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ORIGINAL RESEARCH

# Leucyl and Cystinyl Aminopeptidase as a Prognostic-Related Biomarker in OV Correlating with Immune Infiltrates

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**Background:** It was indicated that tumor intrinsic heterogeneity and the tumor microenvironment (TME) of ovarian cancer (OV) influence immunotherapy efficacy and patient outcomes. Leucyl and cystinyl aminopeptidase (LNPEP) encodes a zinc-dependent aminopeptidase, which has been proved to participant in the vesicle-mediated transport and class I MHC mediated antigen processing and presentation. However, the function of LNPEP in TME of OV and its potential molecular mechanisms have not been determined. Therefore, we aimed to investigate a prognostic biomarker which may be helpful in identifying TME heterogeneity of ovarian cancer.

**Methods:** In this study, bioinformatics databases were used to explore the expression profile and immune infiltration of LNPEP. Bioinformatics analyses of survival data and interactors of LNPEP were conducted to predict the prognostic value of LNPEP in OV. The protein levels of LNPEP were validated by Western blot and immunohistochemistry.

**Results:** Based on the TCGA data, our data displayed that the mRNA expression of LNPEP was markedly down-regulated in ovarian cancer than that 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. GO and KEGG pathway analyses indicated 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. Our data also demonstrated that the expression of LNPEP was strongly correlated with immune infiltration levels, immunomodulators, chemokines and chemokine receptors.

**Conclusion:** In our study, we identified and established a prognostic signature of immune-related LNPEP in OV, which will be of great value in predicting the prognosis of clinical trials and may become a new therapeutic target for immunological research and potential prognostic biomarker in OV.

**Keywords:** LNPEP, ovarian cancer, prognosis, immune infiltration, tumor microenvironment

## Introduction

Ovarian cancer (OV), the most fatal gynecological malignancies, is one of most common causes of cancer-related death in women.[1,2] Despite significant advances have been made in early diagnosis and treatment, the 5-year survival rate is only around 47%.[3] Ovarian cancer has been called the "silent killers" of women.[4] Due to lack of specific clinical symptoms and effective early detection screening, most patients suffer from locally mid-late stage.[5] Up to 70% ovarian cancer patients are diagnosed with advanced stage (III/IV) at the time of presentation, suffered from wild metastasis throughout the abdominal

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cavity with ascites, lymph node metastasis, and peritoneal dissemination.[6,7] Currently, there are numerous therapeutic treatments for patients with ovarian cancer including debulking surgery, chemotherapy, radiation, targeted therapy, immunotherapy and combination strategies.[8] Despite recent development of multiple effective treatments, chemotherapy resistance and tumor recurrence remain the major challenges clinically.[9] Therefore, it is an urgent problem to find new therapeutic modalities to improve the prognosis of patients with ovarian cancer.

*LNPEP* is a protein coding gene, located on chromosome 5q15 in human genome, encodes a zinc-dependent aminopeptidase, which has been shown 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 resides in intracellular vesicles with the insulin-responsive glucose transporter GLUT4 and can then transfer to the cell surface under the action of insulin and/or oxytocin.[12] A recent research reported LNPEP can promote inflammatory gene expression by activation of the Inhibitor of Nuclear Factor Kappa-B pathway.[11] According to the recent reports, the LNPEP gene is also related to 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 of the expression levels of LNPEP and implications in the prognosis and progression of ovarian cancer. Furthermore, the associations of LNPEP with the tumor immune microenvironment (TME) in ovarian cancer have not been determined.

In this study, we use comprehensive bioinformatics and experimental methods to assess the heterogeneity expression pattern and function of LNPEP in different immune cells based on single-cell level using CancerSEA. Several bioformatics tools including TIMER, GEPIA, Gent2, HPA, Cbioportal were used for LNPEP expression pattern. KM plotter was used to display how LNPEP influences the prognosis of ovarian cancer patients. In addition, we investigated the association of LNPEP expression with immune-infiltrating cells and immunomodulators in OV using TIMER database and LinkedOmic. 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 OV patients.

# Materials and Methods

## Patients and Ethics Approval

A total of 60 surgically resected human ovarian cancer specimens and 40 normal ovary tissue were collected in the Obstetrics and Gynecology 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 clinical information was used for research purposes. All patients were informed and signed a consent for scientific purposes. All study procedures were implemented in accordance with the ethical standards of the Helsinki Declaration.

## Western Blot

Clinical samples were lysed with RIPA lysis buffer including protease and phosphatase inhibitor for 10 min under ice. After centrifugation at 12,000×g for 10 min at 4°C, the supernatants were collected and quantitied based on BCA protein detection kit. Next, PVDF membranes were 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 45min at room temperature, the image signals were detected with a chemiluminescence reagent (Thermofisher, WP20005).

## TCGA Database Analysis

All original data were downloaded from The Cancer Genome Atlas (TCGA) (https://cancergenome.nih.gov/). R 3.2.3 was performed to integrate the original data and analysis the difference by the website database. The prognostic difference of the LNPEP gene expression group in 33 kinds of tumors was analyzed with the dichotomy method. To explore the types of cancer that are significantly associated with LNPEP gene expression, we used univariate regression analysis to analyze the prognosis of diversity tumors, including overall survival (OS), disease-specific survival (DSS),

process-free intervention (PFI). A survival curve was drawn, and the selected cancer types were used 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 from about 49,000 pan-cancer individual samples were analyzed across more than 30 different cancer types.[15]

## GEPIA

Gene Expression Profiling Interactive Analysis (GEPIA) is a web resource used 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 "singlegene analysis" in GEPIA to evaluate the mRNA expression levels of LNPEP in OV tissues, compared with normal tissues by Student's *t*-test. $P < 0.05$ was considered statistically significant.

## CancerSEA

CancerSEA (http://biocc.hrbmu.edu.cn/CancerSEA/) is an comprehensive and user-friendly database to analyze distinct functional states of different cancer cells at single-cell level. It was designed to portray 14 functional states of 41,900 cancer single cells from 25 tumor types. Cellular functional states involved stemness, inflammation, EMT, angiogenesis, hypoxia, DNA repair, apoptosis, cell cycle, proliferation, metastasis, DNA damage, differentiation, invasion and quiescence.[17]

## BioGPS

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 basing 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 line.

## TISIDB Database Analysis

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

## Human Protein Atlas Dataset

LNPEP was analyzed at the protein levels in the Human Protein Atlas (HPA) dataset (https://www.proteinatlas.org/), which contained immunohistochemical staining images of 373 clinical OV patients 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 analysis of Gene Ontology (GO) biological process, cellular components, molecular function, 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 cells (TIICs) dependent 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 cell markers 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.

## Genetic Alteration Analysis

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

## GeneMANIA and STRING Web Analysis

GeneMANIA (http://www.genemania.org/) web database performed interaction patterns of differentially regulated gene and showed gene function, protein and genetic interaction, related pathway prediction, co-expression analysis and colocalization and protein domain similarity.[27] STRING web-based network tool (https://string-db.org/) integrates computational methods to predict the gene functions, and was utilized to infer the functional interaction under the direct interaction to score nodes.[28–31]

## Cox Regression Analysis and Kaplan–Meier Survival Analysis

Univariate and multivariate Cox regression analyses wee 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 TCGA databases. The patient 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 (CIs) were tested.

