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Lotus root polysaccharide inhibits the growth of ovarian cancer cells by blocking the cell cycle

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Author contributions

Xi-Feng Zhang designed the study and managed funding; Pei-Yu Dong performed bioinformatic analysis; Sheng-Lin Liang, Long Li and Jing Liu performed experiments. All authors have read and approved the manuscript.

Competing interests

The authors declare no conflicts of interest.

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Peer review information

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Abbreviations

OC, ovarian cancer; LRP, lotus root polysaccharides; MTT, 3-(4,5)-dimethylthiahiazo-(z-y1)-3,5-di-phenytetrazoliumromide; DEmRNA, differentially expressed mRNA; GO, Gene Ontology; EdU, 5-ethynyl-2'-deoxyuridine; KEGG, Kyoto Encyclopedia of Genes and Genomes; GSEA, gene set enrichment analysis; ROS, reactive oxygen species.

Citation

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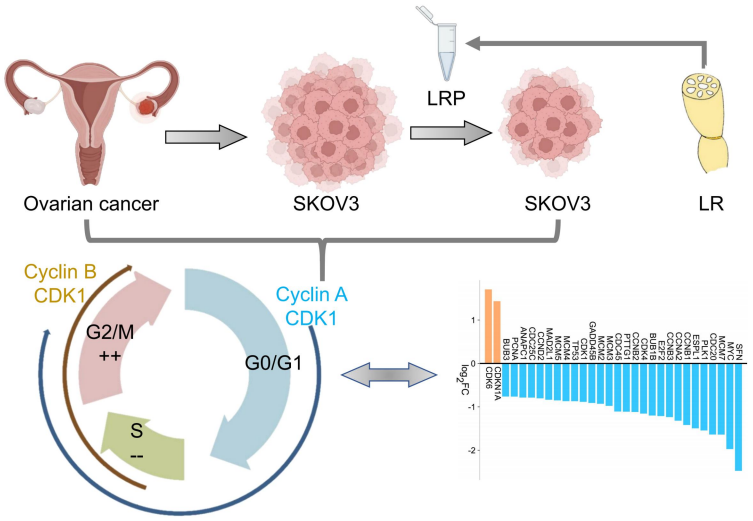
Abstract

Background: Lotus root polysaccharide is a natural antioxidant. As a new anticancer drug, it has anti-proliferation and pro-apoptotic effects in a variety of tumour cells, but its effect on ovarian cancer is not clear. In study, we attempted to elucidate the role and mechanism of lotus root polysaccharide in SKOV3 cells. **Methods:** In this study, the effect of lotus root polysaccharide on mRNA of SKVO3 cells was analyzed by RNA-seq, and verified by Western blot, flow cytometry, fluorescence detection and other techniques. **Results:** The results showed that lotus root polysaccharide could inhibit the proliferation of ovarian cancer cells. Then, a change in gene expression was found by RNA-seq. In the mRNA (differentially expressed mRNA) with these differences, significant changes in the cell cycle were found by Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis. Subsequently, the proportion of cells in S phase decreases and G2/M phase increases, as seen with propidium iodide staining. Gene Set Enrichment Analysis showed inhibition of the cell cycle, and the gene and protein expression of CDK1, CCNA1 and CCNB1 were inhibited. **Conclusion:** Our results show that lotus root polysaccharide can inhibit the growth of SKOV3 cells in vitro by blocking the cell cycle at the G2/M phase, which reveals the potential of lotus root polysaccharide in the treatment of ovarian cancer.

Keywords: lotus root polysaccharide; ovarian cancer; SKOV3; RNA-seq



Highlights

1. The transcriptional expression pattern of lotus root polysaccharides on SKOV3 cells was constructed.
2. The differential genes are mainly concentrated in p53 signaling pathway, DNA replication, cell cycle and so on.
3. Lotus root polysaccharides inhibits the proliferation of SKOV3 cells mainly by inhibiting the expression of CDK1, CCNA1, CCNB1.

Medical history of objective

Lotus (*Nelumbo nucifera* Gaertn.) is a well-known commercial aquatic perennial plant in the lotus family. It was first found in China and India and is now widely planted in Vietnam, Malaysia and other Southeast Asian countries. It has been planted for 3,000 years in China and is an aquatic cash crop for both medicine and food. Polysaccharides can be extracted from the roots and leaves of lotus, and their properties are variable. Studies have shown that lotus root polysaccharides can inhibit the growth of gastric cancer and liver cancer cells.

