between the two groups ($P = 0.0032$, $P = 0.0042$), among which the C2 ovarian cancer samples had higher survival rate and longer median survival time (Figure 3(b) and (c)). The expression differences of 52 ovarian cancer-specific immune-dysregulated lncRNAs in two molecular subtypes were compared, and the results showed that 57.69% (30/52) lncRNAs were significantly different in two subtypes ($P < 0.05$) (Figure 3(d)).

Differential analysis of molecular subtypes in immunology of ovarian cancer

In order to study the differences in immunological characteristics between the two types of samples, we obtained the TGF-beta response, wound healing, IFN-gamma response, and other important immune characteristics scores of TCGA ovarian cancer samples from literature,[18] and the results showed that C1 patients had higher TGF-beta response scores (Figure 4(a)) and lower IFN-gamma response scores and wound healing scores (Figure 4(b) and (c)) compared to C2 patients.

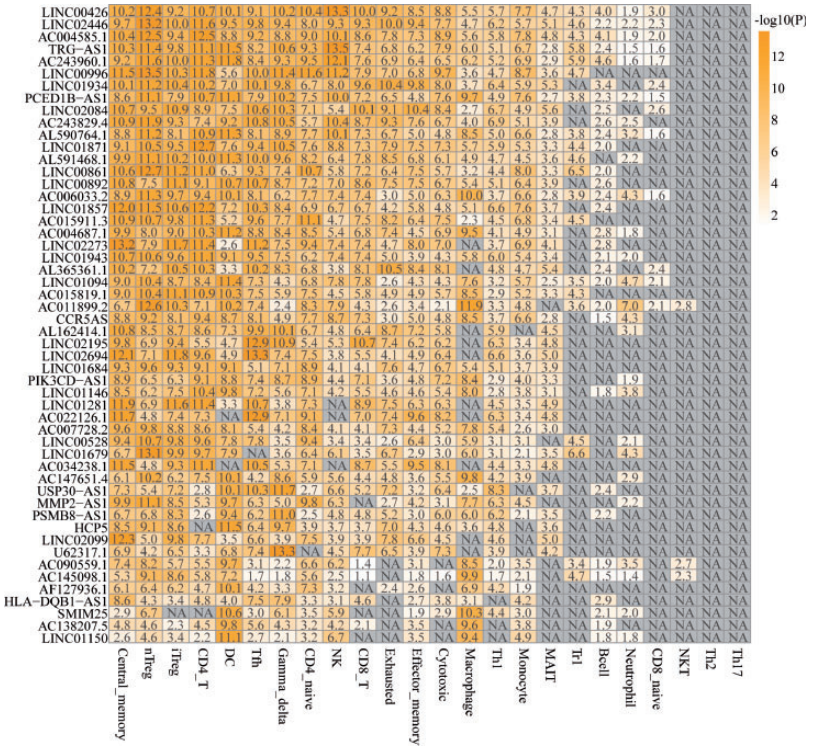

Figure 2. Enrichment significance of dysregulated immune lncRNA and immune cells. (A color version of this figure is available in the online journal.)

The immune scores of 10 immune cells showed that the scores of monocytic lineage, myeloid dendritic cells, endothelial cells, neutrophils, CD8 T cells, and fibroblasts were higher in C1 molecular subtype samples than in C2 molecular subtype samples (Figure S1, $P < 0.05$), T cells, B lineage, NK cells, and cytotoxic lymphocytes have no differences immune scores in C2 molecular subtype samples and C1 molecular subtype samples (Figure S1). To provide basis for further immunotherapy for ovarian cancer, 47 immune checkpoint genes[20] were extracted for the expression of ovarian cancer to compare whether there are differences in the two molecular subtypes. We found that the expression of 18 genes in C1 group was significantly higher than that in C2 group (Figure 4(d)). The expression of five genes in C1 group was significantly lower than that in C2 group (Figure 4(e)). We also tested the difference between the neoantigens in the molecular subtypes of ovarian cancer, and the results showed no significant difference (Figure 4(f)). There is no significant difference in clinical characteristics between the two subtypes (Figure 5(a) to (d)).

Identification of differentially expressed gene analysis and function in ovarian cancer

Limma (3.40.6) was used to calculate the differentially expressed genes (DEGs) between C1 and C2 molecular subtypes. After filtering by threshold FDR < 0.05 and |FC| and > 1.5, there were 456 differentially expressed genes, among

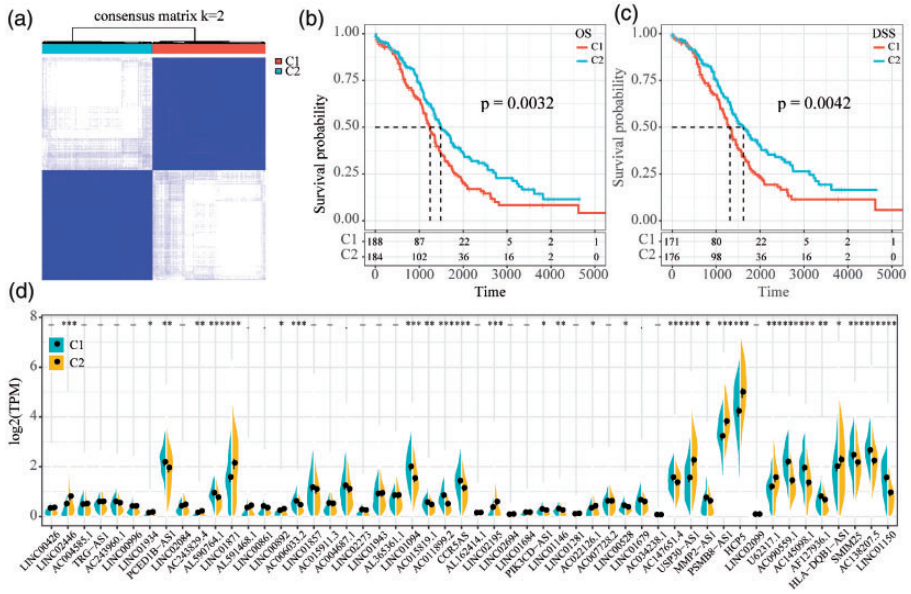

Figure 3. Identification of molecular subtypes in ovarian cancer. (a) Unsupervised clustering of ovarian cancer samples. (b) OS survival curves of two types of ovarian cancer samples. (c) DSS survival curves of two types of ovarian cancer samples. (d) The expression differences of 52 immune-related lncRNAs in molecular subtypes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