## Statistical Analysis

All data of this study were statistically performed by R software 3.6.1. The measurement data are 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 the Pearson's Chi squared test. Univariate and multivariable analyses were involved in using the Cox proportional hazards regression models.

# Result

## 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 liver, bile duct, whereas down-regulated was revealed in breast, central nervous system (CNS), kidney, lung, prostate, rectum and skin according to Timer analysis (Figure 1A, Table S1). Moreover, we estimated LNPEP expression data profiled of 72 paired cancer vs normal tissues by utilizing HG-U133 microarray (GPL570 platform) of Gent2 database (Figure 1B, Table S2). Thus, the results showed that LNPEP was up-regulated in several cancer contain adipose, 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

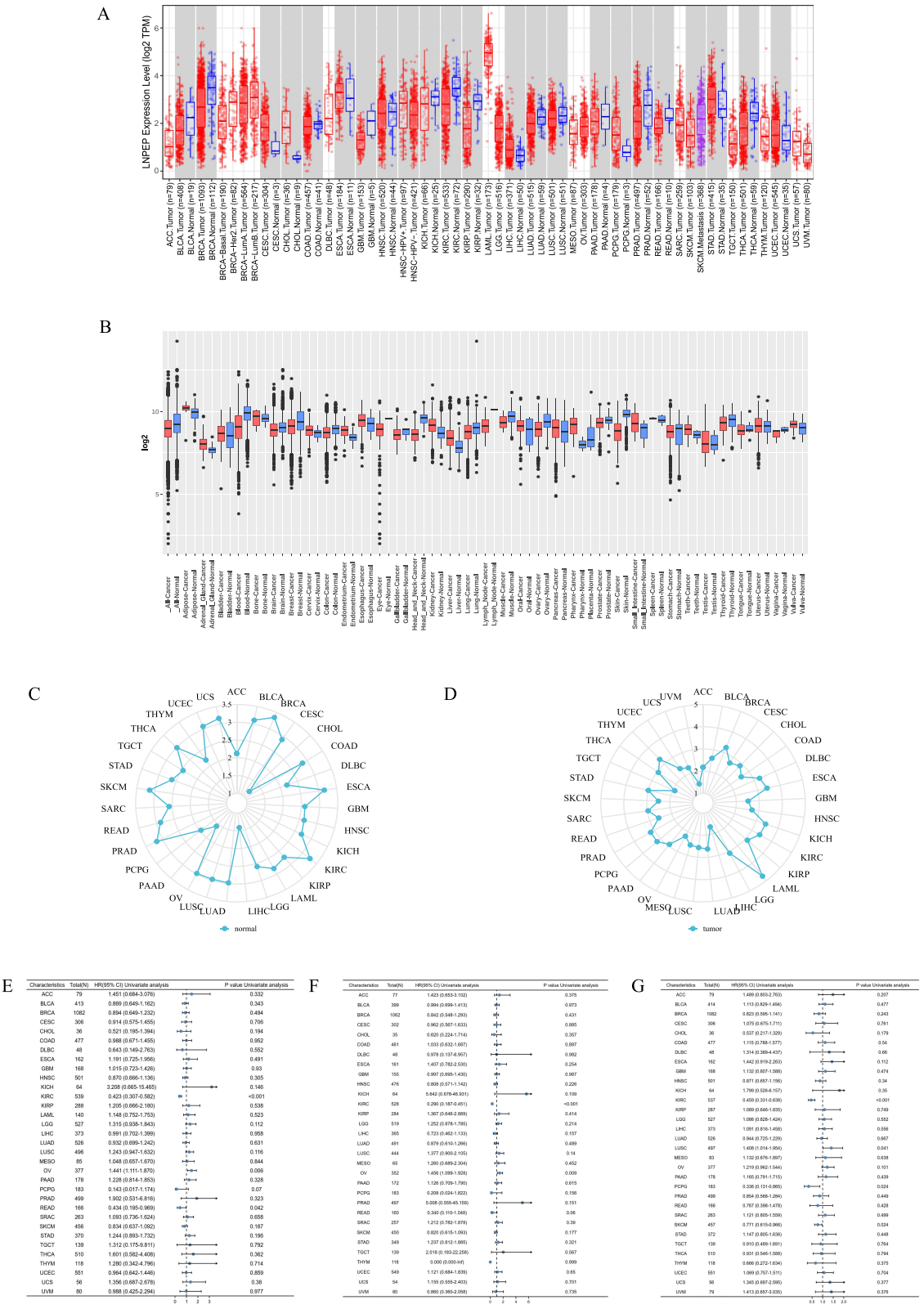


**Figure 1** Pan-cancer LNPEP expression and prognosis. (**A**) LNPEP expression pattern in various tumor types was obtained from the Timer database. (**B**) Expression of LNPEP in different types of cancer derived from GENT2 database. (**C**) LNPEP expression in tumor tissues based on TCGA database. The location of the dot represents the mean value of LNPEP expression. (**D**) LNPEP expression in normal tissues based on 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 ($p=0.024$). $^{**}p < 0.01$; and $^{***}p < 0.001$; ns, not significant.

and vagina (Figure 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 related to the prognosis of KIRC ($P$<0.001), OV ($P$=0.006) and READ ($P$=0.042) (Figure 1E). DSS displayed that LNPEP was markedly correlated with the prognosis of KIRC ($P$<0.001) and OV ($P$=0.009) (Figure 1F). PFI reflected that LNPEP was associated with the prognosis of KIRC ($P$<0.001), PCPG ($P$=0.024) and SKCM ($P$=0.024) (Figure 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 Table S3s and S4. The above results suggested that LNPEP might participate in the process of immune regulation.

## The Expression of LNPEP and Its Association with Clinicopathological Characteristics in OV Patients

Next, the mRNA levels of LNPEP were down-regulated in OV compared to normal ovary tissues which was tested using the data in publically available database. Based on the GEPIA with the criterion of $|Log_2FC|>2$ and $P$<0.05, LNPEP has significantly low expression in OV patients (Figure 2A). Meanwhile, a significant correlation between LNPEP expression and molecular subtypes existed in OV (Figure S2). Subsequently, we analyzed the diagnostic values of LNPEP in OSC with ROC curves. Based on Xiantao Xueshu web tool (https://www.xiantao.love/products), we performed that the area under the curve (AUC) of LNPEP was 0.859 (Figure 2B). In view of highest AUC, LNPEP could have greater potential to be as a diagnostic biomarker for OV patients. As described above, the mRNA levels of LNPEP were downregulated in OV, so we proceed to test the protein level of LNPEP in OV. 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 (Figure 2C). In the HPA dataset, the IHC staining data revealed that LNPEP was mainly localized in the cytoplasmic/membranous and had higher expressions in OV tissues (Figure 2D, Figure 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 immune-histochemical staining of LNPEP in OSC is illustrated in Figure 2E. Additionally, LNPEP expression differed in different immune subtypes of OV. To further evaluate the prognostic value of LNPEP expression, univariate and multivariate Cox regression analyses was performed based on OS, DSS and PFI of OV patients basing on TCGA. As shown in Tables 1–3, LNPEP expression, age class, race, FIGO stage, primary therapy outcome, tumor residual, tumor status were independent prognostic factors for OS, DSS and PFI of OV patients, respectively.