Background

Cancer is one of the most serious diseases and is among those with the highest morbidity and mortality in the world, with more than 8.2 million cancer-related deaths in recent years [1]. Ovarian cancer (OC) was the third most common gynaecological cancer worldwide in 2020 [2]. The incidence of OC in young women has been rising significantly, possibly due to an increased incidence of obesity, metabolic syndrome, oestrogen exposure and infertility [3]. Due to the concealment of OC in its early stages, about 60% of patients have reached an advanced tumour stage (III-IV stage) by the time a definitive diagnosis is made [4]. The lack of biomarkers in early detection and the emergence of drug resistance are the main reasons for the failure of OC treatment. Recently, research based on RNA-seq has expanded to understand the pathological origin and molecular tumorigenesis of OC. Analysis of gene expression and transcriptome changes is also helpful for understanding the classification and progression of OC [5].

At present, chemotherapy is one of the main treatments for OC, and platinum-related chemotherapy is the first-line treatment for OC [6, 7]. However, while most chemotherapeutic drugs inhibit cancer cells, they also have adverse side effects on normal tissues, resulting in a decline in patients' immune function, a serious impact on the human body, and a high cost of treatment [8]. Therefore, the search for a natural medicine with a low price, few side effects and a wide range of therapeutic effects has become a new research direction. Polysaccharides are natural macromolecular polymers, usually composed of more than 10 types of monosaccharides in the form of straight chains or branched chains through glycosidic bonds, and their molecular weights range from tens of thousands to millions of daltons [9]. Bioactive polysaccharides have always been a research hotspot in various scientific fields at home and abroad. Polysaccharides are bioactive substances of plant origin with antioxidant, immunomodulatory, anti-tumour, anti-inflammatory and hypoglycaemic activities [10–12].

Lotus (*Nelumbo nucifera* Gaertn.) is a well-known commercial aquatic perennial plant in the lotus family. It was first found in China and India and is now widely planted in Vietnam, Malaysia and other Southeast Asian countries. It has been planted for 3,000 years in China and is an aquatic cash crop for both medicine and food [13]. Polysaccharides can be extracted from the roots and leaves of lotus, and their properties are variable. Studies have shown that lotus root polysaccharides (LRP) can inhibit the growth of gastric cancer and liver cancer cells [14]. However, the inhibition mechanism of LRP on human OC cells has not been clearly studied. The RNA-seq technique was used to study the inhibitory effect of LRP on SKOV3 cells.

Experimental methods

Lotus root polysaccharide

The hot extraction method according to Yuan et al. [15] was used to extract LRP from the root of lotus. After degreasing of the lotus root powder, extraction of the crude polysaccharide, removal of protein, alcohol precipitation, purification of the LRP, freeze-drying and other steps, the LRP used in this experiment was obtained.

Cell viability

Using 3-(4,5)-dimethylthiahiazo-(z-y1)-3,5-di-phenytetrazolium-romide (MTT) assay (Beijing, China) according to the manufacturer's instructions, the culture medium with different concentrations of LRP was prepared for 24 h, MTT was incubated for 4 h, and formazan solution was added. After shaking and mixing, the absorbance of each well was measured at a wavelength of 490 nm in an ultra-sensitive, ultra-sensitive multi-function microchannel plate detector (Winooski, VT, USA).

Cell cycle detection

The Cell Cycle and Apoptosis Analysis kit (Shanghai, China) was used to detect changes in the cell cycle of SKOV3 caused by 300 μg/mL LRP for 24 h. Fix the collected cells at 70% alcohol at 4 °C for 24 h, propidium iodide staining solution was added and incubated in a warm bath at 37 °C away from light for 30 minutes. Finally, it is tested on the FACSAria III flow cytometer (San Jose, CA, USA). FlowJo_V10 software was used to analyse the results.

Detection of 5-ethynyl-2'-deoxyuridine (EdU) cell proliferation

Using the BeyoClick EdU Cell Proliferation kit with Alexa Fluor 555 (EdU-555) kit (Shanghai, China). Cell culture to appropriate number, according to the instructions, cultivate EdU, fix, penetrate, and click liquid reaction. After sealing, the film was observed and photographed under a Olympus fluorescence microscope (Tokyo, Japan). The SKOV3 that underwent DNA replication during incubation showed red fluorescence, while the nucleus showed blue fluorescence. The ratio of red and blue fluorescence was calculated with ImageJ software (version 1.53e) as the basis of the proliferation rate.

