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High Expression of IGFL1 Predicts Poor Prognosis of Serous Ovarian Carcinoma

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Abstract

Background and objective: Ovarian cancer is a common gynecological tumor that may develop at any age, which has a very poor prognosis. The mortality rate of ovarian cancer ranks first in gynecological malignancies. In regard to the classification of ovarian cancer, patients with serous ovarian carcinoma account for the majority, posing a serious threat to women's lives. Therefore, it is crucial to explore the prognostic indicators of serous ovarian carcinoma. The IGFL family consists of four expressed genes and two pseudogenes. IGFL mRNAs are expressed in many cancers, and studies have previously detected the expression of the IGFL1 gene in the ovaries. This study attempted to analyze the prognostic role of IGFL1 in serous ovarian carcinoma.**Materials and Methods:** The mRNA expression levels of patients with serous ovarian carcinoma were compared by two groups in the TCGA database. Then, the differentially expressed genes (DEGs) were screened by volcano map and Venn analysis, for which the survival analysis was based on the TCGA database. Protein-protein interaction (PPI) networks were then used to identify the interactions. The relationship between differential genes and prognosis was analyzed by human protein profile, quantitative PCR and immunohistochemistry. **Results:** By extracting RNA-seq data from the Cancer Genome Atlas Database (TCGA), the clinical significance of IGFL1 mRNA expression was explored. As a result, IGFL1 levels were found to be highly expressed in serous ovarian carcinoma tissues and related to survival status. Survival analysis showed that patients with serous ovarian carcinoma and higher IGFL1 levels generally had shorter overall survival times compared to those with lower IGFL1 expression levels. Accordingly, high IGFL1 expression can be used as an independent prognostic factor for patients with serous ovarian carcinoma. **Conclusions:** High IGFL-1 expression serves as an independent risk factor for poor prognosis in serous ovarian carcinoma patients.

Keywords: serous ovarian carcinoma**,** prognosis, IGFL-1, biomarker

Introduction

Among all gynecological diseases, ovarian cancer is a relatively common cancer. Compared to other malignant tumors affecting the female reproductive system, the incidence of ovarian cancer ranks third, second only to cervical cancer and endometrial cancer [1][2][3]. Due to multiple risk factors, the mortality rate of ovarian cancer is extremely high [4]. Ovarian cancer is categorized into three main types: epithelial (most common), germ cell, and sex-cord-stromal, with the latter two comprising only about 5% of all ovarian cancers [5][6].

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According to morphology, molecular alterations, and clinical behavior, epithelial ovarian cancer (EOC) is further divided into two groups: type I and type II. Type I tumors are low-grade, slow-growing ovarian carcinomas, while Type II tumors are high-grade and aggressive malignancies [7]. The histological type of epithelial ovarian cancer includes serous ovarian carcinoma, mucinous ovarian cancer and endometrioid ovarian cancer, with serous carcinoma being the most commonly diagnosed [8]. Although an increasing number of studies on ovarian cancer are being conducted, due to limited early screening technology and blurred clinical symptoms of ovarian cancer, most patients are diagnosed with stage III or IV at diagnosis, hence, the cure rate of ovarian cancer has not significantly

changed [9]. Moreover, the overall 5-year survival rate for ovarian cancer remains poor [10][11], while the 5-year survival rate of advanced ovarian cancer is only 29% [12]. Ovarian cancer is aptly called the invisible killer [13]. In order to garner people's attention with respect to ovarian cancer, the United States declared September as National Ovarian Cancer Awareness Month [14].Therefore, it is important to explore possible prognostic biomarkers for ovarian cancer, especially serous ovarian carcinoma.

The human IGFL gene is clustered on chromosome 19 and consists of four expressed genes and two pseudogenes. IGFL mRNA shows a specific expression pattern, which is consistent with that of members of the IGF family and is expressed in many other cancers [15]. In a study on psoriasis, tumor necrosis factor (TNFα) was proposed to enhance the expression of IGFL1 *in vitro* [16]. Although the IGFL gene is rarely expressed, the expression of the IGFL1 gene in the ovary and spinal cord has been previously analyzed via real-time quantitative PCR (RT-PCR). IGFL2 is expressed in the cerebellum, heart, placenta, spleen, stomach, testis and thymus; IGFL3 and IGFL4 are expressed in the cerebellum, however, the role of IGFL1 in ovarian cancer remains unclear [15].