## Genetic Alterations of LNPEP in OV

Genomic alterations induce changes in gene expression. We explored mutation frequency of LNPEP using cBioPortal website. Five datasets (MSK, MSKCC, TCGA Pan-Cancer Atlas, TCGA Firehose legacy and TCGA nature), which included 1737 samples, were selected for analysis. The somatic mutation frequency of LNPEP in OV was 3%, which mainly consisted of missense mutations and synonymous substitution (Figure 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 COSMIC database. Two pie charts clearly showed the distribution of mutation types (Figure 3B and C). Missense substitutions occurred in approximately 34.47% of the samples, synonymous substitutions occurred in 11.69% of the samples, and nonsense substitution occurred in 4.4% of the samples (Figure 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%) (Figure 3C). In addition, altered LNPEP had significant correlation with OS of OV ($P$<0.05) (Figure 3D).

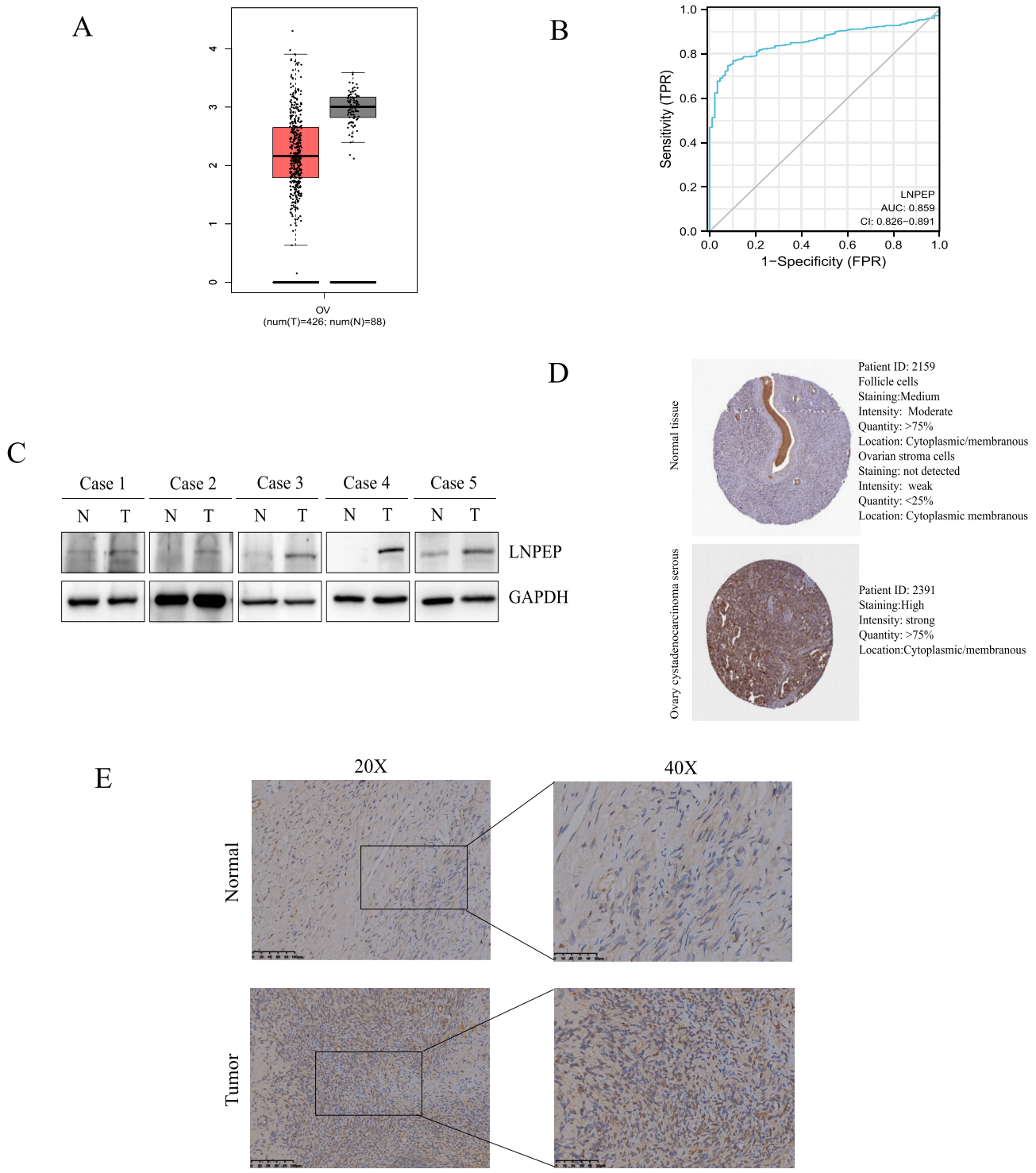


**Figure 2** The diagnostic value of LNPEP expression in OV. (**A**) mRNA expression of LNPEP in tumor tissues and normal tissues based on TCGA samples using the GEPIA database. (**B**) ROC curve analysis of LNPEP for the diagnostic values of OV patients. (**C**) Protein expressions of LNPEP in pairs of OV and adjacent normal tissues samples were determined by Western blot assay (N: normal tissues, T: OV cancer tissues). (**D**) Representative images of IHC with LNPEP in HPA database. (**E**) Representative immunohistochemistry images of LNPEP in OV tissues and corresponding normal tissues.

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

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 OV patients and explore the prognostic value of LNPEP in OV, survival analysis was performed based on information of TCGA database. Results displayed that the upregulation of

**Table 1** Univariate and Multivariate Cox Proportional Hazards Analysis for Overall Survival in OV Cohort

| Variables | Univariated Analysis | | Multivariate Analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value |
| LNPEP: high vs low | 1.441 (1.111–1.870) | **0.006** | 1.312 (0.949–1.814) | 0.1 |
| FIGO stage: I+II vs III+IV | 1.981(1.419–2.764) | **<0.001** | 2.076 (0.490–8.806) | 0.322 |
| Primary therapy outcome: PD+SD vs PR+CR | 0.301 (0.204–0.444) | **<0.001** | 0.350 (0.226–0.542) | **<0.001** |
| Age: ≤60 vs >60 | 1.355 (1.046–1.754) | **0.021** | 1.286 (0.931–1.776) | 0.128 |
| Tumor residual: NRD vs RD | 2.313 (1.486–3.599) | **<0.001** | 1.239 (0.722–2.124) | 0.437 |
| Tumor status: Tumor free vs With tumor | 9.576 (4.476–20.486) | **<0.001** | 18.787 (4.593–76.844) | **<0.001** |

**Note**: Bold data indicate statistically significant.
**Abbreviations**: HR, hazard ratio; CI, confidence interval; PD, progressive response; SD, stable disease; PR, partial response; CR, complete response; NRD, no residual disease; RD, residual disease; OV, ovarian cancer.

