LNPEP as a Prognostic-Related Biomarker in Ovarian Cancer Correlating with Immune Infiltrates

Qian Ma (fccmaq@zzu.edu.cn)  
Zhengzhou University

Lingyi Che  
the First Affiliated Hospital of Zhengzhou University

Wenwen Wang  
the First Affiliated Hospital of Zhengzhou University

Gailing Li  
the First Affiliated Hospital of Zhengzhou University

Ying Zhang  
the First Affiliated Hospital of Zhengzhou University

Yibing Chen  
the First Affiliated Hospital of Zhengzhou University

Zhuoyu Gu  
the First Affiliated Hospital of Zhengzhou University

Xiaoqin Song  
the First Affiliated Hospital of Zhengzhou University

Lei Chang  
the First Affiliated Hospital of Zhengzhou University

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Abstract

Background: Leucyl And Cystinyl Aminopeptidase (LNPEP) encodes a zinc-dependent aminopeptidase, which has been proved to participate in the vesicle-mediated transport and class I major histocompatibility complex-mediated antigen processing and presentation. However, the function of LNPEP in the tumor immune microenvironment of ovarian cancer (OV) and its potential molecular mechanisms have not been elucidated.

Material and Methods: To analyze the expression profile, prognostic value, and immune infiltration of LNPEP, bioinformatics web resources, including Gene Expression Profiling Interactive Analysis Gent2, HPA, Cbiportal, Kaplan–Meier Plotter, and tumor immune estimation resource, were employed. The related genes of LNPEP in OV were mined by TISIDB and LinkedOmics database. The protein-protein interaction network involving LNPEP was constructed by GeneMANIA and STRING database. Additionally, further studies of pathway and LNPEP expression were conducted through GSEA. The protein levels of LNPEP were validated by western-blot assay and immunohistochemistry.

Results: The mRNA expression of LNPEP was noticeably down-regulated in OV than its expression in para-cancer tissues, contrary to the protein level. Importantly, high LNPEP expression was associated with poor prognosis in patients with OV. Furthermore, Cox regression analysis showed that LNPEP was an independent prognostic factor in OV. From the GO and KEGG pathway analyses, it was observed that the co-expressed genes of LNPEP were mainly related to a variety of immune-related pathways, including Th1 and Th2 cell differentiation, Th17 cell differentiation, and immunoregulatory interaction between a lymphoid and a non-lymphoid cell. Our data also demonstrated that LNPEP expression was strongly correlated with immune infiltration rates, immunomodulators, chemokine, and chemokine receptors.

Conclusions: We identified and established a prognostic signature of immune-related LNPEP in OV, which will be important for predicting the prognosis of clinical trials and may become a new therapeutic target for immunological research and potential prognostic biomarker in OV.

Introduction

Ovarian cancer (OV), the most fatal gynecological cancer, is one of the most common causes of cancer-related deaths among women[1, 2]. Despite the development of significant advancements to facilitate early diagnosis and treatment of OV, the 5-year survival rate still falls only around 47%[3]. OV has been termed the “silent killers” of women[4]. As a result of insufficient specific clinical manifestations and effective early detection, the majority of patients suffer from the late intermediate local stage[5]. About 70% of OV patients are diagnosed with advanced-stage OV (III/IV) at the time of presentation, and most often, they have suffered from wild metastasis throughout the abdominal cavity with ascites, lymph node metastasis, and peritoneal dissemination[6, 7]. Currently, there are numerous therapeutic interventions for patients with OV, including treatments that bypass surgery, chemotherapy, radiation, targeted therapy, immunotherapy, and combination strategies[8]. Despite the recent development of many effective
therapies, resistance to chemotherapy and tumor recurrence remain the main clinical challenges[9]. Therefore, it has become pertinent to develop novel therapeutic modalities aimed at improving the prognosis of patients with OV.

LNPEP (Leucyl And Cystinyl Aminopeptidase) is a protein-coding gene, it is located on chromosome 5q15 in the human genome. It encodes a zinc-dependent aminopeptidase, which has been proven to cleave natural peptide hormones in vitro including vasopressin, oxytocin, Lys-bradykinin, Ang-III, dynorphin A, Met-enkephalin, and other peptide hormones[10, 11]. LNPEP is a Single-pass type II membrane protein that localizes to the cell membrane. The protein is contained in intracellular vesicles together with the insulin-responsive glucose transporter GLUT4. Its transfer to the cell surface happens under the action of insulin and/or oxytocin[12]. According to a recent report, LNPEP was observed to promote inflammatory gene expression by activating the Inhibitor of the Nuclear Factor Kappa-B pathway. According to recent reports, the LNPEP gene is also related to the renin-angiotensin system and plays a pivotal role in multiple disorders, including cardiovascular disease, gestational diabetes insipidus, placental trophoblastic tumor, and diabetes mellitus[13, 14]. However, there is no systematic study that reports the expression levels of LNPEP and its implications in the prognosis and progression of OV. Furthermore, the associations of LNPEP with the tumor immune microenvironment (TME) in OV have not been determined.

In this study, we used comprehensive bioinformatics and experimental methods to assess the expression and prognostic effect of LNPEP in OV. From our investigation, it was discovered that LNPEP is significantly dysregulated in OV, and its expression is associated with the prognosis of patients with OV. LNPEP expression was correlated with tumor-infiltrating immune cells and immuno-modulators in OV. Furthermore, the possibility of using LNPEP as a potential therapeutic target in the treatment of OV was also explored. Our study provided novel insights into the functional role of LNPEP in OV, indicating a prognostic biomarker whereby LNPEP affects tumor immune microenvironment, as well as tumor immunotherapy for patients with OV.