In order to further evaluate the clinical significance of IGFL1 in the prognosis of patients suffering from serous ovarian carcinoma, the Cancer Genome Atlas database (TCGA) was explored to evaluate the differential expression of IGFL1 mRNA in serous ovarian carcinoma. Chi-square test and Fisher's exact test were then used to assess its clinical relevance. Survival analysis model was used to determine the correlation between IGFL1 and the survival rate of patients with serous ovarian carcinoma.

Materials and methods

Acquisition of microarray data

The gene expression profile data of serous ovarian carcinoma were obtained from the TCGA database (http://cancergenome.nih.gov/), which was derived from the UCSC Cancer Browser (https://genome-cancer.ucsc.edu). The screened data was normalize prior to application.

Screening of DEGs

The collected data were divided into two groups. The first group included a total of 414 samples, including 23 samples of early serous ovarian carcinoma (including stage I and II) and 391 samples of advanced serous ovarian carcinoma (including stage III and IV). The second group had a total of 383 samples, including 160 samples of long OS160 and 223 samples of short OS223 (median OS was 869 days). R language was used to explore the differentially expressed genes (DEGs).

Venn analysis

The explored differentially expressed genes in both groups were analyzed using the web tool Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn) so as to determine specific genes related to the prognosis of serous ovarian carcinoma.

GO analysis

Functional analysis of the DEGs was carried out via GO (http://www.geneontology.org) based on the biological process [17].

Pathway annotation analysis

In order to identify important pathways related to DEG, pathways based on KEGG, Reactome, and BioCarta were analyzed.

PPI network construction

The online tool called STRING [20] (https://string-db.org) was utilized to assess protein-protein interactions (PPI) and reveal the associations between proteins in the genome. DEGs were then entered into STRING, and the confidence score was set to ≥0.4, with the maximum number of members =50 as the threshold. Each node in the PPI network represented a protein, and each edge represented an interaction involving pairs of proteins. The hub protein served as a node with a relatively large number of edges.

Analysis of IGFL1 expression in the Human Protein Atlas

The Cell Atlas, Tissue Atlas and Pathology Atlas constitutes the Human Protein Atlas (https://www.proteinatlas.org/), which provides numerous transcriptomics and proteomics data related to specific tissues of the human body. This database was used to study the expression of IGFL1 in serous ovarian carcinoma tissues.

Clinical patient samples

Human tissue samples were selected from patients with serous ovarian carcinoma at our department from 2013 to 2015. Written informed consent was obtained from all patients, and this study was approved by the ethics committee of the third Affiliated Hospital of Zhengzhou University (Ethical approval number: Science-2019-LW-103). Relevant

clinical information and overall township and village data were collected from patients via case records and telephone consultations. A total of 36 cases of serous ovarian carcinoma were finally collected, with fresh tissue collected during surgery that was immediately stored in liquid nitrogen (-80 ° C).

Quantitative real-time polymerase chain reaction (qRT-PCR)

A total of 36 serous ovarian carcinoma tumor t issues were used in the study. The total RNA fr om these tissues were extracted using Trizol sol ution (Invitrogen, Waltham, MA, USA) according to the manual. Independently, the RNA of eac h sample were reverse-transcribed using the Pri meScript RT reagent kit (Takara Bio, Otsu, Shiga, Japan). qRT-PCR was performed by employing specific primers as well as the SYBR Green qPC R Master Mix (Takara, Japan). The specific prim ers used were as follows: 5'- AAAGCCTGCCGGTG ACTAAC-3' sense primer and 5'- ACATGTAAACCA TGTAGTTGAGGT -3' antisense primer for GAPDH; and 5'- CTGCATCGTAGCTGTCTTTGC-3' sense pri mer, 5'-AGTTTCCACACGTCTGGGTC-3' antisense pr imer for IGFL1. The expression levels of IGFL1 were then compared in clinical sample using th e $2^{-\Delta\Delta Ct}$ method [18]. The expression levels of IGF L1 and GAPDH in each sample were subsequent ly examined, with GAPDH serving as the interna l control. Finally, the relative expression levels o f IGFL1 were determined using the 2-ΔCt value of IGFL1 divided by that of GAPDH [18].