**Table 2** Univariate and Multivariate Cox Proportional Hazards Analysis of Disease-Specific Survival in OV Cohort

| Variables | Univariated Analysis | | Multivariate Analysis | |
|---|---|---|---|---|
| | HR (95% CI) | *P*-value | HR (95% CI) | *P*-value |
| LNPEP: high vs low | 1.456 (1.099–1.928) | **0.009** | 1.300 (0.941–1.794) | 0.112 |
| Race:Asian Black+African American vs White | 0.592 (0.370–0.946) | **0.028** | 0.774 (0.434–1.380) | 0.385 |
| Primary therapy outcome: PD+SD vs PR+CR | 0.294 (0.198–0.436) | **<0.001** | 0.310 (0.200–0.479) | **<0.001** |
| Tumor residual: NRD vs RD | 2.572 (1.580–4.187) | **<0.001** | 2.192 (1.280–3.754) | **0.004** |

**Note**: Bold indicate statistically significant.
**Abbreviations**: HR, hazard ratio; CI, confidence interval; PD, progressive response; SD, stable disease; PR, partial response; CR, complete response; NRD, no residual disease; RD, residual disease; OV, ovarian cancer.

**Table 3** Univariate and Multivariate Cox Proportional Hazards Analysis of Progression-Free Interval in OV Cohort

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

**Note**: Bold indicate statistically significant.
**Abbreviations**: HR, hazard ratio; CI, confidence interval; PD, progressive response; SD, stable disease; PR, partial response; CR, complete response; NRD, no residual disease; RD, residual disease; OV, ovarian cancer.

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 OV patients (Figure 4A–C). Furthermore, high LNPEP expression was also associated with poor OS/DSS/PFI of OV patients 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–S7A-I), but not those patients who were anatomic neoplasm subdivision (unilateral) (Figures S5J–S7J). In conclusion, high LNPEP expression was associated with poor prognosis in OV patients.

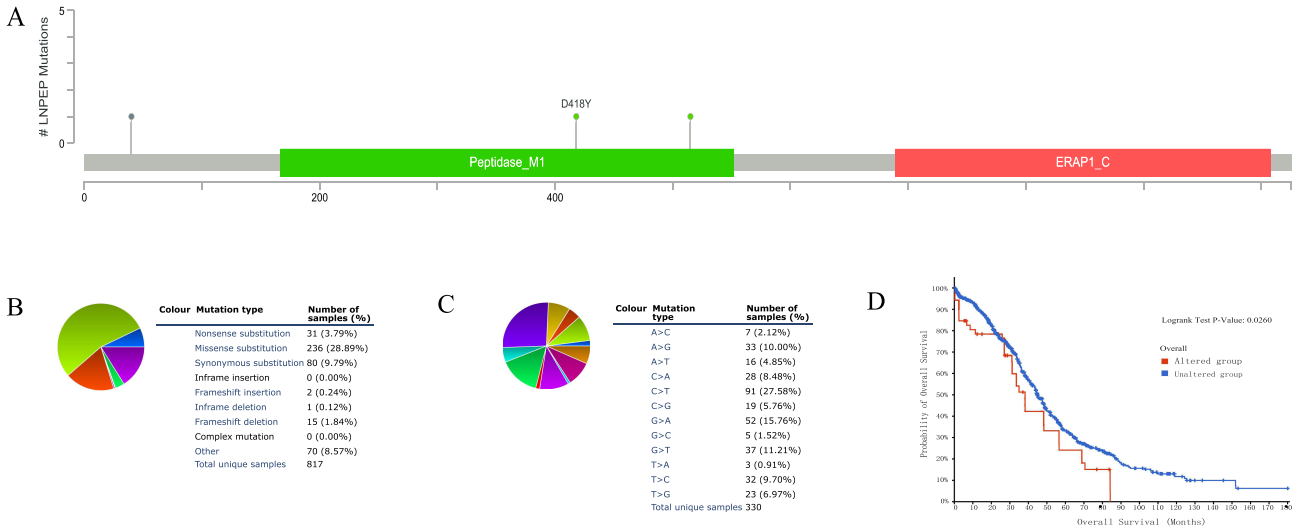


**Figure 3** LNPEP mutation in OV. (**A**) The schematic representation of LNPEP mutations in OV (cBioPortal). (**B** and **C**) The mutation types of LNPEP (%) in OV the Catalogue of Somatic Mutations in Cancer (COSMIC) database. (**D**) Kaplan–Meier curve for OS based on alterations of LNPEP.

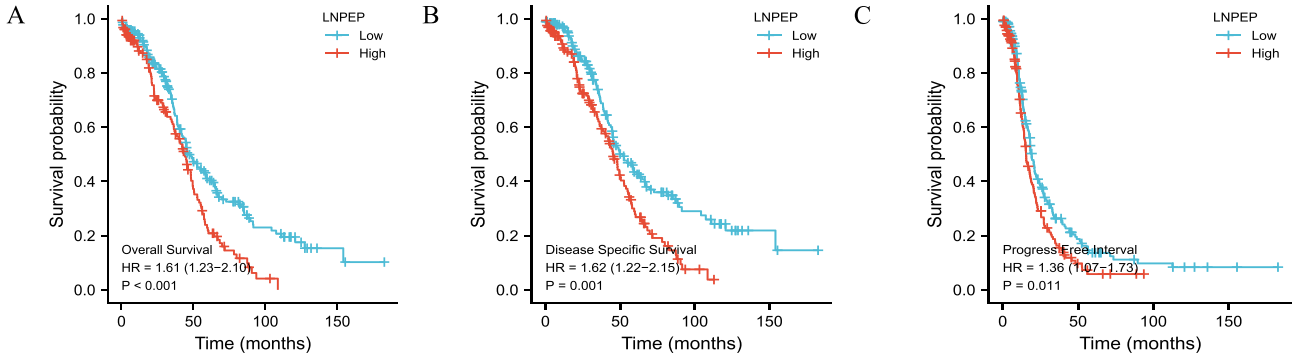


**Figure 4** Analysis of LNPEP expression on the prognosis of OV patients. (**A**–**C**) The relationship between LNPEP and OS, DSS and PFI in OV patients described by Kaplan–Meier plotter, respectively.

## Single-Cell Functional Analysis

CancerSEA database was utilized to further investigate the functional state of PLEK2 in single-cell across various cancer types. Results are summarized in Figure 5A and B. Interestingly, LNPEP was positively correlated with metastasis (cor=0.35, $P<0.05$), angiogenesis (cor=0.34, $P<0.05$), EMT (cor=0.33, $P<0.05$) in OV. As for OV, there is a significant negative correlation between LNPEP expression and invasion (cor=−0.38, $P<0.01$), DNA repair (cor=−0.32, $P<0.05$).