Materials and Methods

Patients and Ethics Approval

A total of 60 surgically-resected human OV specimens and 40 normal ovary tissue were obtained from the Obstetrics and Gynecology Department of the first affiliated hospital of Zhengzhou University between 2019 and 2021. All tissues were immediately frozen in liquid nitrogen, stored at -80°C after resection. The current study was approved by the Ethics Committees of the first affiliated hospital of Zhengzhou University before the use of the clinical information for research purposes. All patients were duly informed of all procedures involved, and they all signed a consent form for scientific purposes. All study procedures were implemented under the ethical standards of the Helsinki Declaration.

Western Blot
Lysis of the clinical samples was conducted with RIPA lysis buffer including protease and phosphatase inhibitor for 10 min under the ice. Afterward, centrifugation was performed at 12,000×g for 10 min at 4°C. Then, the supernatants were collected and quantified based on the BCA protein detection kit. PVDF membranes were then blocked in 5% bovine serum albumin (BSA) and incubated with LNPEP antibodies (BOSTER, A05092-1) at 4°C overnight. Subsequently, after incubation with HRP conjugated secondary antibody for 45 min at room temperature, the image signals were detected with a chemiluminescence reagent (Thermofisher, WP20005).

**The Cancer Genome Atlas (TCGA) Database Analysis**

All original data were downloaded from TCGA (https://cancergenome.nih.gov/). R 3.2.3 was performed to integrate the original data and analyze the difference by the website database. The dichotomy method was used to analyze the prognostic difference of the LNPEP gene expression group in 33 kinds of tumors. To explore the types of cancer that are significantly associated with LNPEP gene expression, we made use of univariate regression analysis to analyze the prognosis of diverse tumors, including overall survival (OS), disease-specific survival (DSS), process-free intervention (PFI). A survival curve was drawn, and the selected cancer types were utilized for further study and analysis.

**Gent2 Database Analysis**

GENT2 (http://gent2.appex.kr/gent2/), an updated gene expression database for normal and tumor tissues, supplied a landscape of gene expression in different types of cancer. Data sourced from about 49,000 pan-cancer individual samples were analyzed across more than 30 different cancer types[15].

**GEPIA and Ge-mini**

Gene Expression Profiling Interactive Analysis (GEPIA), a web resource to obtain the customizable functionalities and predictive analytics based on TCGA and GTEx data, including differential expression analysis, survival analysis, correlation analysis [16]. In our study, we used “single-gene analysis” in GEPIA to evaluate the mRNA expression levels of LNPEP in OV tissues, compared with normal tissues by Student’s t-test. In addition, GE-mini is a vital interactive function and multifunctional visualization instrument that calculates and analyzes gene expression data based on TCGA and GTEx databases. In the study, we used GE-mini to verify the mRNA expression of LNPEP in OV tissues. *P* < 0.05 was considered statistically significant[17].

**BioGPS Database**

BioGPS database (http://biogps.org), a free extensible and customizable gene annotation portal, including transcript expression and phenotypic characteristics, was performed to identify the alternation of the expression of LNPEP in different cancer and paired normal cell lines[18]. Microarray data was performed based on the GeneAtlas U133A and NCI on U133A[19]. One-way ANOVA was used to evaluate the expression of LNPEP in various types of cancer cell lines and normal cell lines.

**TISIDB Database Analysis**
TISIDB database (http://cis.hku.hk/TISIDB), is an online integrated web portal used for tumor and immune system interaction analysis. It collects abundant heterogeneous data. Here, TISIDB provides us with related information for LNPEP with immunomodulators and chemokines. Differences with a p-value < 0.05 were considered to be statistically significant[20].

**Cox Regression Analysis and Kapan-Meier Survival Analysis**

Univariate and multivariate cox regression analysis was used to evaluate the association between LNPEP expression and overall survival (OS), disease-specific survival (DSS), and progression-free interval (PFI) of patients based on the TCGA databases. The patients’ cohorts were divided into high and low LNPEP expression groups with the maximum-electoral log-rank analysis. P-value, hazard ratio (HR), and 95% confidence intervals (CI) were tested.

**Human Protein Atlas Dataset**

LNPEP were analyzed at the protein levels in the Human Protein Atlas (HPA) dataset (https://www.proteinatlas.org/), which contained immunohistochemistry staining images of 373 clinical patients with OV and their survival information with 143 alive and 230 dead patients[21, 22].

**LinkedOmics Database Analysis**

The LinkedOmics database (http://www.linkedomics.org/login.php) is a visual platform that analyses multi-dimensional datasets based on TCGA datasets. LNPEP co-expression was analyzed statistically using Pearson’s correlation coefficient and showed the results via volcano plots, heat maps, and scatter plots in the LinkFinder module. Function module of LinkedOmics revealed an analysis of Gene Ontology biological process (GO), KEGG pathways analysis by using the gene set enrichment analysis (GSEA)[23].

**Tumor Immune Estimation Resource Analysis**

Tumor immune estimation resource (TIMER) (https://cistrome.shinyapps.io/timer/) is a public web server that systematically assesses tumor infiltration immune cell (TIICs) depended on gene expression across 21 cancer types from TCGA[24]. We mainly explored LNPEP expression in pan-cancer and the correlation between gene expression and abundance of immune cell infiltration, including B cells, CD8 + T cells, CD4 + T cells, macrophages, neutrophils, and dendritic cells (DCs) in the TME of human cancer via the SangerBox website. Then, compared with purity in OV, we also performed the correlation modules between RNA-seq expression profile data of LNPEP in OV and immune cells marker including B cells, CD8 + T cells, CD4 + T cells, macrophage, monocyte, tumor-associated macrophages (TAM), M1 macrophage, M2 macrophage, neutrophils, dendritic cell, follicular helper T cells (Tfh) cells, T-helper 1 (Th1) cells, Type 2 helper T cell (Th2), T-helper 9 (Th9) cells, T-helper 17 (Th17) cells, T-helper 22 (Th22) cells, Tregs cells, exhausted T cells.