Immunohistochemistry

The expression of IGFL1 protein in paraffin embedded tissues of 34 cases of serous ovarian carcinoma was detected by immunohistochemistry (Thermo ; 1:100). First, the sections were treated with 3% H2O2 and 5% bull serum albumin (BSA), after which they were incubated with primary antibody overnight at 4 ℃. Following incubation for 1 hour with horseradish peroxidase (HRP) - bound secondary antibody at 37 ℃, the sections were washed and stained with hematoxylin and observed under a microscope (Olympus, Shinjuku, Japan).

Data analysis

Bioinformatics analyses were conducted using the R software (version 3.4; R Foundation for Statistical Computing, Vienna, Austria). The Chi-square test and Fisher Chi-square test were used to compare the clinicopathological factors, and the Student's t-test and one-way ANOVA were then used to analyze continuous variables. A survival analysis was done using a Kaplan-Meier analysis and log rank t-test. Moreover, univariate and multivariate logistic regression models were done to confirm the correlations between the expression levels of IGFL1 with the corresponding clinical features. Statistics related to the clinical samples were then analyzed using Prism 7 (GraphPad Software Inc., La Jolla, USA). Statistical analyses were conducted using ANOVA, and a chi-square test was performed with SPSS 26.0 for Windows (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant.

Results

Screening specific genes associated with poor prognosis

The gene expression profile data of serous ovarian carcinoma were obtained from the TCGA database(http://cancergenome.nih.gov/). In order to determine specific genes associated with poor prognosis in patients with serous ovarian carcinoma, the short (< 1000 days) OS group and long (> 1000 days) OS group were divided according to OS (figure 1A, $P<0.0001$). The data were then divided into early (including stage I and II) and late (stage III and IV), and a total of 283 DEGs, including 245 upregulated genes and 38 downregulated genes (Figures 1B and 1C), were identified in a volcano plot. The corresponding expression levels of 278 DEGs obtained are shown in the corresponding Heatmap (Figure 1D). A total of 74 DEGs were observed, including 15 upregulated and 59 downregulated transcription factors (FIG. 1F) using the volcanic plot (FIG. 1E) to determine the DEGs between the long OS and short OS (median OS was 869 days). The expressions of 171 DEGs are shown in the associated Heatmap (Figure 1G). Finally, a Venn diagram was established to display 2 DEGs according to two filtering methods (Figure 1H).

Proven DEGs are associated with poor prognosis of TCGA

The expression level of DEGs can be tested in TCGA to verify the prognostic significance of DEGs. As expected, Kaplan-Meier survival analysis showed that the differential gene IGFL1 was significant in tissue expression ($P = 0.036$), which may play a key role in the development of serous ovarian carcinoma. The IGFL1 gene was then chosen in order to study its relationship with the prognosis of patients (Figure 2).