Reactive oxygen species (ROS) assay

The treated cells were removed from the cell culture medium and incubated in a diluted 2,7-dichlorodihydrofluorescein diacetate, in a 37 °C cell incubator, for 20 minutes. The cells were washed three times with serum-free cell culture medium. Excitation and emission wavelengths of 488 nm and 525 nm were used to detect the fluorescence intensity before and after stimulation, and the concentration of 300 μg/mL was photographed. ImageJ software (version 1.53e) was used to quantify the average fluorescence intensity.

Western blotting

Western blotting was used to detect protein quantity [16, 17]. In short, the sample was lysed in RIPA lysis solution to extract the total protein and then denatured. Next, the sample proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel electrophoresis at a constant voltage of 100 V. In this study, the proteins were transferred from sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels to polyvinylidene fluoride membranes using a semi-dry transfer method. Then, the membranes were blocked for 4 h and incubated with primary antibodies overnight at 4 °C. The membranes were washed three times with Tris buffered saline and incubated with secondary antibodies (1:1,200 dilution) at room temperature for 1.5 h. Finally, the BeyoECL plus kit (Shanghai, China) was used to detect the protein signal. Glyceraldehyde-3-phosphate dehydrogenase was used as a reference protein, and blots were analysed using ImageJ software (version 1.53k).

RNA-seq

mRNA sequencing was performed with the Hiseq 4000 platform by Novogene (Beijing, China). Sequence data were controlled, trimmed and filtered through fastp (version 0.19.5). Then, STAR was used to compare high-quality readings with the human reference genome

(GRCh38). The differentially expressed mRNA (DEmRNA) threshold was $\log_2$(fold change) ≥ 0.9 and P value < 0.05. FeatureCounts (version 1.6.3) software was used to calculate the expression of each gene and transcript. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed at https://www.bioinformatics.com.cn, an online platform for data analysis and visualization. The STRING database (https://string-db.org/) was applied to construct a protein-protein interaction network between differentially expressed genes, which was visualized by Cytoscape (http://www.cytoscape.org/). In order to understand the function of differential genes more widely, enrichment analysis of the GO cellular component, KEGG, Reactome and Wiki pathways databases was carried out by using the functional profiling function of g:Profiler set to user threshold = 0.01.

Results

Exposure of LRP inhibits SKOV3 growth and hinders cell cycle progression

To study whether LRP will affect SKOV3, we used the MTT cell proliferation assay to detect the effects of LRP on the proliferation of SKOV3 (Figure 1A). It was found that the inhibitory effect was concentration-dependent, but LRP had no obvious cytotoxic effect on SKOV3. LRP could increase the ROS content of SKOV3 cells and increased with increasing concentration (Figure 1B–D). The cluster dendrogram shows that the experimental results of 300 μg/mL LRP treated SKOV3 are statistically significant (Figure 1E). The cell cycle distribution was detected by flow cytometry. The results showed that SKVO3 was blocked in G2/M phase after exposure to LRP (Figure 1F, G). The EdU kit is a pyrimidine analogue, which can be integrated into double-stranded DNA in the process of DNA synthesis [18]. Therefore, the proliferation ability of SKOV3 was detected by using the EDU kit. It showed that the proportion of EDU-positive cells in the LRP treatment group was significantly lower than that in the control group, which further verified results of MTT and cell cycle assays (Figure 1H, I).