**Mutation Analysis Database**

The mutation frequency and types of LNPEP in OV were utilized to evaluate using the cBioPortal database (http://www.cbioproject.org/) [25] and Catalogue of Somatic Mutations in Cancer (COSMIC)
database ([http://cancer.sanger.ac.uk](http://cancer.sanger.ac.uk))[26].

**GeneMANIA and STRING web analysis**

GeneMANIA ([http://www.genemania.org/](http://www.genemania.org/)) web database performed interaction patterns of differentially regulated genes and showed gene function, protein and genetic interaction, related pathway prediction, co-expression analysis, colocalization, and protein domain similarity[27]. STRING web-based network tools ([https://string-db.org/](https://string-db.org/)), which integrates computational methods to predict the gene functions, were utilized to infer the functional interaction under the direct interaction to score nodes[28, 29].

**Statistical Analysis**

All data of this study were statistically analyzed with R software 3.6.1, and GraphPad Prism version 8.0. The measurement data were summarized as the mean ± SD. Differential expression levels of LNPEP mRNA between the OV tissues and the adjacent normal tissues were evaluated by using Student’s t-test. A two-tailed \( p \)-value < 0.05 was considered as the threshold to identify statistically significant.

Correlations between LNPEP expression and clinicopathological characteristics were performed by Pearson’s Chi-squared test. Univariate and multivariable analyses were involved in using the Cox proportional hazards regression models.

**Results**

**the mRNA expression value of LNPEP in different cancer types**

Firstly, we performed pan-cancer analysis derived from TCGA by adopting Timer2.0 and Gent2 to estimate the expression pattern of LNPEP. Compared with normal tissues, LNPEP was displayed to be upregulated in the liver, bile duct, whereas down-regulated was revealed in the breast, central nervous system (CNS), kidney, lung, prostate, rectum, skin according to Timer analysis (Fig. 1A). Moreover, we estimated LNPEP expression data profiled of 72 paired cancer compared to normal tissues by utilizing HG-U133 microarray (GPL570 platform) of Gent2 database (Fig. 1B). Thus, it can be observed from the results LNPEP was up-regulated in several cancer cases concerning adipose tissue, adrenal gland, cervix, endometrium, esophagus, kidney, oral, liver, pancreas, pharynx, small intestine, and vulva. Conversely, down-regulation of LNPEP was reported in the field of blood, colon, head and neck, lung, muscle, ovary, prostate, skin, teeth, and vagina (Fig. 1C and D). To evaluate the correlation between LNPEP gene expression and the patient prognosis in 33 tumors, we used gene expression profile data and single-factor regression analysis to draw forest plots. In addition, OS showed that LNPEP was notably correlated to the prognosis of KIRC \( (p < 0.001) \), OV \( (p = 0.006) \), and READ \( (p = 0.042) \) (Fig. 1E). DSS displayed that LNPEP was markedly correlated with the prognosis of KIRC \( (p < 0.001) \) and OV \( (p = 0.009) \) (Fig. 1F). PFI reflected that LNPEP was associated with the prognosis of KIRC \( (p < 0.001) \), PCPG \( (p = 0.024) \), and SKCM \( (p = 0.024) \) (Fig. 1G). The expression of LNPEP in different cancer cell lines and normal tissues was explored via the BioGPS database. Ten cancer cell lines with the highest LNPEP expression level are
displayed in Figure S1A. In normal cells, the LNPEP expression level was higher in immune cells (Figure S1B). Detailed information is shown in Supplementary Tables S3 and S4. The above results suggest that LNPEP might participate in the process of immune regulation.

**Expression of LNPEP and its association with Clinico-pathological Characteristics in Patients with OV**

Next, the mRNA level of LNPEP was down-regulated in OV compared to normal ovary tissues which were tested using the data sourced from the available database. Based on the GEPIA with the criterion of $|\text{Log2FC}|>2$ and $p<0.05$, LNPEP has significantly low expression in patients with OV (Fig. 2A). Similarly, we also found that LNPEP downregulation arose from the GE-mini database (Fig. 2B). Subsequently, we analyzed the diagnostic values of LNPEP in OSC with ROC curves. Based on the Xiantao Xueshu web tool (https://www.xiantao.love/products), we performed a plot of the area under the curve (AUC) of LNPEP which was estimated to be 0.859 (Fig. 2C). From the high AUC value derived, it was speculated that LNPEP could have a greater potential to be a diagnostic biomarker for patients with OV. As described above, the mRNA levels of LNPEP were downregulated in OV, so we proceeded to test the protein level of LNPEP in OV. The protein levels for LNPEP in OV and normal ovary tissue were subjected to western blot analysis. The result showed that LNPEP expression was upregulated in OV, as compared to the mRNA levels (Fig. 2D). In the HPA dataset, the IHC staining data revealed that LNPEP was mainly localized in the cytoplasmic/membranous tissues and had it showed higher expressions in OV tissues (Figs. 2E, S3A, and S3B). To validate the above findings and investigate the clinicopathological roles and distribution of LNPEP expression in OV, immunohistochemical analysis of the 60 paraffin-embedded OV tissue blocks was performed. Representative immunohistochemical staining of LNPEP in OSC is illustrated in Fig. 2F. In addition, LNPEP expression differed in different immune subtypes of OV. Meanwhile, a significant correlation between LNPEP expression and molecular subtypes existed in OV (Figure S2). To further evaluate the prognostic value of LNPEP expression, univariate and multivariate Cox regression analysis was performed based on the OS, DSS, and PFI of patients with OV based on TCGA. As shown in Tables 1, 2, and 3, LNPEP expression, age class, race, FIGO stage, primary therapy outcome, tumor residual, and tumor status were termed as independent prognostic factors for the determination of the OS, DSS, and PFI of patients with OV, respectively.
### Table 1
Univariate and multivariate Cox proportional hazards analysis for overall survival in the OV cohort.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>HR (95%CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>LNPEP:high vs low</td>
<td>1.441 (1.111–1.870)</td>
<td><strong>0.006</strong></td>
</tr>
<tr>
<td>FIGO stage: I + II vs III + IV</td>
<td>1.981(1.419–2.764)</td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td>Primary therapy outcome: PD + SD vs PR + CR</td>
<td>0.301 (0.204–0.444)</td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td>Age: &lt;=60 vs &gt; 60</td>
<td>1.355 (1.046–1.754)</td>
<td><strong>0.021</strong></td>
</tr>
<tr>
<td>Tumor residual: NRD vs RD</td>
<td>2.313 (1.486–3.599)</td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td>Tumor status: Tumor free vs With tumor</td>
<td>9.576 (4.476–20.486)</td>
<td><strong>&lt; 0.001</strong></td>
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</tbody>
</table>