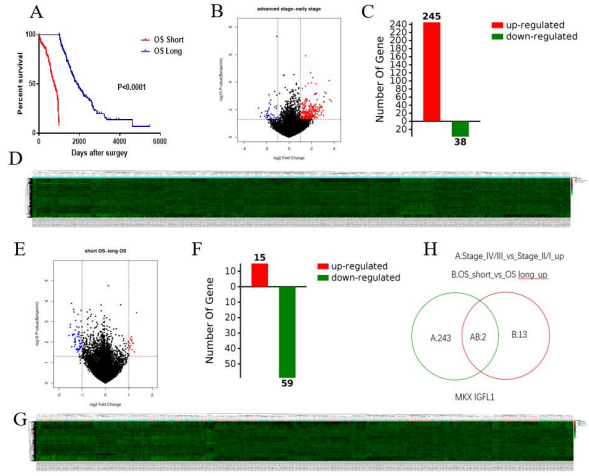

Figure 1

Figure 1. Differentially expressed genes (DEGs) were screened by The Cancer Genome Atlas (TCGA) database
(A) According to RNA-seq data from the TCGA database, patients with serous carcinoma (SC) were divided into two groups according to whether the survival time exceeded 1000d. Hierarchical clustering analysis of candidate genes related to early and advanced stage of disease. (B) The volcano plot was made based on RNA-seq data in the TCGA database, in which the X-axis represents the folding rate of log2-transformed. The Y-axis represents the adjusted P value of log10-transformed. The red dots represent DEGs based on fold changes greater than 2. Herein, the volcano plot displays the different genes when comparing patients at early stage (I and II) and advanced stage (III and IV). (C) Through a volcanic plot analysis, 245 upregulated and 38 downregulated genes were found. (D) TCGA database of candidate genes associated with FIGO stage heatmap. (E) DEGs were selected using a volcano plot when comparing 160 samples of long OS and 223 samples of short OS (median OS was 869 days) from the TCGA database. (F) The volcano plot indicated 15 upregulated and 59 downregulated genes. (G)Heatmap of candidate genes related to survival in the TCGA database. (H) The Venn diagram representing the distribution of DEGs in different groups. Finally, two differentially expressed genes were screened out.

Figure 2

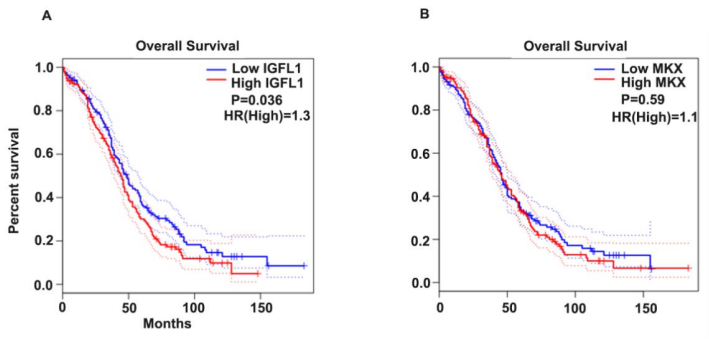

Figure 2. TCGA database screening out two genes(IGFL1 and MKX) and the Kaplan Meier curve

GO and KEGG analyses

GO enrichment can analyze the function of the selected DEG from different biological processes, cell components and molecular functions to further understand the biological role of IGFL1 in the occurrence and development of serous ovarian carcinoma. The results demonstrated that IGFL1 was involved in a variety of biological processes, including the regulation of cell differentiation, apoptotic process , immune system process , positive regulation of cell population proliferation , cell migration , MAPK cascade , positive regulation of MAPK cascade , cell growth , MAP kinase activity , positive regulation of epithelial cell proliferation , activation of MAPK activity , phosphatidylinositol 3-kinase signaling , and positive regulation of activated T cell proliferation (Figure 3 A). It can be inferred that IGFL1 may affect the development of serous ovarian carcinoma by regulating the immune

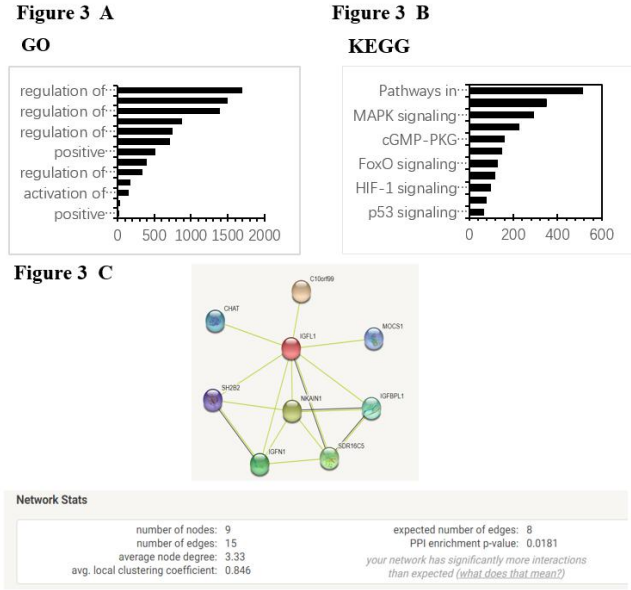

Figure 3. IGFL1 function analysis

(A) GO analysis of IGFL1. (B) KEGG analysis of IGFL1. (C) Protein - protein interaction (PPI) networks showing the interaction between DEGs. Each node represents a gene, the mutual relationship between the edges.