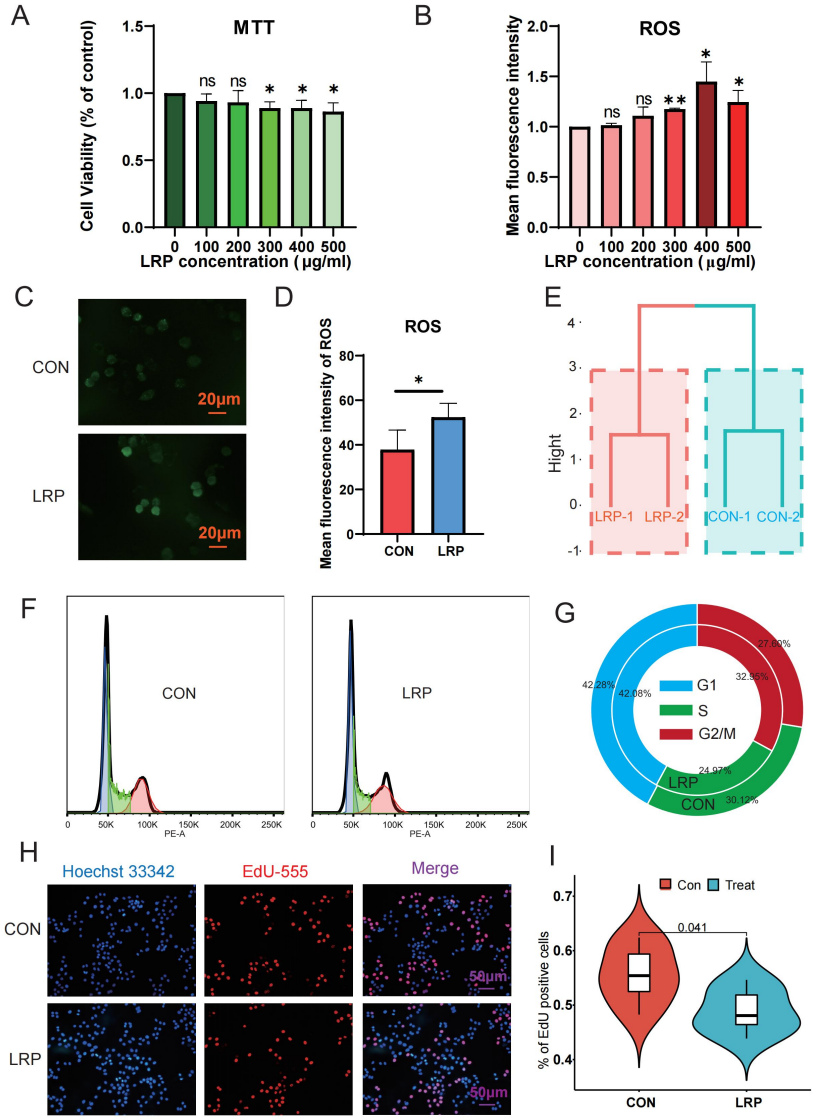


Figure 1 LRP inhibits cell cycle and proliferation of SKOV3 cells. (A) Effects of different concentrations of LRP on the viability of SKOV3 cells. (B) Effects of different concentrations of LRP on ROS of SKOV3 cells. (C, D) Pictures of the effect of 300 μg/mL LRP on ROS of SKOV3 cells and statistical results of average fluorescence intensity; Bar = 20 μm. (E) The cluster dendrogram shows that the experimental results of 300 μg/mL LRP treated SKOV3 are statistically significant. (F, G) After treatment with LRP for 24 hours, the cell cycle distribution of SKOV3 cells was G1 phase in blue, S phase in green, and G2/M phase in red. (H) Immunofluorescence photos of EdU positive cells (red) and nuclei (blue) 24 hours after LRP treatment; Bar = 50 μm. (I) Statistical results of the proportion of positive cells after 24 hours of LRP treatment ; Wilcoxon test = 0.041. The results were showed as mean ± SEM (n ≥ 3); *p < 0.05, ** p < 0.01 vs. the control group. LRP, lotus root polysaccharides; EdU, 5-ethynyl-2'-deoxyuridine; MTT, 3-(4,5)-dimethylthiahiazo-(z-y1)-3,5-di-phenytetrazoliumromide; ROS, reactive oxygen species.