### Table 2
Univariate and multivariate Cox proportional hazards analysis of disease-specific survival in OV cohort.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th>Multivariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95%CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>LNPEP:high vs low</td>
<td>1.456 (1.099–1.928)</td>
<td><strong>0.009</strong></td>
</tr>
<tr>
<td>Race: Asian Black + African American vs White</td>
<td>0.592 (0.370–0.946)</td>
<td><strong>0.028</strong></td>
</tr>
<tr>
<td>Primary therapy outcome: PD + SD vs PR + CR</td>
<td>0.294 (0.198–0.436)</td>
<td><strong>&lt; 0.001</strong></td>
</tr>
<tr>
<td>Tumor residual: NRD vs RD</td>
<td>2.572 (1.580–4.187)</td>
<td><strong>&lt; 0.001</strong></td>
</tr>
</tbody>
</table>
### Table 3
Univariate and multivariate Cox proportional hazards analysis of progression-free interval in OV cohort.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Univariate analysis</th>
<th></th>
<th></th>
<th>Multivariate analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LNPEP: high vs low</td>
<td>1.219 (0.962–1.544)</td>
<td>0.101</td>
<td></td>
<td>HR (95%CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>FIGO stage: I + II vs III + IV</td>
<td>1.573 (0.918–2.694)</td>
<td>0.099</td>
<td></td>
<td>HR (95%CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Primary therapy outcome: PD + SD vs PR + CR</td>
<td>0.457 (0.325–0.642)</td>
<td>&lt;0.001</td>
<td></td>
<td>0.752 (0.521–1.086)</td>
<td>0.128</td>
</tr>
<tr>
<td>Tumor residual: NRD vs RD</td>
<td>1.695 (1.219–2.358)</td>
<td>0.002</td>
<td></td>
<td>0.885 (0.594–1.320)</td>
<td>0.550</td>
</tr>
<tr>
<td>Tumor status: Tumor free vs With tumor</td>
<td>10.045 (5.758–17.526)</td>
<td>&lt;0.001</td>
<td></td>
<td>12.988 (6.395–26.377)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### Genetic Alterations of LNPEP in OV

Genomic alterations induce changes in gene expression. We explored the mutation frequency of LNPEP using the cBioPortal website. Five datasets (MSK, MSKCC, TCGA Pan-Cancer Atlas, TCGA Firehose legacy, and TCGA nature), which included 1,737 samples, were selected for analysis. The somatic mutation frequency of LNPEP in OV was observed to be 3%. It mainly consisted of missense mutations and synonymous substitution (Fig. 3A). We observed that OSC patients possessed a high frequency of gene alterations (Figure S4). In addition, the mutation types of LNPEP were further evaluated in the COSMIC database. Two pie charts were used to show the distribution of mutation types clearly (Figs. 3B and 3C). Missense substitutions occurred in approximately 34.47% of the samples, while synonymous substitutions occurred in 11.69% of the samples. Lastly, nonsense substitution occurred in 4.4% of the samples (Fig. 3B). The substitution mutations mainly occurred at C > T (27.08%), followed by G > A (16%), G > T (11.38%), and A > G (10.15%) (Fig. 3C). In addition, altered LNPEP had a significant correlation with the OS of OV (p < 0.05) (Fig. 3D).

### The Prognostic and Diagnostic Values of LNPEP in Patients with OV

Survival time was determined from the date of primary tumor surgery to the time of death or last follow-up. To assess the effect of LNPEP expression on the OS, DSS, and PFI of patients with OV, and to explore the prognostic value of LNPEP in OV, survival analysis was performed based on information from the TCGA database. Results displayed that the upregulation of LNPEP expression was associated with poor OS (HR = 1.44 (1.11–1.87), log-rank p = 0.006), DSS (HR = 1.46 (1.10–1.93), log-rank p = 0.009), PFI (HR = 1.36 (1.07–1.73), log-rank p = 0.011) of patients with OV (Fig. 4A–C). Furthermore, high LNPEP expression was also associated with poor OS/DSS/PFI of patients with OV who were white, high grade
histologic, anatomic neoplasm subdivision (bilateral), age, tumor residual (NRD and RD), and tumor status (with tumor and tumor-free) (Figures S5A–I, S6A–I, and S7A–I), but not those patients who were under the anatomic neoplasm subdivision (unilateral) (Figures S5J, S6J, and S7J). In conclusion, high LNPEP expression was associated with a poor prognosis of patients with OV.