Meanwhile, in order to explore the biological pathway related to IGFL1 in serous ovarian carcinoma, a pathway analysis was conducted. The results showed that the main pathways involved in IGFL1 activity included the p53 signaling pathway，EGFR tyrosine kinase inhibitor resistance， HIF-1 signaling pathway，AMPK signaling pathway，FoxO signaling pathway， mTOR signaling pathway， cGMP-PKG signaling pathway， Ras signaling pathway， MAPK signaling pathway， PI3K-Akt signaling pathway ，and Pathways in cancer (Figure 3 B).

Protein-protein interaction (PPI) network

A PPI network of IGFL1 was then constructed to define the interactions between IGFL1 and other hub proteins. The PPI network was built based on the information in the STRING protein query in the public database. The PPI network consisted of 9 interacting nodes with 15 edges. Accordingly, it was concluded that C10orf99 、 MOCS1 、 IGFBPL1 SDR16C5 、IGFN1 、NKAIN1 、SH2B2 、CHAT and IGFL1 were closely linked (Figure 3 C).

The expression of IGFL1 in human protein map and Immunohistochemistry

The human protein atlas database contains a plethora of detailed information, including 20 common tumor types and 44 different normal tissues and organs. In addition, the expression of immunohistochemical proteins in different tissues was also inquired and downloaded. Therefore, the corresponding information may be downloaded from the human proteomic database to confirm the histological level of IGFL1 to further understand and explore the clinical significance of IGFL1 expression in patients with serous ovarian carcinoma (Figure 4). The results demonstrated that IGFL1 expression level was associated with the prognosis of serous ovarian carcinoma. Compared to patients with low IGFL1 expression, patients with high IGFL1 expression had a poorer prognosis.

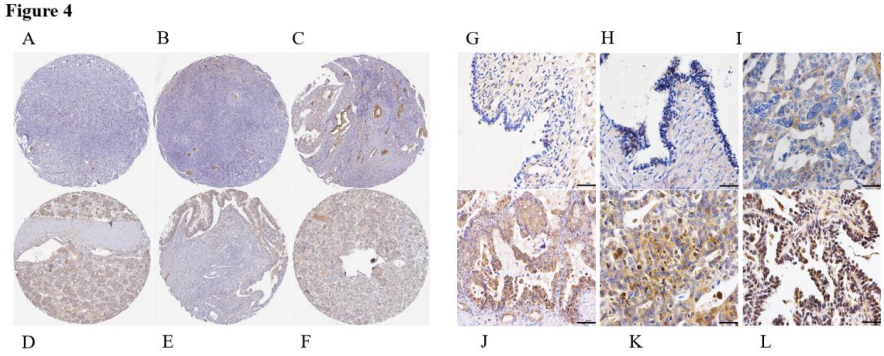

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Figure 4. Expression of IGFL1 in human protein atlas in SC patient tissues

(A)(B)(C) Expression of IGFL1 in normal tissues in the Human Protein Atlas. (D)(E)(F) Expression of IGFL1 in serous ovarian carcinoma samples in the Human Protein Atlas. (G)(H) Expression of IGFL1 in benign tumor tissues by Immunohistochemistry. (I)(J) Expression of IGFL1 in early stage serous ovarian carcinoma tissues by Immunohistochemistry. (K)(L) Expression of IGFL1 in advanced stage serous ovarian carcinoma tissues by Immunohistochemistry.