LRP exposure changes SKOV3 mRNA expression

In order to clarify the potential mechanism of inhibition of SKOV3 proliferation by lotus root polysaccharide exposure, transcriptional sequencing was used to determine RNA expression. The PCA map showed that SKOV3 gene expression was significantly altered after LRP treatment (Figure 2A). The volcanic map shows that 1372 mRNA was up-regulated, and 1378 mRNA was down regulated after treatment (Figure 2B). The heat map of the first 500 DEmRNAs expression profiles showed that after LRP treatment, more genes in SKOV3 were significantly down-regulated (Figure 2C). These results showed that compared with the Con, the SKOV3 cells treated with LRP were inhibited as a whole, which was consistent with the phenotypic results. In order to further understand the molecular mechanism of SKOV3 inhibition induced by LRP, GO and KEGG pathway analysis was performed on DEmRNAs. GO terms related to proliferation were enriched in biological processes, cellular components and molecular functions (Figure 2D). Among the first 20 KEGG pathways analysed, the highest degree of enrichment was in the p53 signalling pathway, DNA replication and the cell cycle (Figure 2E). Show more related pathways by secondary classification of KEGG enrichment results. Secondary enrichment analysis of all KEGG pathways showed that cellular processes were significantly enriched, especially cell growth and death (Supplementary Figure S1). Functional profiling with g:Profiler showed that the enrichment results for several databases were related to the cell cycle (Supplementary Figure S2).