Relationship between LNPEP Expression and Immune Cell Infiltration in Pan-Cancer Patients

Tumor-infiltrating lymphocytes are self-governed predictors of tumor sentinel lymph node status and survival rate response to therapies. We investigated the relationship between LNPEP expression and immune infiltration using TIMER2.0. The correlation coefficients between LNPEP expression and the abundances of seven immune infiltrates (CD8 + T cells, CD4 + T cells, B cells, neutrophils, macrophages, monocytes, and dendritic cells) were analyzed using Spearman tests (tumor purity adjusted). To explore the pan-cancer correlation between LNPEP expression and immune infiltration, we first evaluated the abundance of immune cell infiltration. As shown in Fig. 5A–G, the profiles of LNPEP associated with various immune infiltration displayed that it was positively correlated with immune cell infiltration levels of neutrophils. However, the data indicated that it was contradicted in CD8 + T cells, CD4 + T cells, B cells, macrophages, monocyte, and DCs. Correlation analyses using data from published work, which evaluated the 24 immune cells, showed that LNPEP was positively correlated with the infiltration levels of mast cells, T cells, T helper cells, Tcm, Th2 cells, whereas it was negatively correlated with those of NK CD56 bright cells (Fig. 5H).

LNPEP is Correlated with Immune Infiltration in OV

Afterward, we then performed a correlation analysis between LNPEP and tumor-infiltrating immune cells to evaluate the immunotherapy effect. Tumor purity acted as a critical factor, which effected the analysis of immune infiltration based on the genomic approach. We found LNPEP expression had a slightly negative correlation with tumor purity (r=-0.149, p = 1.81E-02). In addition, LNPEP expression had significant positive correlations with five immune infiltrates, including CD8 + T cells (r = 0.274, p = 1.17E-05), B cells (r = 0.161, p = 1.09E-02), macrophage (r = 0.274, p = 1.16E-05), monocytes (r = 0.227, p = 3.13E-04), dendritic cells (r = 0.128, p = 4.39E-02) (Fig. 6A). In addition, we also comprehensively clarified the correlations between LNPEP expression and related immune cell gene markers. Correlation coefficients were adjusted for tumor purity. The genetic markers of immune cells were used to analyze and identify the immune cells, including CD8 + T cells, general T cells, B cells, monocytes, M1 and M2 macrophages, neutrophils, dendritic cells, Th1, Th2, Tfh, Th17, Treg and T cell exhaustion (Table 4). Interestingly, LNPEP expression was critically correlated with the expression of markers of specific immune cells, such as CD8 + T cells marker, FCGR3B (r = 0.208, p < 0.001), SIGLEC5 (r = 0.248, p < 0.001), FPR1 (r = 0.146, p < 0.05); T cells marker, CD2 (r = 0.133, p < 0.05); B cells marker, CD19 (r = 0.145, p < 0.05); monocytes marker, CD115 (r = 0.162, p < 0.05) and CD86 (r = 0.193, p < 0.01); TAM marker, CD68 (r = 0.23, p < 0.001), IL10 (r = 0.232, p < 0.001); M1 macrophages marker, PTGS2 (r = 0.175, p < 0.01), CD163 (r = 0.267, p < 0.001), IRF5 (r =
0.146, \( p < 0.05 \); M2 Macrophages marker, VSIG4 \( (r = 0.163, p < 0.05) \), MS4A4A \( (r = 0.17, p < 0.01) \); neutrophils marker, CD11b \( (r = 0.198, p < 0.01) \), CCR7 \( (r = 0.185, p < 0.01) \); dendritic cells marker, ITGAM \( (r = 0.198, p < 0.01) \), and NRP1 \( (r = 0.189, p < 0.01) \). Moreover, the expression of LNPEP was associated with the expression of markers of specific subsets of T cells in OV, which included Th1 marker, TBX21 \( (r = 0.181, p < 0.01) \), STAT4 \( (r = 0.177, p < 0.01) \), STAT1 \( (r = 0.269, p < 0.001) \), tumor necrotic factor (TNF) \( (r = 0.135, p < 0.05) \); Th2 marker, STAT5A \( (r = 0.215, p < 0.001) \), QRS1 \( (r = 0.23, p < 0.001) \), STAT6 \( (r = 0.161, p < 0.01) \); Th17 marker, STAT3 \( (r = 0.395, p < 0.001) \); Treg marker, FOXP3 \( (r = 0.286, p < 0.001) \), STAT5B \( (r = 0.344, p < 0.001) \), and CCR8 \( (r = 0.195, p < 0.01) \); T cell exhaustion marker, CTLA4 \( (r = 0.152, p < 0.05) \), HAVCR2 \( (r = 0.212, p < 0.001) \), PDCD1 \( (r = 0.121, p < 0.05) \), LAG3 \( (r = 0.126, p < 0.05) \), GAMB \( (r = 0.166, p < 0.05) \). However, LNPEP expression did not reveal any significant correlation with CD4+ T cells. LNPEP was significantly correlated with immune stimulators, such as CD80 \( (\rho = 0.217, p = 0.000136) \), IL2RA \( (\rho = 0.214, p = 0.000163) \), MICB \( (\rho = 0.207, p = 0.000265) \), and PVR \( (\rho = 0.205, p = 0.00031) \) (Fig. 6B). The expression of LNPEP was also associated with immune inhibitors, including CD274 \( (\rho = 0.148, p < 3.3E-05) \), CTLA \( (\rho = 0.148, p = 0.00933) \), IDO1 \( (\rho = 0.126, p = 0.0269) \), TGFB1 \( (\rho = 0.148, p = 0.00927) \) (Fig. 6C). LNPEP expression was significantly correlated with CCL4 \( (\rho = 0.132, p = 0.0212) \), CCL11 \( (\rho = 0.199, p = 0.00045) \), CXCL13 \( (\rho = 0.141, p = 0.0132) \), and CXCL14 \( (\rho = 0.209, p = 0.00023) \) (Fig. 6D). Meanwhile, LNPEP expression was significantly associated with chemokine receptors, including CCR1 \( (\rho = 0.175, p = 0.00211) \), CCR4 \( (\rho = 0.19, p = 0.000825) \), CCR8 \( (\rho = 0.166, p = 0.00363) \), and CXCR6 \( (\rho = 0.188, p = 0.000947) \) (Fig. 6E). In summary, comprehensive analysis indicated that LNPEP may function as an immunoregulatory factor in OV.
Table 4
Correlation analysis between LNPEP and related genes of immune cells in TIMER database.