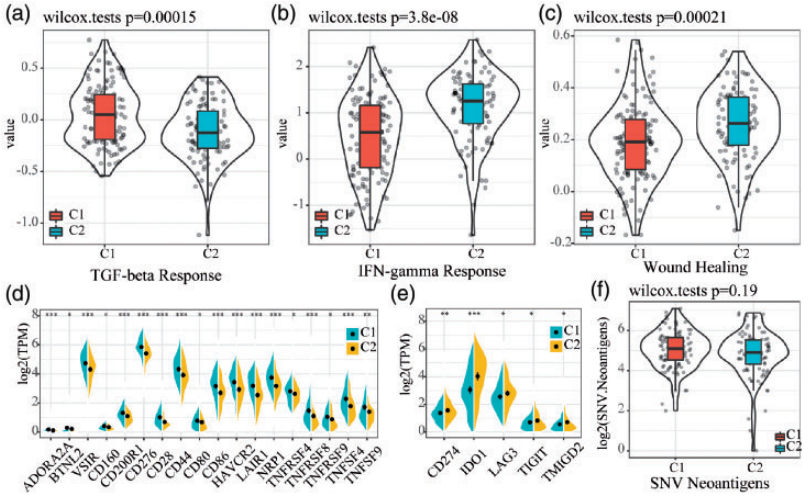

Figure 4. Analysis of immunological differences in ovarian cancer subtype. (a) Differences in TGF-beta response between molecular subtypes. (b) Differences in FN-gamma response between molecular subtypes. (c) Differences in wound healing between molecular subtypes. (d) Immunocheckpoint genes with higher expression in C1 group. (e) Immunocheckpoint genes with higher expression in C2 group. (f) There is no differences of SNV neoantigens between molecular subtypes. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (A color version of this figure is available in the online journal.)

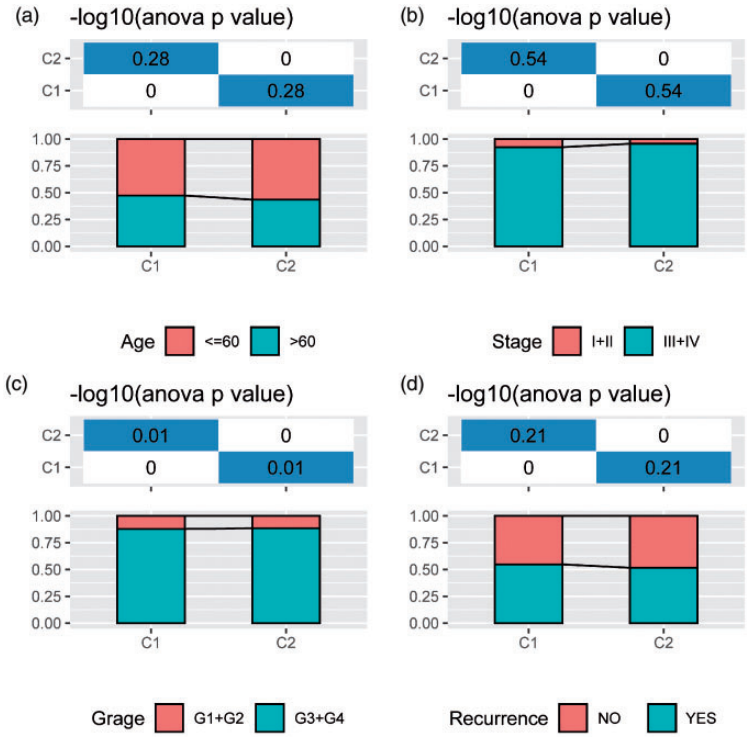

Figure 5. Analysis of clinical features differences in ovarian cancer subtype. (a) Differences in age between molecular subtypes. (b) Differences in stage between molecular subtypes. (c) Differences in grade between molecular subtypes. (d) Differences in recurrence between molecular subtypes. (A color version of this figure is available in the online journal.)