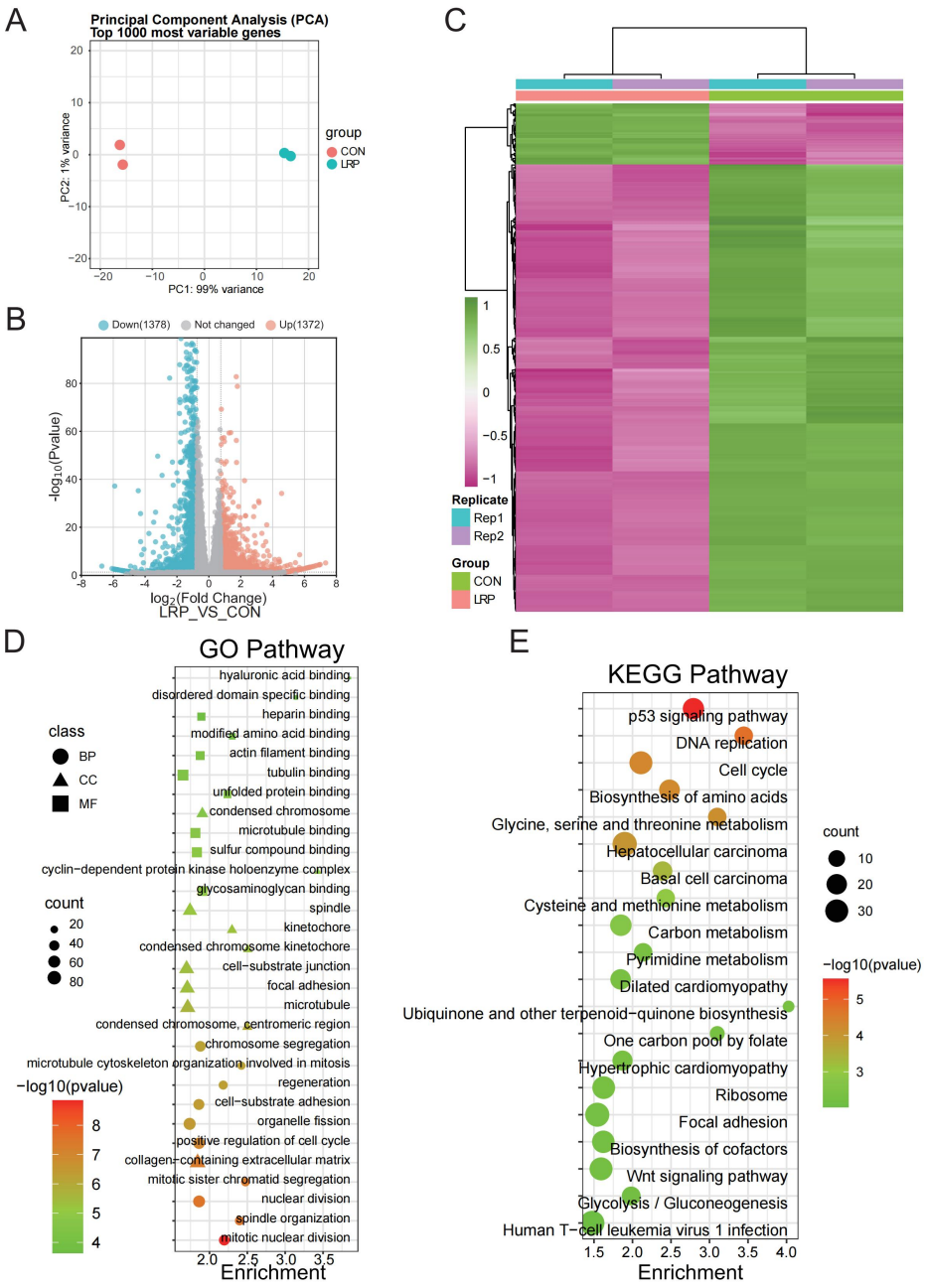


Figure 2 LRP change the expression of mRNA in SKOV3 cells. (A) Correlation analysis between groups (CON = control group; Treat = 300 μg/ml LRP). (B) The MA plot showed the distribution of DEmRNA between the LRP treated group and the control group. (C) Heat map analysis of the top 500 DEmRNAs. (D) The bubble diagram confirmed the GO concentration of DEmRNAs. (E) The bubble chart shows the condensed analysis of DEmRNAs pathways by the KEGG. LRP, lotus root polysaccharides; DEmRNA, differentially expressed mRNA; GO, Gene Ontology; EdU, 5-ethynyl-2'-deoxyuridine; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological processes; CC, cellular components; MF, molecular functions.

LRP inhibits the cell cycle of SKOV3 by down-regulating the expression of cell cycle-related genes

From the perspective of abundant KEGG pathways and GO conditions, cell cycle pathways play an indispensable role in LRP-induced cell cycle arrest. In addition, the function of DEmRNAs was studied by gene set enrichment analysis (GSEA) (Figure 3A). GSEA analysis showed that the checkpoints of KEGG_CELL_CYCLE and KEGG_DNA_REPLICATION in the LRP-treated group were lower than those in the control group. In the treatment group, gene expression at the checkpoint was inhibited. The genes enriched by KEGG_CELL_CYCLE and KEGG_DNA_REPLICATION are shown in a string diagram to show the expression and interrelationship (Figure 3B). We separately screened the DEmRNAs in the cell cycle signalling pathway and showed the expression models of these DEmRNAs by heat map (Figure 3C). By using a string database to predict protein-protein interactions, the results show that cyclin A, cyclin B and CDK1 in DEmRNAs are key genes in the regulatory network of cell cycle arrest (Figure 3D). These data suggest that LRP exposure affects the expression of mRNA involved in the cell cycle distribution. Western blotting results further showed the expression of SKOV3-related genes CCNA1, CCNB1 and CDK1 was reduced after exposure to lotus root polysaccharide (Figure 3E–G).