<table>
<thead>
<tr>
<th>Description</th>
<th>Gene markers</th>
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<td>CD8 + T cell</td>
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<td>SIGLEC5</td>
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<td>FPR1</td>
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<td>**</td>
<td>0.146</td>
</tr>
<tr>
<td>T cell (general)</td>
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<tr>
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<td>CD58</td>
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<td>IL10</td>
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<td></td>
<td>CD163</td>
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<td>***</td>
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<td>IRF5</td>
<td>0.233</td>
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<td>MS4A4A</td>
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<td>CCR7</td>
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<td>BDCA-4(NRP1)</td>
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<td>Description</td>
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<td>0.188 0.181 **</td>
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<tr>
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<td>STAT4</td>
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<tr>
<td></td>
<td>STAT1</td>
<td>0.27 0.269 ***</td>
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<tr>
<td></td>
<td>TNF-a(TNF)</td>
<td>0.188 0.181 **</td>
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<td></td>
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<td>GATA3(QRSL1)</td>
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<td></td>
<td>STAT6</td>
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<td>STAT5B</td>
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<td></td>
<td>TGFB(TGFB1)</td>
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<td>T cell exhaustion</td>
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<td>TIM3 (HAVCR2)</td>
<td>0.232 0.212 ***</td>
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<tr>
<td></td>
<td>PD-1(PDCD1)</td>
<td>0.181 0.121 *</td>
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<td></td>
<td>LAG3</td>
<td>0.126 0.126 *</td>
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<td></td>
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<tr>
<td></td>
<td>GZMB</td>
<td>0.14 0.166 *</td>
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**LNPEP Co-Expression Network in OV**

The results of the co-expression pattern of LNPEP displayed that 1,914 genes were positively correlated with LNPEP, while 1,279 genes were negatively correlated with LNPEP (Fig. 7A). Heat maps showed the top 50 genes positively and negatively associated with LNPEP (Figs. 7B, C). KEGG pathway showed that co-expressed genes of LNPEP mainly participated in the ECM-receptor interaction, allograft rejection, Th1, and Th2 cell differentiation, Th17 cell differentiation, osteoclast differentiation, graft-versus-host disease, malaria, natural killer cell-mediated cytotoxicity, leishmaniasis, and TNF signaling pathway. (Fig. 7D). GO term annotation indicated enrichment in adaptive immune response, leukocyte cell-cell adhesion, T cell activation, cellular defense response, negative regulation of cell adhesion, cell adhesion mediated by integrin protein, lymphocyte-mediated immunity, leukocyte migration, regulation of cell-cell adhesion, tolerance induction, (Fig. 7E). In addition, we evaluated the pathway through which LNPEP may involve
using GSEA in OV based on TCGA. The results indicated that LNPEP was significantly associated with cell adhesion and extracellular matrix (Figure S8A and S8B). These results suggested that the LNPEP expression network significantly affected the immune microenvironment and ligand-receptor interactions in OV.

**Protein-Protein Interaction (PPI) Network Analysis for LNPEP**

An aberrant expression of LNPEP may be involved in the process of diverse cancer types. Herein, we implemented two web resources, GeneMANIA, and STRING, to investigate the PPI network associated with LNPEP. It is well known that the protein alters a wide variety of biological processes and cellular signaling regarding protein interaction and signal transduction pathway[30, 31]. GeneMANIA, as an integrated network, focuses on functional prediction and performs an interaction network analysis based on a network-based gene ranking algorithm. Meanwhile, STRING is more inclined to the physical and functional interactions of the gene set. As Fig. 8A showed, GeneMANIA supplied the PPI-associated protein, including ORC6, CMTR1, KIF3B, LMNB2, PGD, NEK2, MTHFD2, PTTG1, EZH2, NCAPH, PKMYT1, RCHY1, SRPK1, RBM38, DMRTC2, ASXL1, SF1, PLAS2, PIAS4, and PIAS1. STRING analysis provided a predicted PPI network, which shares interaction with TNKS2, TNKS, STX4, VAMP2, RAB10, RAB14, RAB8A, TBC1D4, SLC2A4, RAB28 (Fig. 8B). The PPI network stats are the number of nodes: 11; the number of edges: 32; average node degree: 5.82; local clustering coefficient: 0.913 and PPI enrichment $p < 0.05$. The related parameters predicted PLAGL2 which is involved in the progression and prognosis of cancer.

**T Cell Exhaustion Analysis**

T cell exhaustion is a common feature of chronic infections and cancers in patients. Most patients with tumors possess a large number of exhausted T cells. Herein, we evaluated the relationship between LNPEP and marker genes of exhausted T cells, immune stimulator-related genes, immune inhibitor-related genes, chemokines, and receptors. The results determined that LNPEP was positively correlated with marker genes of exhausted T cells, immune stimulator-related genes, and immune inhibitor-related genes in OV (Figs. 9A and B). Additionally, LNPEP expression was also positively correlated with chemokines and chemokine receptors, such as CCL4 and CCL11, and their receptors CCR1 and CCR4 (Figs. 9C and D).