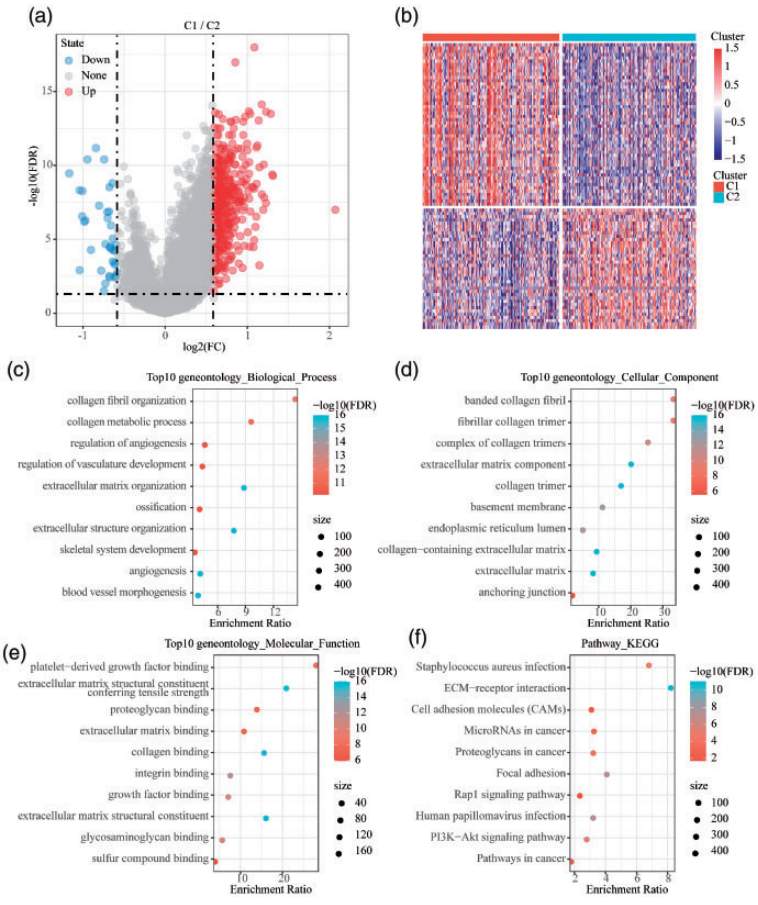

Figure 6. Analysis and functional identification of differentially expressed gene in ovarian cancer subtype. (a) Volcanogram of different genes between molecular subtypes of ovarian cancer. (b) Heat map of different genes between molecular subtypes of ovarian cancer. (c) Top10 BP enrichment of differential genes in ovarian cancer molecular subtypes. (d) Top10 CC enrichment of differential genes in ovarian cancer molecular subtypes. (e) Top10 MF enrichment of differential genes in ovarian cancer molecular subtypes. (f) KEGG enrichment of differential genes in ovarian cancer molecular subtypes. (A color version of this figure is available in the online journal.)

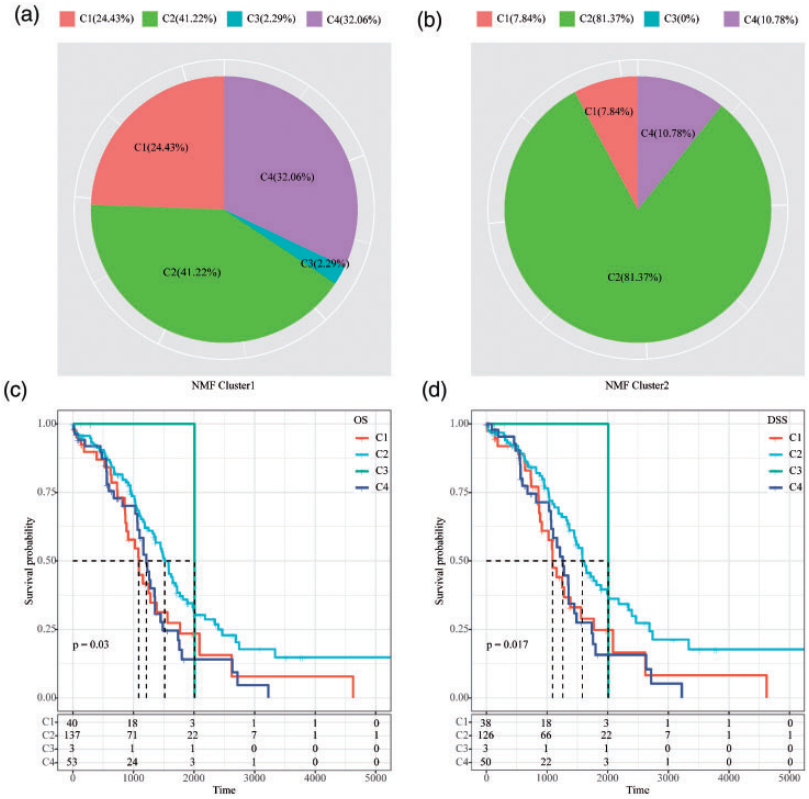

Figure 7. Comparison with existing immune subtypes. (a) The distribution of C1 molecular subtypes identified by us in the four existing immune subtypes. (b) The distribution of C2 molecular subtypes identified by us in the four existing immune subtypes. (c) The OS KM curve of immune subtypes has been reported in the TCGA dataset. (d) DSS KM curve of immune subtypes has been reported in the TCGA dataset. (A color version of this figure is available in the online journal.)

which 419 were up-regulated and 37 were down-regulated (Figure 6(a) and (b)).

Functional enrichment analysis of KEGG and GO was performed on 456 differentially expressed genes by R software package WebGestaltR (0.4.3), among which there were 320 significantly different genes commented to BP (FDR < 0.05) (Figure 6(c)), 51 significantly different genes commented to CC (FDR < 0.05) (Figure 6(d)), and 31 significantly different genes commented to MF (Figure 6(e)). KEGG enrichment results showed that there were 25 KEGG pathways with significant differences (FDR < 0.05) (Figure 6(f)), among which the ECM-receptor interaction, PI3K-Akt signaling pathway, pathways in cancer, proteoglycans in cancer and other pathways for tumor development were significantly enriched.

Comparison with published immune subtypes

Six subtypes of immune infiltration were identified from the literature, namely C1 (wound-healing), C2 (inF-R predominated), C3 (inflammation), C4 (lymphocyte depletion), C5 (immunologically silenced), and C6 (TGF-beta predominated).[18] The samples of molecular subtype C1 identified by us accounted for 24.43%, 41.22%, 2.29%, and 32.06%, respectively, in the C1–C4 immune subtype (Figure 7(a)). The samples of molecular subtype C2 accounted for 7.84%, 81.37%, and 10.78%, respectively, in the C1, C2, and C4 immune subtypes (Figure 7(b)). Survival analysis showed that among the four immune subtypes, C1 and C4 had a poor prognosis, while C2 had a good prognosis (Figure 7(c) and (d)). Interestingly, C2 ovarian cancer samples significantly increased in the C2 immune subtype, accounting for 81.37%, which was associated with a good prognosis. Our C1 subtype contains only 56.49% of the existing immune C1 and C4, which also explains the poor prognosis of C1.