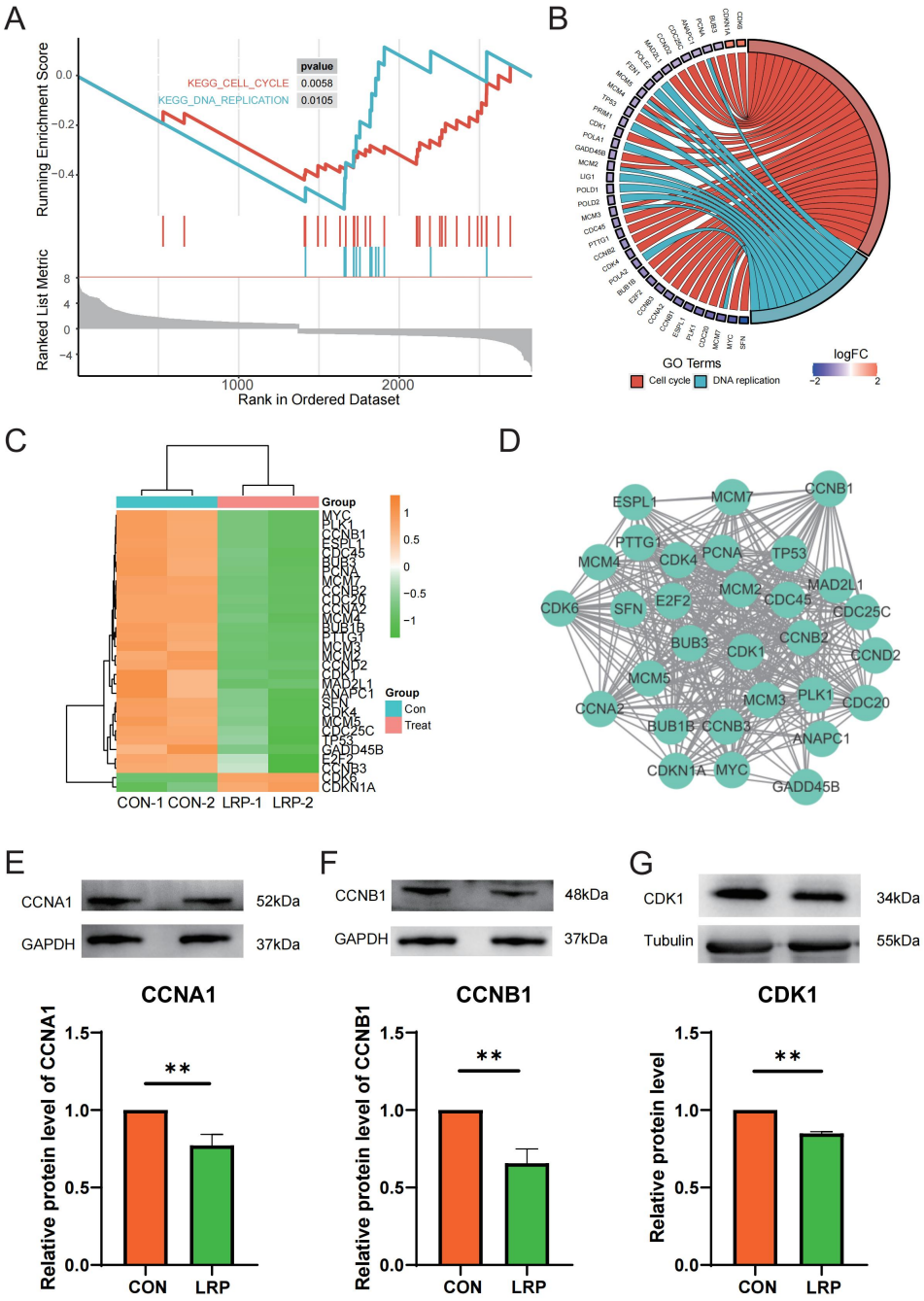


Figure 3 LRP affects the cell cycle of SKOV3 cells. (A) The results of GSEA between the groups. (B) The string diagram shows the cell cycle and DNA replication in the KEGG process. (C) Hierarchical cluster heat map showing DEmRNAs of cell cycle. (D) Cytoscape shows the protein-protein interaction network of cell cycle-related DEmRNAs. (E–G) The expression of CCNA1, CCNB1, CDK1 in stem cells was detected by Western blotting. The cells were treated with 300 μg/mL LRP for 24 hours. The results were showed as mean ± SEM (n ≥ 3); *$p < 0.05$, **$p < 0.01$ vs. the control group. LRP, lotus root polysaccharides; GSEA, gene set enrichment analysis; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEmRNA, differentially expressed mRNA; GO, Gene Ontology.

Discussion and conclusion

As one of the major diseases harmful to women's health, OC is a going concern all over the world. OC is mostly sporadic, and the cause is unknown: only 20%–25% of patients have a genetic family history [19]. At present, surgery and chemotherapy are the main treatment methods. The goal of surgery is satisfactory tumour cell reduction after R0 resection [20]. The postoperative first-line chemotherapy regimen is a platinum-containing regimen, such as paclitaxel plus carboplatin, docetaxel plus carboplatin, liposome doxorubicin plus carboplatin and so on [21, 22]. Our provides a new perspective for the anti-OC mechanism of LRP, and LRP is expected to become an effective drug for the treatment of OC.

Many studies have shown that polysaccharides contribute to the development of functional foods for cancer treatment. Natural polysaccharides have significant biological activity and anti-tumour effects [23]. Clinically, medicinal plant polysaccharides mainly include ginseng polysaccharides, *Astragalus* polysaccharides, *Ganoderma lucidum* polysaccharides, lotus polysaccharides, lentinan polysaccharides, rumen cocci polysaccharides and so on, which have attracted much attention because of their remarkable anti-tumour activity [24]. *Balanophora polyandra* polysaccharides are natural polysaccharides that can inhibit the proliferation of OC in rats and SKOV3 in vitro [25]. Polysaccharide isolated from polysaccharide isolated also plays a key role in inhibiting OC [26, 27]. *Bangia fuscopurpurea* is a new polysaccharide extracted from *Artemisia maculata*. It has been proved that it can increase the accumulation of ROS in A2780OC cells and decrease the mitochondrial membrane potential, thus inducing apoptosis of A2780OC cells [28]. An acidic polysaccharide isolated from *Codonopsis pilosula* can inhibit the proliferation of HO-8910 cells [29]. Lotus root belongs to the *Magnolia* subclass, mountain dragon eye. The medicinal value is quite high, and its roots, leaves, flowers and fruits are all treasures, which can be nourished and used as medicine. Lotus root is the plump underground stem of lotus, which is edible. The LRP extracted from the root of lotus in this study can inhibit the proliferation of SKOV3 and increase the expression of ROS. There are few reports on the role of LRP in OC. We studied the effect of LRP on OC cells and found that LRP also exerts its anti-tumour activity through its anti-proliferation effect.