**Discussion**

OV is one of the deadliest gynecological cancers that afflict women in the world today. All over the world, >29,000 women are diagnosed with OV, a disease that causes >184,800 deaths every year[32]. Similarly, 52,100 new cases of OV are expected in 2025, with 22,500 anticipated deaths in China alone[33]. Despite enormous advances in the early screening, diagnosis, and treatment of primary tumors (stage I + II), the metastatic spread represents a major cause of death. The 5-year relative survival rate for patients with OV is < 50% in developed countries[34]. It can be observed from the histological subtypes, that ovarian serous
carcinoma (OSC) presents the highest mortality rate, with the 5-year overall survival rate < 30%[35]. As a result of the specificity of location, OV is undetectable at the early phase. The poor prognosis of OV relates to the near-universal recurrence of tumors, despite aggressive multimodality treatment via surgical resection, radiotherapy, and chemotherapy. Consequently, there is a pressing need for effective therapeutic targets and promising prognostic biomarkers for the early diagnosis and treatment of patients with OV.

In our study, a systematic evaluation of the regulatory mechanism, and clinical significance of LNPEP in OV was conducted. Primarily, we assessed the expression and prognostic significance of LNPEP in pan-cancer and found that its expression was low in nine tumors, including BLCA, BRCA, GBM, KIRC, KIRP, LUAD, PRAD, SKCM, and THCA. In comparison, high LNPEP expression was observed in two tumors, including CHOL and LIHC. In addition, LNPEP expression was notably related to KIRC, OV, READ, PCPG, and SKCM across the OS, DSS, PFI, respectively. Furthermore, we explored the expression, mutation, prognostic value of LNPEP and identified the functional profiles of LNPEP played in OV progression and prognosis. According to the analysis of the TCGA data and RT-PCR, it was observed from the results that the mRNA levels of LNPEP were significantly downregulated in OV tissues. Subsequently, we employed the use of IHC analysis based on the HPA database and western blot technology to evaluate the protein level of LNPEP in OV. The results from this analysis were opposed to the mRNA expression levels. Interestingly, we speculated that post-translational modification, such as phosphorylation, N-glycosylated, glycosylation, and ubiquitination, altered the expression of LNPEP. However, the high expression of LNPEP predicted a poor OS, DSS, and PFI in patients with OV. These observations underpinned our hypothesis that LNPEP was related to ovarian carcinogenesis, and may function as a potential biomarker in the prognosis of OV.

Meanwhile, based on the KEGG and GO molecular function analysis, the expression of LNPEP was involved in various immune-related processes, including adaptive immune response, leukocyte cell-cell adhesion, T cell activation, lymphocyte activation involved in immune response, immune response-regulating signaling pathway, regulation of innate immune response, and type2 immune response. These immune-related processes of LNPEP served a critical regulatory role in immune responses and immune-related processes. LNPEP as a single-pass type II membrane protein is mostly present in the endosomal vesicles, where it is suspected to contribute to specific tumor antigen cross-presentation that localizes to the surface of the cell membrane as a receptor for angiotensin IV[13]. However, the role of LNPEP in the regulation of antitumor immunity, as well as its clinical significance in cancers remains unknown. Further exploration of novel immune-modulators has the potential to contribute to a better understanding of the dynamic activities in the TME. The relationships between LNPEP and immune infiltration in OV were analyzed using the TIMER and TISIDB databases. The results revealed that LNPEP expression is significantly positively correlated with immune infiltration of the immune cell, including CD8 + T cells, B cells, macrophages, neutrophils, and dendritic cells. Also, LNPEP expression positively correlated with immune-stimulators, such as CD80, IL2RA, MICB, and PVR. Additionally, the correlation between LNPEP expression and gene markers of diverse immune cells based on Timer analysis performed showed that
LNPEP expression was associated with M1, M2 macrophage cells, and functional T cells, including Th1, Th2, TfH, Th17, Treg, and exhausted T cells.

It has been observed that CD8+ T cells are the best representative killer cells in the T lymphocyte population. They confer T cell-mediated cellular immunity, especially in anti-tumor immunity[36, 37]. It can be observed from our study, that, LNPEP expression showed a positive correlation with CD8+ T cells, macrophage, B cells, neutrophil, monocyte, and dendritic cells using the TIMER2 database. This finding may be able to elucidate the protective role of LNPEP in most tumor types. Tregs have been shown to exert suppressive effects and attribute to tumor cells that have escaped the attack of cytotoxic CD8+ T cells[38, 39]. Hence, we indicated that Treg infiltration levels positively correlated with LNPEP, whilst observing the limited function of cytotoxic CD8+ T cells, despite a case where their number is large.

Notably, exhausted T cells are present in patients with chronic infections and cancer, and they are indicated by a loss of functional capabilities in these patients. A majority of patients with cancer usually have a large amount of T-cells, but most of these T cells are exhausted[40]. It was revealed from our study, that LNPEP was positively correlated with marker genes of exhausted T cells, immune-activating genes, and immune-suppressive genes in OV such as CTLA4, TIM3, PD-1, LAG3, and GZMB. These results laid claim to the fact that LNPEP may be an important inducer of canonical features of T cell exhaustion, and it could also function as an immune checkpoint. Therefore, we speculated that LNPEP could be involved in critical and different roles in the regulation of the TME. This makes LNPEP important to the identification of specific stages and grades of OV. However, in vivo and in vitro experiments should be performed in testing the antitumor activity targeted LNPEP. Additionally, clinical trials should be performed to validate the immune checkpoint function of LNPEP.

In conclusion, a comprehensive assessment of LNPEP was performed. It indicated its potential-related role as an indicator of prognosis and its immune-regulation effect in OV. LNPEP expression not only correlates with immune cell infiltration but also correlates with immune-modulators and chemokines. As a potentially new immune checkpoint, LNPEP may be a target for immunotherapy of patients with OV.