Classification of immune lncRNA in endometrial carcinoma and cervical cancer

In order to verify the functional effects of these 52 immune-related lncRNAs, we used these 52 lncRNAs to classify endometrial cancer samples and cervical cancer sample in TCGA database, and divided TCGA endometrial cancer samples into two categories, C1 and C2 containing 432 and 109 samples, respectively (Figure 8(a)). TCGA cervical cancer samples were also divided into two categories, C1 (75 samples) and C2 (216 samples), respectively (Figure 8 (d)). In addition, survival analysis of TCGA endometrial cancer samples was conducted based on this classification information, and we found that there were significant survival differences in OS time and DSS time between the two groups ($P = 0.039$, $P = 0.021$), among which the C2 endometrial cancer samples had higher survival rate and longer median survival time (Figure 8(b) and (c)). Survival analysis of TCGA cervical cancer samples showed that there were significant survival differences in OS time and DSS time between the two groups ($P = 0.036$, $P = 0.039$), among which the C2 endometrial cancer samples had higher

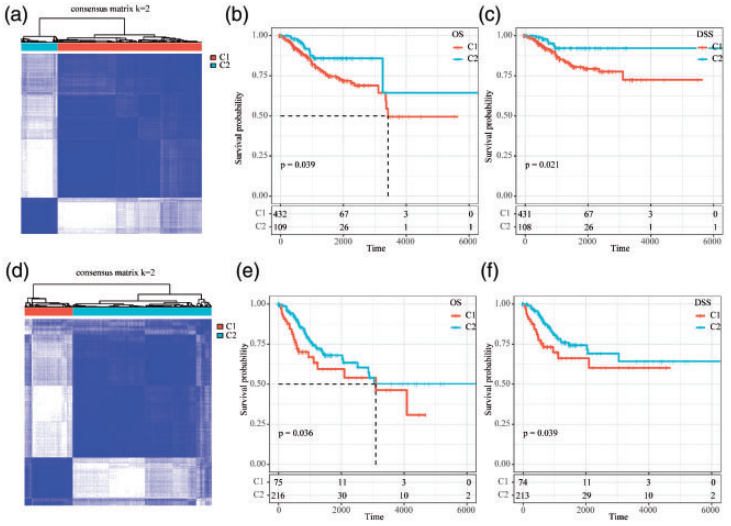

Figure 8. Identification of subtypes of endometrial carcinoma and cervical cancer. (a) Unsupervised clustering of endometrial carcinoma samples. (b) OS survival curves of two types of endometrial carcinoma samples. (c) DSS survival curves of two types of endometrial carcinoma samples. (d) Unsupervised clustering of cervical cancer samples. (e) OS survival curves of two types of cervical cancer samples. (f) DSS survival curves of two types of cervical cancer samples. (A color version of this figure is available in the online journal.)

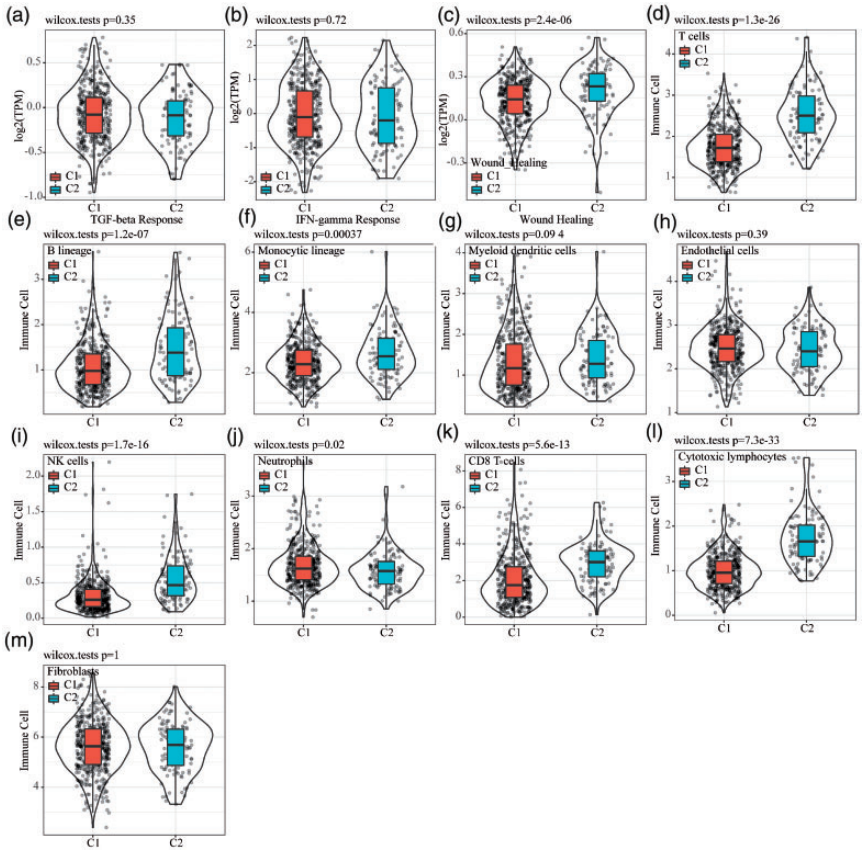

Figure 9. Immunological analysis of endometrial carcinoma molecular subtypes. (a) Differences in TGF-beta response between molecular subtypes of endometrial carcinoma. (b) Differences in FN-gamma response between molecular subtypes of endometrial carcinoma. (c) Differences in wound healing between molecular subtypes of endometrial carcinoma. (d) Differences in T cells scores between endometrial carcinoma molecular subtypes. (e) Differences in B lineage scores between endometrial carcinoma molecular subtypes. (f) Differences in monocytic lineage scores between endometrial carcinoma molecular subtypes. (g) Differences in myeloid dendritic cells scores between endometrial carcinoma molecular subtypes. (h) Differences in endothelial cells scores between endometrial carcinoma molecular subtypes. (i) Differences in NK cells scores between endometrial carcinoma molecular subtypes. (j) Differences in neutrophils scores between endometrial carcinoma molecular subtypes. (k) Differences in CD8 T cells scores between endometrial carcinoma molecular subtypes. (l) Differences in cytotoxic lymphocytes scores between endometrial carcinoma molecular subtypes. (m) Differences in fibroblasts scores between endometrial carcinoma molecular subtypes. (A color version of this figure is available in the online journal.)

survival rate and longer median survival time (Figure 8(e) and (f)).