## 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 relation of 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, neutrophil, macrophages and dendritic cells) were analysed 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 Figure S8A–F, the profiles of LNPEP associated with various immune infiltration displayed that it was positively correlated with immune cell infiltration levels of neutrophils. However, data indicated that it was obviously 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

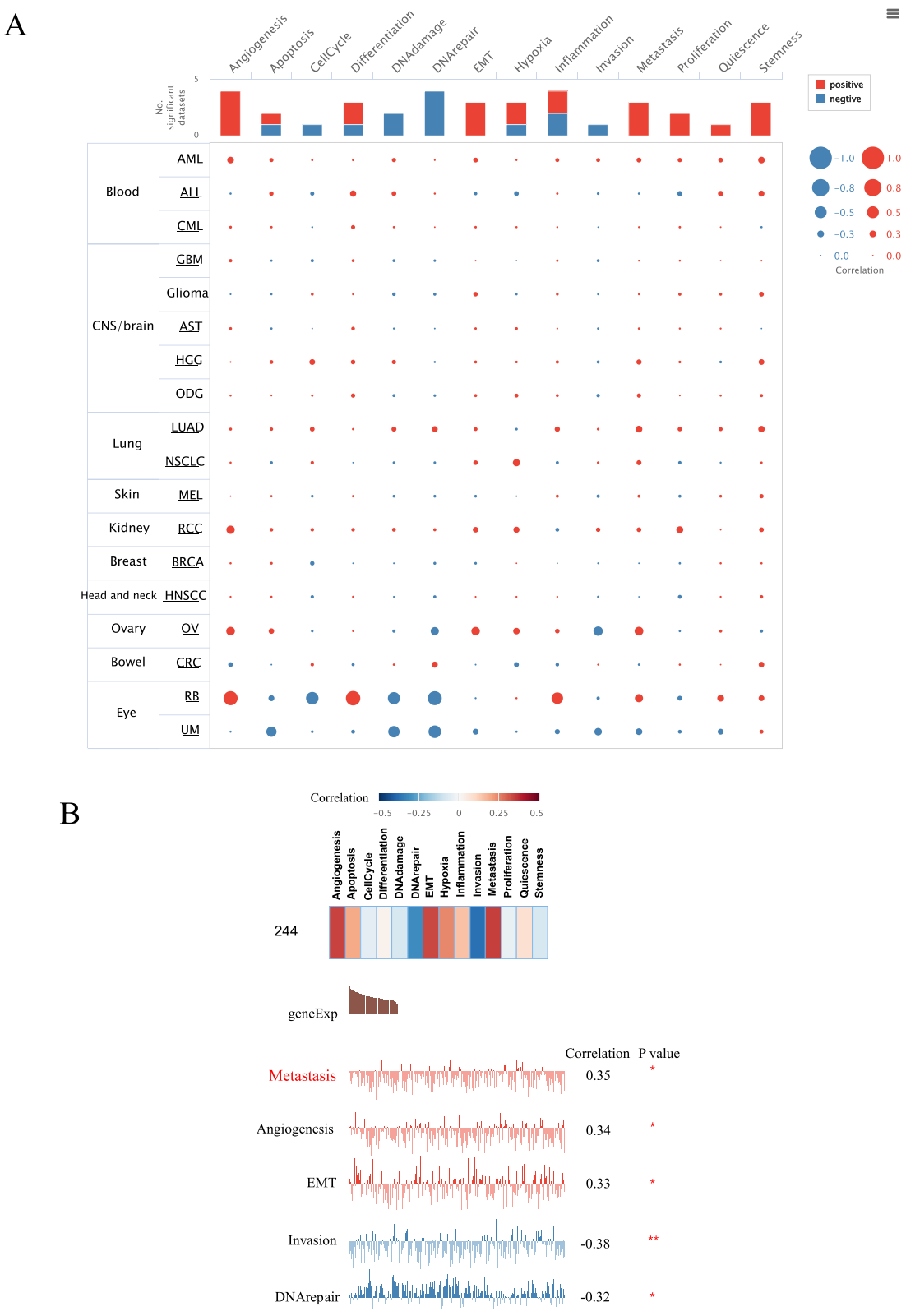


**Figure 5** Sing-cell functional analysis of LNPEP using CancerSEA. (**A**) The functional state of LNPEP across various types of cancer. (**B**) The LNPEP-related overview of functional states in OV. Red and blue bar represent a positive correlation and a negative correlation, respectively. Significance was set at $p<0.05$.

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 (Figure S8G).

## LNPEP is Correlated with Immune Infiltration in OV

Next, 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 tumour purity (r=−0.149, *P*=1.81E-02). In addition, LNPEP expression had significant positive correlations with five immune infiltrates, including CD8+T cells, B cells, macrophage, monocytes, dendritic cells (Figure 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 gene 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, T cells, B cells, monocytes, TAM, M1 macrophages, M2 macrophages, neutrophils and dendritic cells. Moreover, the expression of LNPEP was associated with the expression of markers of specific subsets of T cells in OV, which included Th1, Th2, Tfh, Th17, Treg, T cell exhaustion. However, LNPEP expression did not reveal any significant correlation with CD4+ T cells. LNPEP was significantly correlated with immune stimulators, such as CD80, IL2RA, MICB, and PVR (Figure 6B). The expression of LNPEP was also associated with immune inhibitors, including CD274, CTLA, IDO1, TGFB1 (Figure 6C). LNPEP expression was significantly correlated with CCL4, CCL11, CXCL13, and CXCL14 (Figure 6D). Meanwhile, LNPEP expression was significantly associated with chemokine receptors, including CCR1, CCR4, CCR8, and CXCR6 (Figure 6E). In summary, comprehensive analysis indicated that LNPEP may function as an immunoregulatory factor in OV.

## LNPEP Co-Expression Network in OV

We employed the LinkedOmics database to identify the LNPEP-related co-expressed genes and predicted the function in OV. As displayed in the volcano plot, 1914 genes were positively correlated with LNPEP, while 1279 genes were negatively correlated with LNPEP (Figure 7A). Heat maps showed the top 50 genes positively and negatively associated with LNPEP (Figure 7B and C). The results above mentioned that LNPEP has a potential role involved in the transcriptome. GSEA tool was further conducted to identify the significant GO and KEGG pathway. GO results showed that LNPEP-related co-expressed genes were mainly involved in adaptive immune response, protein complex involved in cell adhesion and structural constituent of ribosome (Figure 7D–F). KEGG pathway analysis revealed enrichment in ribosome, associated with ECM–receptor interaction (Figure 7G). 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 S9A and B). These results suggested that the LNPEP expression network significantly affected the immune microenvironment and ligand–receptor interactions in OV.

## Protein–Protein Interaction Network Analysis for LNPEP

As 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 protein–protein interaction (PPIs) network associated with LNPEP. It is well known that protein alters a wide variety of biological processes and cellular signalling regarding as protein interaction and signal transduction pathway.[2,3] 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 genes set. As Figure 8A shows, GeneMANIA supplied the PPIs associated protein included 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 (Figure 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 LNPEP involved in the progression and prognosis of cancer.