**Abbreviations**

AUC, area under curve; BSA, bovine serum albumin; CI, confidence interval; COMIC, catalogue of somatic mutations; CNS, central nervous system; DC, dendritic cell; DSS, disease-specific survival; FDR, false discovery rate; GSEA, gene set enrichment analysis; GEPIA, gene expression profiling interactive analysis; HPA, human protein atlas; KEGG, Kyoto Encyclopedia of Genes and Genomes; LNPEP, Leucyl And Cystinyl Aminopeptidase; MsigDB, Molecular Signatures Database; OSC, ovarian serous carcinoma; OS, overall survival; OV, ovarian cancer; PFI, progression-free interval; TCGA, the cancer genome atlas; TAM, tumor-associated macrophages; TIICs, tumor infiltration immune cells;

**Declarations**
Data Sharing Statement

All original data generated or analyzed during the study are included in the article and Supplementary Materials. Further inquiries can be directed to the corresponding author.

Consent for Publication

All authors have approved the manuscript for submission.

Disclosure

The authors declare no conflicts of interest in this work.

Funding

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Authors contribution

QM, LC applied for grant support, initiated and conducted the study, interpreted the data and contributed to the manuscript. WW, LYC, LC revised the manuscript and handled manuscript submission and revision. YZ, XS and YC provided technical support, data analysis and revised the manuscript. All authors have read and approved the final manuscript for publication.

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Figures

Figure 1

Pan-cancer LNPEP expression and prognosis.

(A) LNPEP expression pattern in various tumor types was obtained from the Timer Database. (B) Expression of LNPEP in different types of cancer derived from GENT2 database. (C) LNPEP expression in tumor tissues based on TCGA database. The location of the dot represents the mean value of LNPEP expression. (D) LNPEP expression in normal tissues based on the GTEx database. The location of the dot displays the mean value of LNPEP expression. (E) OS analysis revealed that LNPEP was significantly correlated with the prognosis of KIRC (p<0.001) and READ (p=0.042) (F) DSS displayed that LNPEP was observably correlated with the prognosis of KIRC (p<0.001) and OV (p=0.009) (G) The prognosis of PFI showed that LNPEP was markedly correlated with the prognosis of KIRC (p<0.001), PCPG (p=0.024), SKCM (p=0.024). *p < 0.05; **p < 0.01; and ***p < 0.001; NS, not significant.
Figure 2

The diagnostic value of LNPEP expression in OV

(A) mRNA expression of LNPEP in tumor tissues and normal tissues based on TCGA samples using the GEPIA database. (B) The mRNA expression of LNPEP in OV was obtained from GE-mini. T and N represent the OV tissues and normal tissues, respectively. (C) ROC curve analysis of LNPEP for the
diagnostic values of patients with OV. (D) Protein expressions of LNPEP in pairs of OV and adjacent normal tissue samples were determined by western blot assay (N: normal tissues, T: OV cancer tissues). (E) Representative images of IHC with LNPEP in the HPA database. (F) Representative immunohistochemistry images of LNPEP in OV tissues and corresponding normal tissues.

Figure 3

LNPEP mutation in OV

(A) The schematic representation of LNPEP mutations in OV (cBioPortal). (B–C) The mutation types of LNPEP (%) in OV the Catalogue of Somatic Mutations in Cancer (COSMIC) database. (D) Kaplan–Meier curve for OS based on alterations of LNPEP.
Figure 4

Analysis of LNPEP expression on the prognosis of patients with OV.

(A–C) The relationship between LNPEP and OS, DSS, and PFI in patients with OV is described by Kaplan–Meier plotter, respectively.
Figure 5

Relationship between LNPEP expression and immune cell infiltration in pan-cancer patients

(A-G) Associations of LNPEP expression to tumor purity and immune infiltration in various cancers. Association heatmap of immune cell infiltration based on CD8+ T cells (A), CD4+ T cells (B), B cells (C),
neutrophils (D), macrophages (E), monocyte (F), DCs (G), and other immune cell (H) in multiple cancer types. *p < 0.05; **p < 0.01; ***p < 0.001.

**Figure 6**

**LNPEP is associated with immune infiltration in OV.** (A) Correlation between LNPEP expression and the abundance of tumor-infiltrating immune cells in OV using the TIMER database. (B, C) Correlation between LNPEP expression and immune-stimulators (B) and immune inhibitors (C) in OV available from the TISIDB database. (D, E) Correlation between LNPEP expression and chemokines (D) and chemokine receptors (E) in OV available from the TISIDB database.

**Figure 7**

**LNPEP co-expressed genes and functional enrichment analysis**

(A) Volcano map of co-expressed profiling of LNPEP in OV by the LinkedOmics database. (B, C) Heat maps of top 50 positively (B) and 50 negatively (C) correlated genes with LNPEP are displayed. (D, E)
LNPEP co-expression genes were annotated by Gene Ontology (GO) analysis (D) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (E) and available at LinkedOmics.

**Figure 8**

(A) GeneMANIA performed the interactions for LNPEP linked with each other based on physical and genetic interactions, pathway analysis, co-expression, and localization. (B) The protein interaction network in LNPEP was conducted in the STRING database.
Figure 9

The correlation between LNPEP and immune-regulation-related genes

(A) The heatmap represents the correlation between LNPEP expression and immune-activating genes in OV. (B) The heatmap represents the correlation between LNPEP expression and immunosuppressive status-related genes. (C) The heatmap
represents the correlation between LNPEP expression and chemokine receptor genes. (D) The heatmap represents the correlation between LNPEP expression and chemokine genes. \( *p < 0.05; **p < 0.01; \) and \( ***p < 0.001. \)

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.csv
- TableS2.xls
- FigureS1.pdf
- FigureS2.pdf
- FigureS3.pdf
- FigureS4.pdf
- FigureS5.pdf
- FigureS6.pdf
- FigureS7.pdf
- FigureS8.pdf