Immunological differences in endometrial carcinoma and cervical cancer subtypes

In order to study the differences in immunological properties between the two types of samples in endometrial carcinoma, we found that there was no difference in TGF-beta response and IFN-gamma response scores in C1 patients compared with C2 patients (Figure 9(a) and (b)), while C1 patients had significantly lower wound healing scores (Figure 9(c)). The immune scores of the 10 immune cells also showed that T cells, B lineage, monocytic, myeloid dendritic cells, NK cells, neutrophils, CD8 T cells, and thirdly cytotoxic lymphocytes were different in the two most lymphocytes molecular subtypes ($P < 0.05$) (Figure 9(d) to (m)). In cervical cancer subtype, C2 had no difference in TGF-beta response, but obviously higher IFN-gamma response scores and lower wound healing scores in comparison to C1 type (Figure 10(a) to (c)). The immune scores of the 10 immune cells showed that T cells, B lineage, monocytic lineage, myeloid dendritic cells, NK cells, neutrophils, CD8 T cells, and cytotoxic lymphocytes were different in the two most molecular subtypes ($P < 0.05$) (Figure 10(d) to (m)).

Functional analysis of differentially expressed genes in endometrial carcinoma and cervical cancer subtypes

The differentially expressed genes (DEGs) between endometrial cancer and cervical cancer C1 and C2 molecular subtypes were calculated by limma (3.40.6). After filtering by the TCGA data set with threshold FDR < 0.05 and $|FC|$ and > 1.5, a total of 685 differentially expressed genes were found, among which 399 were up-regulated and 286 were down-regulated in endometrial cancer (Figure 11(a) and (b)). KEGG and GO functional enrichment analysis showed that there were 260 significant differences in annotation to BP (FDR < 0.05) (Figure 11(c)), 14 significant differences in annotation to CC (FDR < 0.05) (Figure 11(d)),

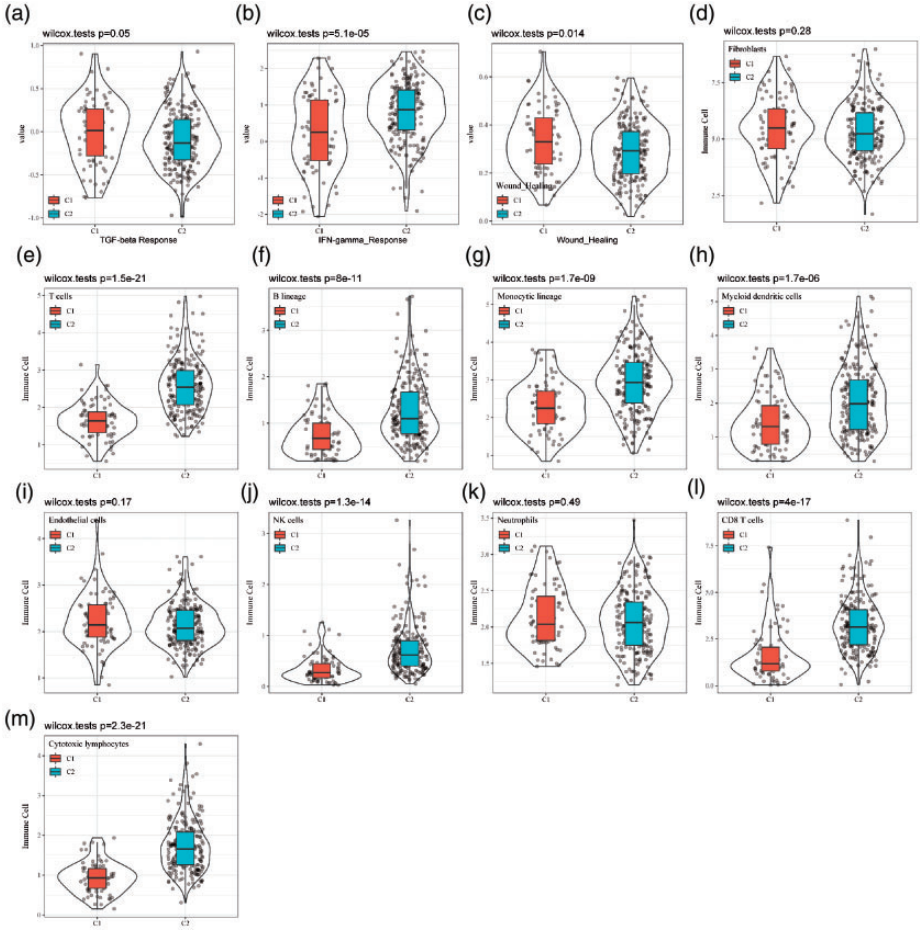

Figure 10. Immunological analysis of cervical cancer molecular subtypes. (a) Differences in TGF-beta response between molecular subtypes of cervical cancer. (b) Differences in FN-gamma response between molecular subtypes of cervical cancer. (c) Differences in wound healing between molecular subtypes of cervical cancer. (d) Differences in T cells scores between cervical cancer molecular subtypes. (e) Differences in B lineage scores between ENDOMETRIAL CANCER molecular subtypes. (f) Differences in monocytic lineage scores between cervical cancer molecular subtypes. (g) Differences in myeloid dendritic cells scores between cervical cancer molecular subtypes. (h) Differences in endothelial cells scores between cervical cancer molecular subtypes. (i) Differences in NK cells scores between cervical cancer molecular subtypes. (j) Differences in neutrophils scores between cervical cancer molecular subtypes. (k) Differences in CD8 T cells scores between cervical cancer molecular subtypes. (l) Differences in cytotoxic lymphocytes scores between cervical cancer molecular subtypes. (m) Differences in fibroblasts scores between cervical cancer molecular subtypes. (A color version of this figure is available in the online journal.)

31 significant differences in annotation to MF (FDR < 0.05) (Figure 11(e)). There were 29 KEGG pathways with significant differences (FDR < 0.05) (Figure 11(f)), among which immune-related pathways such as natural killer cell-mediated cytotoxicity, T cell receptor signaling pathway, Th17 cell differentiation, Th1 and Th2 cell differentiation, and NF-Kappa B signaling pathway are significantly enriched. There are also 734 differentially expressed genes that were found, among which 158 were up-regulated and 576 were down-regulated in cervical cancer (Figure 12(a) and (b)). KEGG and GO functional enrichment analysis showed that there were 687 significant differences in annotation to BP (FDR < 0.05) (Figure 12(c)), 73 significant differences in annotation to CC (FDR < 0.05) (Figure 12(d)), 68 significant differences in annotation to MF (FDR < 0.05) (Figure 12(e)). There were 43 KEGG pathways with significant differences (FDR < 0.05) (Figure 12(f)), among which, Th1 and Th2 cell differentiation, cytokine-cytokine receptor interaction, Th17 cell differentiation, and natural killer cell-mediated cytotoxicity signaling pathway are significantly enriched.