Through MTT detection, we found that the inhibition of SKOV3 by LRP showed a concentration gradient, and when the concentration was 300 μg/mL, it could inhibit proliferation. In order to further determine the inhibitory effect, we used EdU-555 to detect the effect of 300 μg/mL LRP on SKOV3 DNA synthesis and found that the inhibition rate was similar to that of MTT. Moreover, LRP can increase the ROS content of SKOV3. However, further study is required to elucidate the molecular mechanisms.

RNA-seq results showed that LRP significantly changed the gene expression of SKOV3, which proved to have a significant effect. The results of GO enrichment showed that LRP had significant effects on molecular function, biological processes and cellular components of SKOV3. The mitotic cycle of the cell cycle can be divided into three stages: the pre-DNA synthesis stage (G1 phase), the DNA synthesis stage (S phase) and the post-DNA synthesis stage (G2 phase) [30]. During the cell process, cyclin A and cyclin B activate CDK1 to regulate mitosis and meiosis [31]. Mitosis is triggered by the activation of CDK1, CDK1 can promote the conversion of G2/M phase. During the transition period from G2 to M, both CyclinA and CyclinB were expressed in a certain period. Both cyclins can bind and activate CDK1 [32]. Protein-protein interaction results show that CDK1 is associated with each gene in the cell cycle and is itself a key gene in the cell cycle. Propidium iodide staining showed that the DNA synthesis of OC cells was inhibited, and the cell cycle was blocked in G2/M phase after LRP treatment. Through GSEA analysis, we found that DNA replication and cell cycle cell progression in SKOV3 treated with LRP were inhibited. Most genes related to the cell cycle are suppressed more intuitively through string maps and heat maps. The expression of CCNB1, a gene related to cell cycle progression in G2max M, was decreased at both gene and protein levels. Our studies have shown down-regulation of the expression of these genes and proteins, which inhibits the cell cycle in SKOV3 cells at G2max/M phase, thus inhibiting cell proliferation. To summarize, the results of this study show that LRP inhibit the proliferation of SKOV3 cells by blocking cell cycle in G2/M phase (Figure 4).

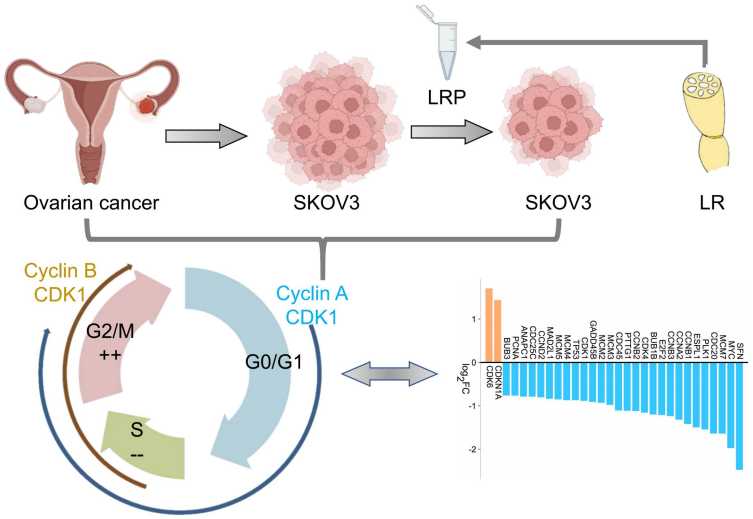


Figure 4 Mechanism diagram of cell cycle arrest by LRP. LRP inhibits cell proliferation by blocking SKOV3 in G2/M phase. Created with BioRender.com. LRP, lotus root polysaccharides; LR, lotus root.

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