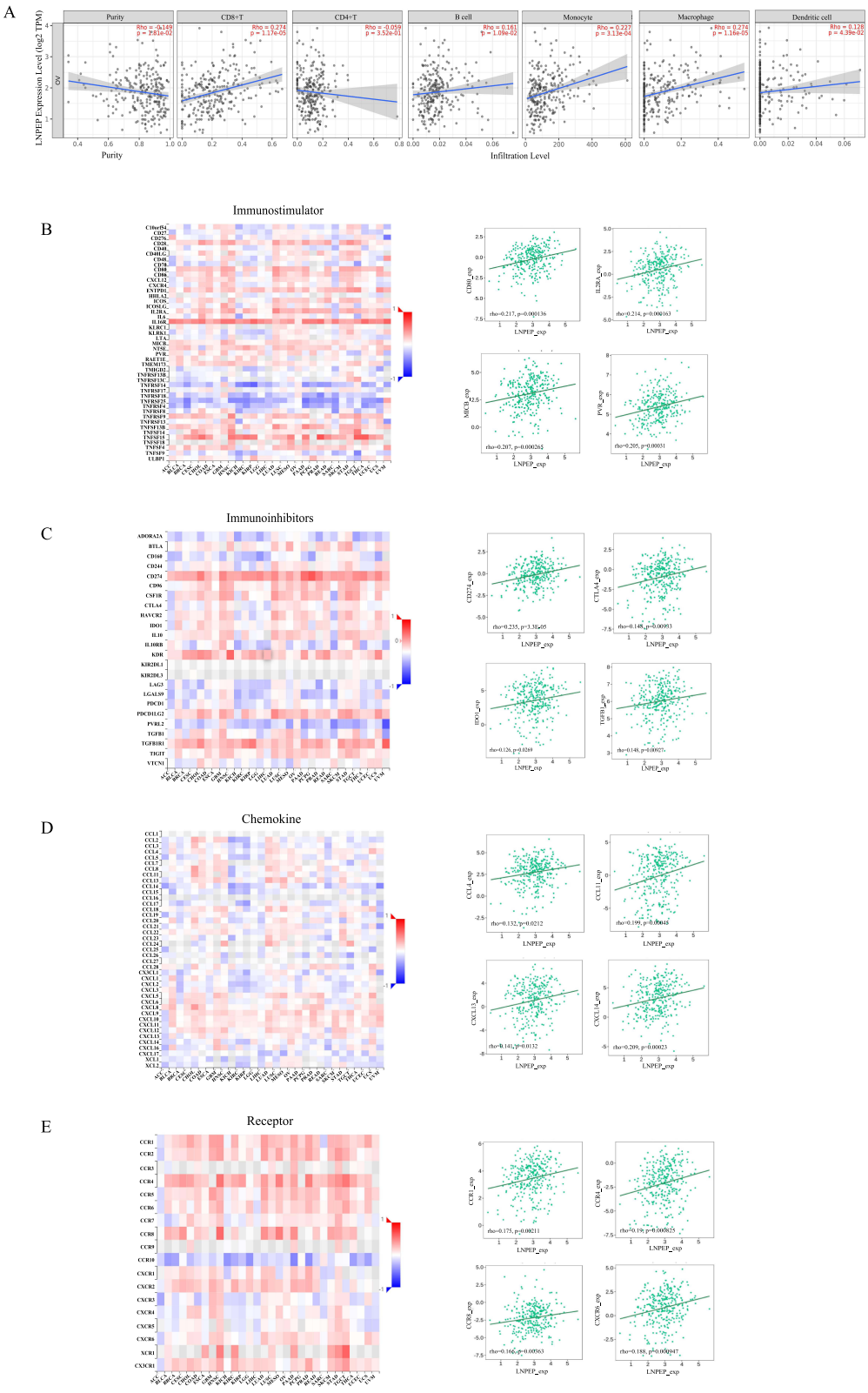


**Figure 6** LNPEP is associated with immune infiltration in OV. (**A**) Correlation between LNPEP expression and the abundance of tumor infiltrating immune cells in OV using the TIMER database. (**B** and **C**) Correlation between LNPEP expression and immunostimulators (**B**) and immunoinhibitors (**C**) in OV available from the TISIDB database. (**D** and **E**) Correlation between LNPEP expression and chemokines (**D**) and chemokine receptors (**E**) in OV available from the TISIDB database.

**Table 4** Correlation Analysis Between LNPEP and Related Genes of Immune Cells in TIMER Database

| Description | Gene Markers | OV (n=303) | | | |
|---|---|---|---|---|---|
| | | None | | Purity | |
| | | rho | *P* | rho | *P* |
| CD8+ T cell | *CD8A* | 0.161 | ** | 0.114 | 0.0731 |
| | *CD8B* | 0.155 | ** | 0.094 | 0.14 |
| | *FCGR3B* | 0.244 | *** | 0.208 | *** |
| | *SIGLEC5* | 0.245 | *** | 0.248 | *** |
| | *FPR1* | 0.166 | ** | 0.146 | * |
| T cell (general) | *CD3D* | 0.125 | * | 0.067 | 0.290 |
| | *CD3E* | 0.172 | ** | 0.119 | 0.0617 |
| | *CD2* | 0.135 | * | 0.133 | * |
| B cell | *CD19* | 0.209 | ** | 0.145 | * |
| Monocyte | *CD14* | 0.147 | * | 0.104 | 0.101 |
| | *CD115*(CSF1R) | 0.184 | ** | 0.162 | * |
| | *CD86* | 0.215 | *** | 0.193 | ** |
| TAM | *CD68* | 0.246 | *** | 0.23 | *** |
| | *IL10* | 0.248 | *** | 0.232 | *** |
| M1 macrophage | *COX*(PTGS2) | 0.206 | *** | 0.175 | ** |
| | *CD163* | 0.27 | *** | 0.267 | *** |
| | *IRF5* | 0.233 | *** | 0.146 | * |
| M2 macrophage | *VSIG4* | 0.181 | ** | 0.163 | * |
| | *MS4A4A* | 0.186 | ** | 0.17 | ** |
| Neutrophils | *CD11b*(ITGAM) | 0.218 | *** | 0.198 | ** |
| | *CCR7* | 0.201 | *** | 0.185 | ** |
| Dentritic cell | *CD11c*(ITGAX) | 0.28 | *** | 0.265 | *** |
| | *BDCA-4*(NRP1) | 0.227 | *** | 0.189 | ** |
| Th1 | *T-bet* (TBX21) | 0.182 | ** | 0.135 | * |
| | *STAT4* | 0.194 | *** | 0.177 | ** |
| | *STAT1* | 0.27 | *** | 0.269 | *** |
| | *TNF-A*(TNF) | 0.188 | *** | 0.181 | ** |
| Th2 | *STAT5A* | 0.256 | *** | 0.215 | *** |
| | *GATA3*(QRSL1) | 0.243 | *** | 0.23 | * |
| | *STAT6* | 0.204 | *** | 0.191 | ** |
| Tfh | *BCL6* | 0.143 | * | 0.158 | * |
| Th17 | *STAT3* | 0.401 | *** | 0.395 | *** |
| Treg | *FOXP3* | 0.292 | *** | 0.286 | *** |
| | *CCR8* | 0.201 | *** | 0.195 | ** |
| | *STAT5B* | 0.363 | *** | 0.344 | *** |
| | *TGFB*(TGFB1) | 0.284 | *** | 0.297 | *** |
| T cell exhaustion | *CTLA4* | 0.185 | ** | 0.152 | * |
| | *TIM3* (HAVCR2) | 0.232 | *** | 0.212 | *** |
| | *PD-1*(PDCD1) | 0.181 | ** | 0.121 | * |
| | *LAG3* | 0.126 | * | 0.126 | * |
| | *GZMB* | 0.14 | * | 0.166 | * |

**Note**: ***$P<0.001$; **$P<0.01$; *$P<0.05$.

## 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, immunestimulator-related genes, immunoinhibitor-related genes, chemokines and receptors. The