Discussion

In our work, a grand total of 52 lncRNAs were recognized as aberrant immune lncRNAs specific to ovarian cancer. We redefined two different molecular subtypes, C1(188) and C2(184 samples), among which C2 grouped ovarian cancer samples have higher survival probability and a longer median life expectancy ($P < 0.05$) with activated IFN-gamma response, wound healing, and cytotoxic lymphocytes signal; 456 differentially expressed genes were recognized in C1 and C2 subtypes, among which 419 were up-regulated and 37 were down-regulated, in TCGA dataset. Functional enrichment analysis implied that these genes were actively plunge into an ECM-receptor interaction, PI3K-Akt signaling pathway interaction KEGG pathway. Compared with the existing immune subtype, the Cluster2 sample showed a significant increase in the proportion of the existing C2 immune subtype, accounting for 81.37%, which was associated with good prognosis. Our C1 subtype contains only 56.49% of the existing immune C1 and C4, which also explains the poor prognosis

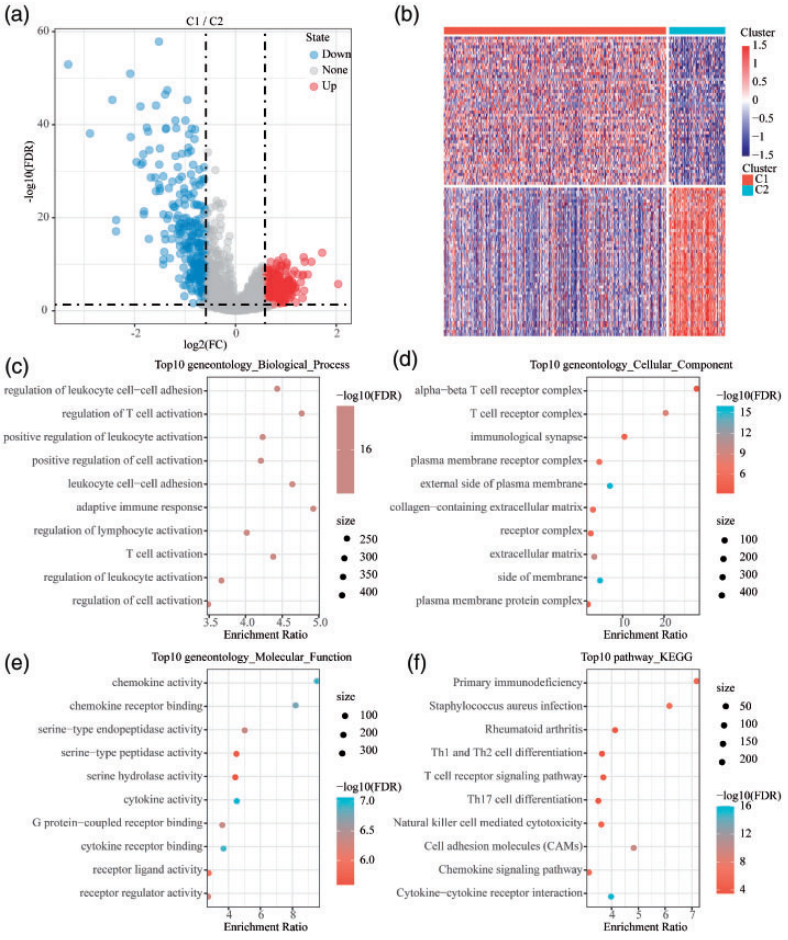

Figure 11. Analysis and functional identification of differentially expressed gene in endometrial carcinoma. (a) Volcanogram of different genes between molecular subtypes of endometrial carcinoma. (b) Heat map of different genes between molecular subtypes of endometrial carcinoma. (c) Top10 BP enrichment of differential genes in endometrial carcinoma molecular subtypes. (d) Top10 CC enrichment of differential genes in endometrial carcinoma molecular subtypes. (e) Top10 MF enrichment of differential genes in endometrial carcinoma molecular subtypes. (f) KEGG enrichment of differential genes in endometrial carcinoma molecular subtypes. (A color version of this figure is available in the online journal.)