Verification of clinical patients

In order to further clarify the clinical significance of IGFL1 expression, the tissue samples of 36 patients with serous ovarian carcinoma were analyzed by real-time quantitative PCR and immunohistochemistry. The patients' detailed clinical data included their age, subdivision, longest dimension, lymph node metastasis, FIGO stage, sample type, IGFL1 expression, Histological types and vital status. IGFL1 was observed to be highly expressed in advanced serous ovarian carcinoma (Figure 5 A), and high expression of IGFL1 in serous ovarian carcinoma was found to be associated with poor prognosis(P=0.0022, Figure 5 B). Afterward, 36 serous ovarian carcinoma samples were divided into "high" and "low" according to the median IGFL1 level of 0.149717. Here, IGFL1 was found to be closely associated with age (P = 0.019), FIGO stage (P = 0.026), and histological type (P ≤ 0.001) . Therefore, IGFL1 can be used as a prognostic indicator for patients with serous ovarian carcinoma.

Figure 5

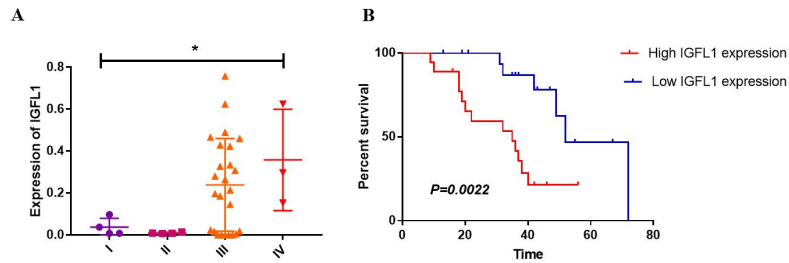

Figure 5. The relationship between the expression of L1 and the clinical characteristics and total survival rate (OS) of SC patients.

(A)Different groups' FIGO staging and IGFL1 mRNA expression level. (B) IGFL1 expresses overall survival in patients with clinical effect (p=0.0022, n = 36).

The logit model was analyzed, and the univariate analysis showed that IGFL1 overexpression (or = 1.530, P = 0.031), age (or = 5.200, P = 0.023), stage (or = 6.400, P = 0.036) and histological type (or = 44.200, P = 0.001) were associated with the progression of serous ovarian carcinoma. According to the multivariate analysis, histological type (or = 84.316, P = 0.002) and high expression of IGFL1 (or = 2.360, P = 0.005) were found to be independent predictors of tumor progression in patients with serous ovarian carcinoma . These results suggest that IGFL1 expression plays an important role in predicting tumor progression in patients suffering from serous ovarian carcinoma.

Discussion

Although much attention is given toward the research of advanced serous ovarian carcinoma, the prognosis of advanced serous ovarian carcinoma has not been greatly improved due to certain factors, such as poor early screening methods and low early disease diagnosis rate. So far, the treatment of ovarian cancer mainly revolves around surgery and chemotherapy [19]. However, serous ovarian carcinoma is increasingly resistant to chemotherapy and is also prone to recurrence. The death risk of patients with serous epithelial carcinoma is 1.7 times higher than that of patients with other histological types [20]. Therefore, an effective index is urgently required in order to predict the prognosis of serous ovarian carcinoma and provide a new direction in treatment. Due to cancer research, high-throughput technology, which is an important tool in life science research, has been widely used to diagnose early cancer as well as evaluate tumor prognosis [21]. By conducting a transcriptome analysis, many new tumor markers and therapeutic targets have been identified. However, biomarkers related to the prognosis of serous ovarian carcinoma have not been fully studied. Therefore, screening

and identifying new indicators related to the prognosis of serous ovarian carcinoma will help predict the survival time of patients with serous ovarian carcinoma and prolong their survival period.