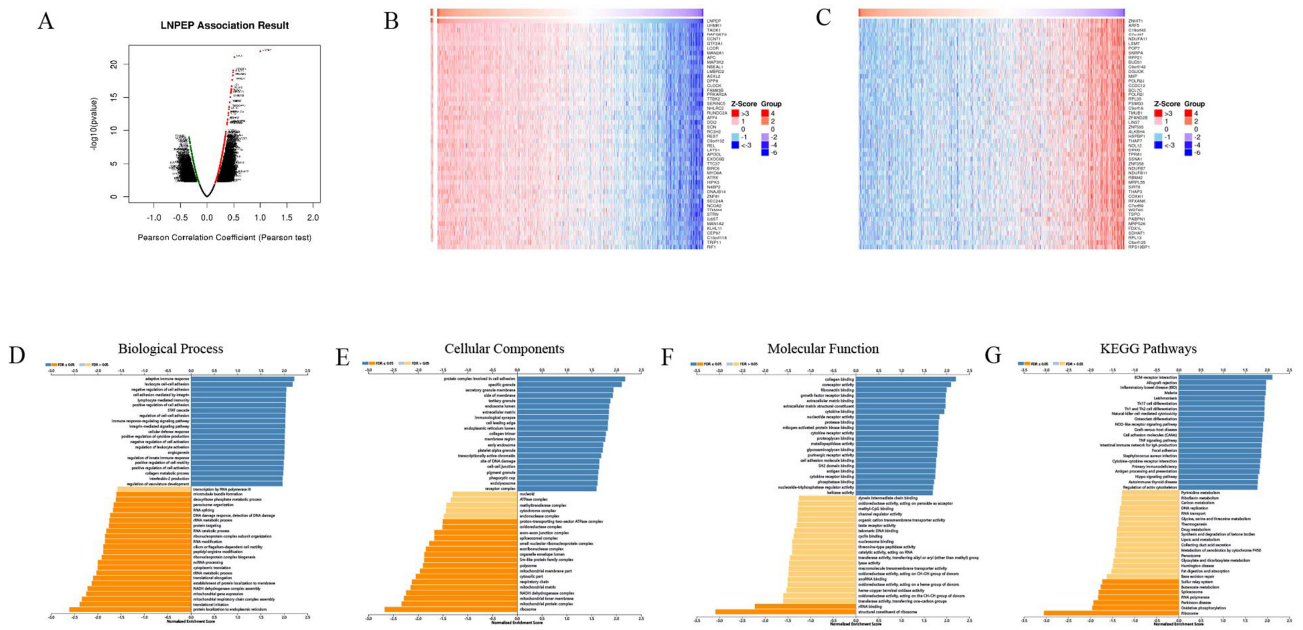


**Figure 7** LNPEP co-expressed genes and functional enrichment analysis. (**A**) Volcano map of co-expressed profiling of LNPEP in OV by the LinkedOmics database (Spearman correlation test). (**B** and **C**) Heat map of top 50 positively (**B**) and 50 negatively (**C**) correlated genes with LNPEP are displayed. (**D**–**G**) LNPEP co-expression genes were annotated by Gene Ontology (GO) analysis (**D**–**F**) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (**G**) and available at LinkedOmics. (**D**) Biological process, BP. (**E**) Cellular components, CC. (**F**) Molecular function, MF.

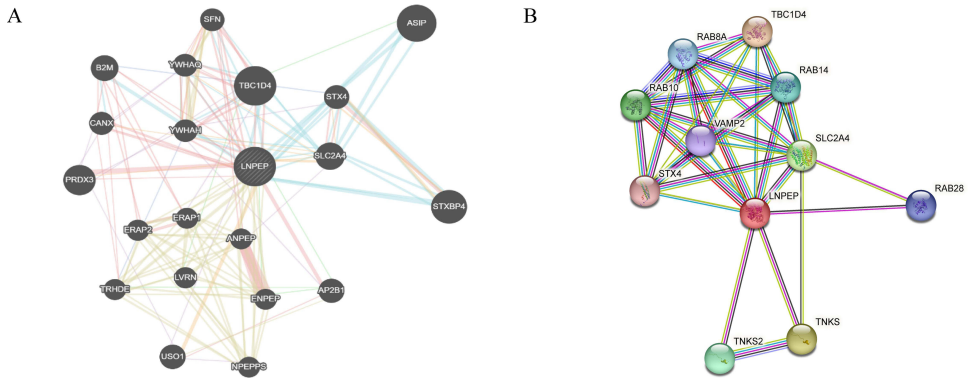


**Figure 8** Interaction network of LNPEP gene arised from GeneMANIA and STRING database. (**A**) GeneMANIA performed the interactions for LNPEP linked with each other based on physical and genetic interactions, pathway analysis, co-expression, and localization. (**B**) The protein interaction network in LNPEP was conducted in the STRING database.

results determined that LNPEP was positively correlated with marker genes of exhausted T cells, immunestimulator-related genes and immunoinhibitor-related genes in OV (Figure 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 (Figure 9C and D).

## Discussion

LNPEP was reported to be involved in various biological processes, including preeclampsia, psoriasis, septic shock and other immune-related disease.[12,13,32,33] It also degraded peptide hormones such as oxytocin, vasopressin and angiotensin III, and played a role in maintaining homeostasis during pregnancy.[12,34] Blockade of LNPEP-angiotensin IV signaling reactivated the neuronal peptides in the brain and cleaves Met-enkephalin and dynorphin.[10] However, how LNPEP might be correlated with OV immune microenvironment and lead to tumor heterogeneity has not been fully clarified.