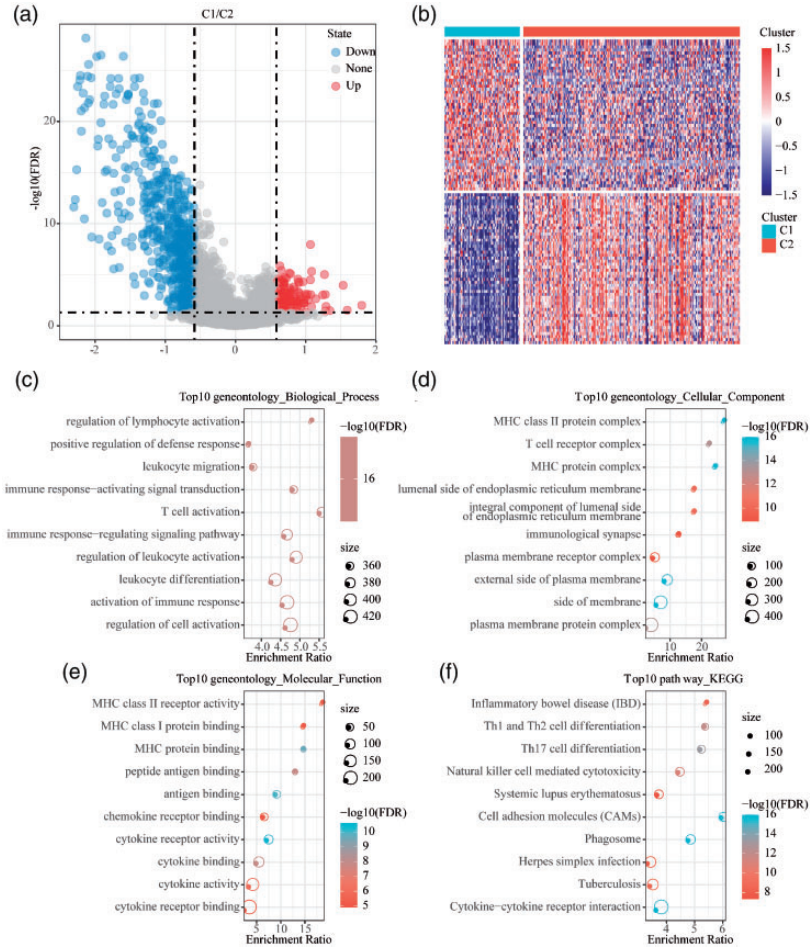

Figure 12. Analysis and functional identification of differentially expressed gene in cervical cancer. (a) Volcanogram of different genes between molecular subtypes of cervical cancer. (b) Heat map of different genes between molecular subtypes of cervical cancer. (c) Top10 BP enrichment of differential genes in cervical cancer molecular subtypes. (d) Top10 CC enrichment of differential genes in cervical cancer molecular subtypes. (e) Top10 MF enrichment of differential genes in cervical cancer molecular subtypes. (f) KEGG enrichment of differential genes in cervical cancer molecular subtypes. (A color version of this figure is available in the online journal.)

of C1. Furthermore, 52 immune-related lncRNAs were used to assign the TCGA-endometrial cancer data set into two categories, and C2 had a good prognosis. The differentially expressed genes were highly correlated with immune-cell-related pathways.

With the advances in large-scale sequencing technology and bioinformatics methods, lncRNAs have been revealed to be engaged in carcinogenesis and cancer development.[21] The molecular atlas shows that ovarian cancer is a complex and heterogeneous disorder with unique molecular subsets and clinical characteristics. Many works have also proved the importance of lncRNA in ovarian cancer, encompassing its role as a driver of tumor inhibition and carcinogenic property, microrRNA competitors, and diagnostic biomarkers.[22,23] In the near future, the engagement of lncRNAs in immune regulation has been broadly characterized.[24–26] Hence, we hypothesized that immune-related lncRNAs could be applied to investigate various immune types and characterize their mechanisms in ovarian cancer. In our work, 52 immune-associated prognostic lncRNAs were obtained, and two immune-related lncRNA clusters, C1 (188) and C2 (184), were constructed using 392 ovarian cancer samples from TCGA. Further, significant survival differences were found between the two groups.

Fascinatingly, we found two clusters of ovarian cancer cases on the basis of immune-related lncRNAs, which differed significantly in immunological characteristics. It has been reported that lncRNA may also play a key role in transcriptional regulation of gene expression during innate immune response.[6] LncRNA EGFR activates T-regulatory cells differentiation, thereby contributing to immune evasion from hepatocellular carcinoma.[27] shMALAT1 treatment in diffuse large B cell lymphoma decreased PD-L1 level and inhibited apoptosis of CD8+ T cells.[28] We analyzed immune characteristic score and immune cell types in two clusters. The results showed that the cluster 1 had higher TGF-beta response score, and lower IFN-gamma response and wound healing score than the cluster 2. Monocytic lineage, myeloid dendritic cells, endothelial cells, neutrophils, CD8 T cells, and fibroblasts score had significantly difference in two molecular subtypes ($P < 0.05$). The above results show that in ovarian cancer samples, the two groups of molecular subtypes have significant immunological differences,

which may lead to significant differences in the prognosis of the two groups.

Strong robustness is necessary for the classification of molecular subtypes to be applied in clinical practice. In this study, in order to prove the robustness of 52 immune-related lncRNAs, we applied these lncRNAs to TCGA endometrial carcinoma samples and also divided them into two categories, among which C1 had a poor prognosis and C2 had a good prognosis. Differential genes are highly correlated with immune-cell-related pathways, which also prove the importance of these immune-related lncRNAs from the side.

There are several limitations that need to be noted in the study. First, lncRNA should be studied in further experiments, which may provide new therapeutic targets for ovarian cancer. Secondly, due to data limitations, immune-related lncRNAs were only validated in an independent patient data set, and more patient data sets are expected to validate the performance of immune-related lncRNAs to accelerate clinical application. In future research, we will continue to study and solve these problems.

Conclusions

In summary, our study identified immune lncRNA-mediated molecular subtypes associated with clinical outcomes in patients with ovarian cancer.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; XJL, JhG conducted conception and design of the research. JW, JC performed acquisition of data. JHY analyzed data. ZJJ contributed to the statistical analysis. XJL drafted the manuscript. JHG revised manuscript for important intellectual content. All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID ID