The gene expression data of serous ovarian carcinoma from the TCGA database were analyzed, and a gene related to serous ovarian carcinoma prognosis was screened. In this paper, the main role of IGFL1 was analyzed and verified in the prognosis of patients suffering from serous ovarian carcinoma. First, the TCGA database was used to screen the mRNA expression profiles of early and late, long OS group and short OS group. Accordingly, IGFL1 and MKX were found to be potential indicators of poor prognosis in view of the relationship between mRNA expression and prognosis. Moreover, Kaplan-Meier survival analysis showed that the differential gene IGFL1 was significant in tissue expression (P = 0.036), therefore, IGFL1 was selected for further analysis. In order to further evaluate the overall role of IGFL1 in tumor progression, GO and GSEA analyses were performed, which demonstrated that the gene was related to the following regulatory pathways: cell differentiation, apoptotic process, immune system process， positive regulation of cell population proliferation, cell migration， MAPK cascade， positive regulation of MAPK cascade， cell growth， MAP kinase activity，positive regulation of epithelial cell proliferation， activation of MAPK activity， phosphatidylinositol 3-kinase signaling，and positive regulation of activated T cell proliferation. In view of these findings, it can be inferred that IGFL1 may affect the development of serous ovarian carcinoma by regulating the immune system. Finally, the collected clinical samples were analyzed and verified, in which patients with high IGFL1 expression were found to have a poorer survival rate. In addition, IGFL1 expression was observed to be higher in patients at an advanced stage. Therefore, the high expression of IGFL1 serves as the reason for the poor prognosis of serous ovarian carcinoma.

In an article on the study of inflammatory skin and IGFL gene expression, the expression of mIGFL was noted to be further upregulated during the inflammatory response of the skin as well as following skin injury. Specifically, *in vitro*, tumor necrosis factor alpha enhances the expression of IGFL1 [16]. IGFL may promote the development of serous ovarian carcinoma through the inflammatory response pathway. Previous studies have also shown that inflammation is a high-risk factor for ovarian cancer, and the inflammatory repair of ovulation wounds helps increase the spread of cancer cells to periovarian tissues [22]. Since the IGFL gene has structural homology with the IGF family, it is speculated that IGFL and the IGF family have similar effects [15]. Members of the IGF family are known to be involved in major biological processes: metabolic control, growth and reproduction [23]. IGF1 plays a vital role in metabolism and proliferation, and IGF1 is a survival agent and growth factor for a variety of normal and malignant cells [24]. Under certain circumstances, the anti-apoptotic effect of IGF1 can promote cancer cell growth or adipocyte expansion [24]. The mechanism of IGF1 in tumors is as follows： IGF-1 activates phosphatidylinositol-3-kinase (PI-3K)/Akt signaling cascade and mitogen-activated protein kinase (MAPK). Activated Akt can suppress apoptosis by inhibiting the activation of the interleukin-1β-converting enzyme [25]. Over-activation of PI-3K/Akt triggers NF-κB signaling and accelerates the aging process while impairment of PI-3K/Akt signaling leads to activation of FoxO factors, extending lifespan [26]. In this regard, studies have shown that high expression of IGF1 is associated with a high risk of colorectal cancer [27], and overexpression of IGF1 or IGF1R leads to the migration of cancer cells [28]. In addition, studies have shown that the overexpression of IGF1 may serve as the cause of ovarian cancer with tumor recurrence [29]. Elevated serum IGF1 levels were also found to double the risk of prostate cancer [30]. Furthermore, IGFL1 may be involved in tumor regulation in the same way as IGF1.

Age, FIGO staging, ascites cytology, histological type and grade, scope of surgery and number of residual tumors are all known important independent prognostic indicators for ovarian cancer [20]. In this study, the level of IGFL1mRNA expression in serous ovarian carcinoma tissue was found to be negatively correlated with the prognosis of serous ovarian carcinoma. The higher the expression of IGFL1, the worse the prognosis of serous ovarian carcinoma. This is the first study to show that IGFL1 has an adverse effect on serous ovarian carcinoma through bioinformatics screening. The detection of this marker can be of great help in the prognosis of SC and to prolong the survival period of patients, which may become an ideal target for the treatment of serous ovarian carcinoma.

Conclusion

Due to the lack of clinical sample verification, this study has certain limitations. This study has not yet

discussed the pathogenic mechanism associated with the obtaining findings. In the future, additional *in vivo* and *in vitro* experiments are required for further exploration. In short, the prognostic value of IGFL1 for serous ovarian carcinoma patients should be given focused attention. High IGFL1 expression has been shown to be a clear sign in patients with poor prognosis.

Data Availability

All data generated or analyzed during this study are included in this published article.Raw and processed data are available upon request.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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