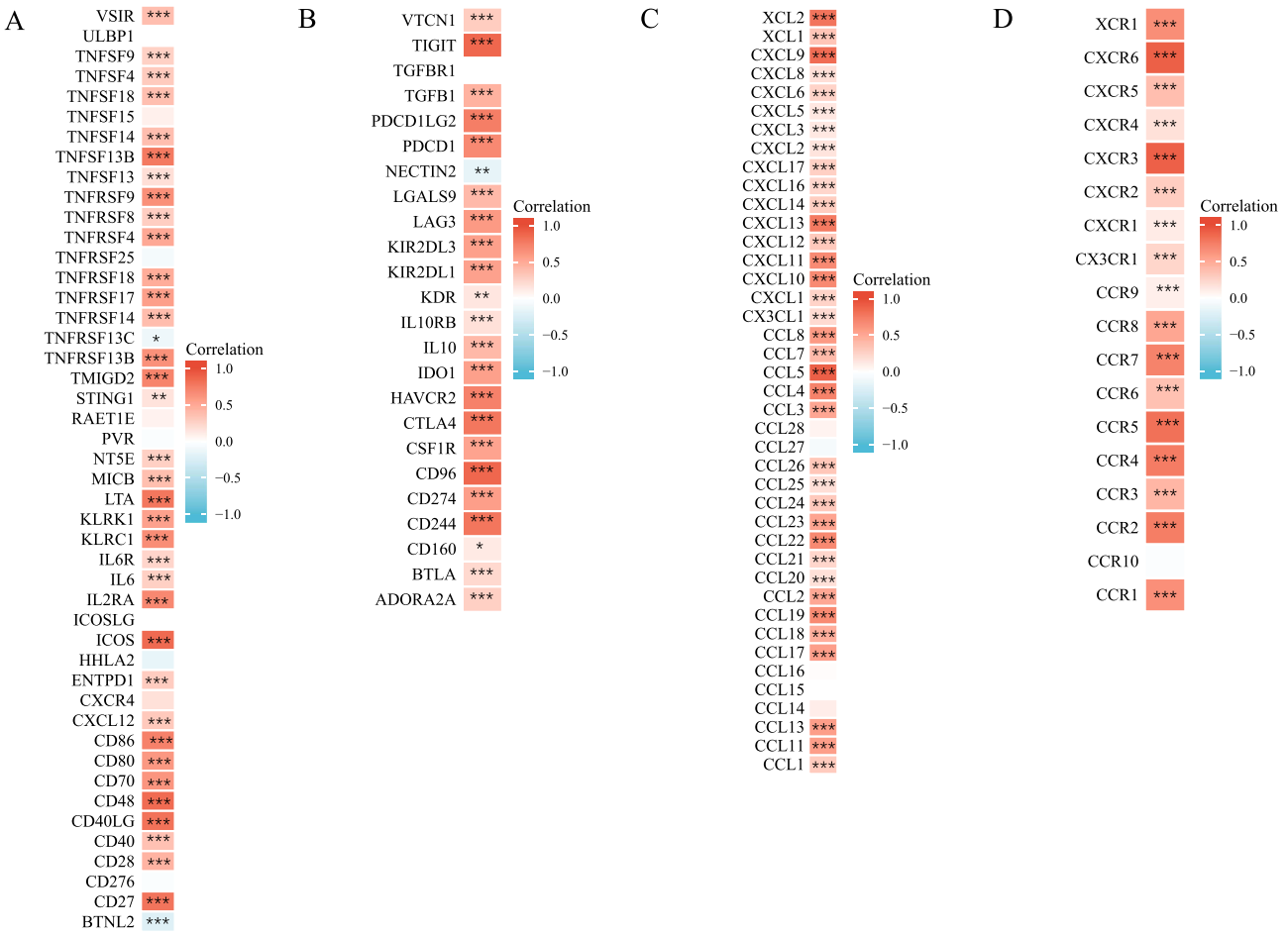


**Figure 9** The correlation between LNPEP and immunoregulation-related genes. (**A**) The heatmap represents the correlation between LNPEP expression and immune activating genes in OV. (**B**) The heatmap represents the correlation between LNPEP expression and immunosuppressive status related genes. (**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 ***$p < 0.001$.

In our study, we systematically evaluated the regulatory mechanism and clinical significance of LNPEP in ovarian cancer. Firstly, 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, 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 function profiles of LNPEP played in ovarian cancer progression and prognosis. According to the analysis of TCGA data, the results showed that mRNA levels of LNPEP were significantly downregulated in OV tissues. Subsequently, we used IHC analysis based on HPA database and Western blot technology to evaluate the protein level of LNPEP in OV, and the results were opposed to the mRNA expression levels. Interestingly, we speculated that post-translational modification altered the expression of LNPEP, such as phosphorylation, N-glycosylated, glycosylation, ubiquitination, etc. Moreover, the high expression of LNPEP predicted worse OS, DSS, PFI in patients with ovarian cancer. Therefore, we found strong evidence that LNPEP might be used as a prognostic biomarker in ovarian cancer patients.

To demonstrate the functional role of LNPEP, scRNA-seq database-CancerSEA was further implemented. We found LNPEP positively related to many functions in various cancer types. However, LNPEP was positively correlated to metastasis, angiogenesis, and EMT, negatively correlated to invasion and DNA repair in OV. The distinct functional roles of LNPEP in OV is most likely be due to inherent clinical heterogeneity or small amount of single cells collected basing

on scRNA-seq data. These observations supported our hypothesis that LNPEP was related to ovarian carcinogenesis and may function as a potential candidate marker for predicting the prognosis.

Meanwhile, basing on the KEGG and GO analysis, the expression of LNPEP was involved in various immune-related processed, 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, type 2 immune response, which served a critical regulatory role of LNPEP in immune response and immune-related process. LNPEP is known as a single-pass type II membrane protein that mostly present in the endosomal vesicles, where it could contribute to specific tumor antigen cross-presentation that localizes to the surface of the cell membrane as a receptor for angiotensin IV.[13] However, whether LNPEP is associated with regulating antitumor immunity and clinical significance in cancers remains unknown. Further exploring novel immunomodulators contributes to a better understanding the dynamic interaction in Tumor microenvironment (TME). The relationships between LNPEP and immune infiltration in OV were analyzed using 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, neutrophil, dendritic cells as well as positively correlated with immunostimulator. In addition, correlation between LNPEP expression and gene markers of diverse immune cells performed that LNPEP expression was associated with M1, M2 macrophage cells and functional T cells, including Th1, Th2, Tfh, Th17, Treg and exhausted T cells.

CD8+ T cells are the best representative killer cells in the T lymphocyte population. They are T cell-mediated cellular immunity, especially in anti-tumor immunity.[35,36] In our study, we showed that LNPEP expression was positively correlated with CD8+ T cells, macrophage, B cells, neutrophil, monocyte and dendritic cells using TIMER database. This finding may explain the protective role of LNPEP in most tumor types. Tregs have shown to exert suppressive effects and attribute to tumor cells escaped from the attack of cytotoxic CD8+ T cells.[37,38] Hence, we indicated that Treg infiltration levels were positively correlated with LNPEP, observing that, the function of cytotoxic CD8+ T cells is limited, even if their number is large.

Notably, exhausted T cells refer to the loss of functional capabilities in patients with chronic infections and cancer. A majority of patients with cancer usually have a large amount of T cells, but most of them are exhausted.[39] In our study, we revealed that LNPEP was positively correlated with marker genes of exhausted T cells, immune activating genes, and immunosuppressive genes in OV such as CTLA4, TIM3, PD-1, LAG3, and GZMB. These results mentioned LNPEP may be an important inducer of canonical features of T cell exhaustion and as an immune checkpoint. Therefore, we speculated that LNPEP could involve in critical and different roles in the regulation of the TME, which is needed to identify in specific stages and grade of OV. However, in vivo and in vitro experiment should be performed in testing the antitumor activity targeted LNPEP. Additionally, clinical trials should to be done to validate the immune checkpoint function of LNPEP.

Accordingly, these above data suggest that LNPEP is closely linked to OV immune infiltration and effect TME in OV. These findings emphasized LNPEP expression with various types of immune cell infiltration and T cell exhaustion. Therefore, LNPEP high-expression pattern might contribute to cancer dissemination and immune escape, leading to worse prognosis of OV.

## Conclusion

In conclusion, we conducted a comprehensive assessment of LNPEP, indicating its potential-related role as an indicator of prognosis and its immunoregulation effect in OV. LNPEP expression not only correlates with immune cell infiltration but also correlates with immunomodulators and chemokines. As a potential new immune checkpoint, LNPEP may be a target for OV immune therapy.

## 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.

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## Disclosure

The authors declare that they have no competing interests in this work.

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