Jinghai Gao https://orcid.org/0000-0002-7986-8333

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Torre LA, Trabert B, DeSantis CE, Miller KD, Samimi G, Runowicz CD, Gaudet MM, Jemal A, Siegel RL. Ovarian cancer statistics, 2018. *CA Cancer J Clin* 2018;**68**:284–96
2. Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. *Lancet* 2014;**384**:1376–88
3. Mizrahi A, Czerniak A, Levy T, Amiur S, Gallula J, Matouk I, Abu-Lail R, Sorin V, Birman T, de Groot N, Hochberg A, Ohana P. Development of targeted therapy for ovarian cancer mediated by a plasmid expressing diphtheria toxin under the control of H19 regulatory sequences. *J Transl Med* 2009;**7**:69
4. Chen W, Zheng R, Baade PD, Zhang S, Zeng H, Bray F, Jemal A, Yu XQ, He J. Cancer statistics in China, 2015. *CA Cancer J Clin* 2016;**66**:115–32
5. Yao RW, Wang Y, Chen LL. Cellular functions of long noncoding RNAs. *Nat Cell Biol* 2019;**21**:542–51
6. Li Z, Rana TM. Decoding the noncoding: prospective of lncRNA-mediated innate immune regulation. *RNA Biol* 2014;**11**:979–85
7. Atianand MK, Caffrey DR, Fitzgerald KA. Immunobiology of long noncoding RNAs. *Annu Rev Immunol* 2017;**35**:177–98
8. Chen YG, Satpathy AT, Chang HY. Gene Regulation in the immune system by long noncoding RNAs. *Nat Immunol* 2017;**18**:962–72
9. Jiang MC, Ni JJ, Cui WY, Wang BY, Zhuo W. Emerging roles of lncRNA in cancer and therapeutic opportunities. *Am J Cancer Res* 2019;**9**:1354–66
10. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell* 2012;**21**:309–22
11. Colvin EK, Howell VM, Mok SC, Samimi G, Vafaee F. Expression of long noncoding RNAs in cancer-associated fibroblasts linked to patient survival in ovarian cancer. *Cancer Sci* 2020;**111**:1805–17
12. Shang A, Wang W, Gu C, Chen C, Zeng B, Yang Y, Ji P, Sun J, Wu J, Lu W, Sun Z, Li D. Long non-coding RNA HOTTIP enhances IL-6 expression to potentiate immune escape of ovarian cancer cells by upregulating the expression of PD-L1 in neutrophils. *J Exp Clin Cancer Res* 2019;**38**:411
13. Tomczak K, Czerwińska P, Wiznerowicz M. The cancer genome atlas (TCGA): an immeasurable source of knowledge. *Contemp Oncol* 2015;**19**:A68–77
14. Bhattacharya S, Andorf S, Gomes L, Dunn P, Schaefer H, Pontius J, Berger P, Desborough V, Smith T, Campbell J, Thomson E, Monteiro R, Guimaraes P, Walters B, Wiser J, Butte AJ. ImmPort: disseminating data to the public for the future of immunology. *Immunol Res* 2014;**58**:234–9
15. Li Y, Jiang T, Zhou W, Li J, Li X, Wang Q, Jin X, Yin J, Chen L, Zhang Y, Xu J, Li X. Pan-cancer characterization of immune-related lncRNAs identifies potential oncogenic biomarkers. *Nat Commun* 2020;**11**:1000
16. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015;**43**:e47
17. Wang J, Vasaikar S, Shi Z, Greer M, Zhang B. WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. *Nucleic Acids Res* 2017;**45**:W130–7
18. Thorsson V, Gibbs DL, Brown SD, Wolf D, Bortone DS, Ou Yang TH, Porta-Pardo E, Gao GF, Plaisier CL, Eddy JA, Ziv E, Culhane AC, Paull EO, Sivakumar IKA, Gentles AJ, Malhotra R, Farshidfar F, Colaprico A, Parker JS, Mose LE, Vo NS, Liu J, Liu Y, Rader J, Dhankani V, Reynolds SM, Bowlby R, Califano A, Cherniack AD, Anastassiou D, Bedognetti D, Mokrab Y, Newman AM, Rao A, Chen K, Krasnitz A, Hu H, Malta TM, Noushmehr H, Pedamallu CS, Bullman S, Ojesina AI, Lamb A, Zhou W, Shen H, Choueiri TK, Weinstein JN, Guinney J, Saltz J, Holt RA, Rabkin CS, Lazar AJ, Serody JS, Demicco EG, Disis ML, Vincent BG, Shmulevich I. The immune landscape of cancer. *Immunity* 2018;**48**:812–30.e14
19. Becht E, Giraldo NA, Lacroix L, Buttard B, Elarouci N, Petitprez F, Selves J, Laurent-Puig P, Sautès-Fridman C, Fridman WH, de Reyniès A. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. *Genome Biol* 2016;**17**:218
20. Danilova L, Ho WJ, Zhu Q, Vithayathil T, De Jesus-Acosta A, Azad NS, Laheru DA, Fertig EJ, Anders R, Jaffee EM, Yarchoan M. Programmed cell death ligand-1 (PD-L1) and CD8 expression profiling identify an immunologic subtype of pancreatic ductal adenocarcinomas with favorable survival. *Cancer Immunol Res* 2019;**7**:886–95
21. Gao Y, Li X, Zhi H, Zhang Y, Wang P, Wang Y, Shang S, Fang Y, Shen W, Ning S, Chen SX, Li X. Comprehensive characterization of somatic

mutations impacting lncRNA expression for pan-cancer. *Mol Ther Nucleic Acids* 2019;**18**:66–79
22. Du W, Feng Z, Sun Q. LncRNA LINC00319 accelerates ovarian cancer progression through miR-423-5p/NACC1 pathway. *Biochem Biophys Res Commun* 2018;**507**:198–202
23. Wang X, Yang B, She Y, Ye Y. The lncRNA TP73-AS1 promotes ovarian cancer cell proliferation and metastasis via modulation of MMP2 and MMP9. *J Cell Biochem* 2018;**119**:7790–9
24. Huang D, Chen J, Yang L, Ouyang Q, Li J, Lao L, Zhao J, Liu J, Lu Y, Xing Y, Chen F, Su F, Yao H, Liu Q, Su S, Song E. NKILA lncRNA promotes tumor immune evasion by sensitizing T cells to activation-induced cell death. *Nat Immunol* 2018;**19**:1112–25
25. Xu H, Jiang Y, Xu X, Su X, Liu Y, Ma Y, Zhao Y, Shen Z, Huang B, Cao X. Inducible degradation of lncRNA Sros1 promotes IFN-γ-mediated activation of innate immune responses by stabilizing Stat1 mRNA. *Nat Immunol* 2019;**20**:1621–30
26. Mowel WK, Kotzin JJ, McCright SJ, Neal VD, Henao-Mejia J. Control of immune cell homeostasis and function by lncRNAs. *Trends Immunol* 2018;**39**:55–69
27. Jiang R, Tang J, Chen Y, Deng L, Ji J, Xie Y, Wang K, Jia W, Chu WM, Sun B. The long noncoding RNA lnc-EGFR stimulates T-regulatory cells differentiation thus promoting hepatocellular carcinoma immune evasion. *Nat Commun* 2017;**8**:15129
28. Wang QM, Lian GY, Song Y, Huang YF, Gong Y. LncRNA MALAT1 promotes tumorigenesis and immune escape of diffuse large B cell lymphoma by sponging miR-195. *Life Sci* 2019;**231**:116335

(Received July 19, 2020, Accepted October 13, 2020)