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Studies on the Influence of Liriopes Radix Nanovesicles on Inflammatory and Immune-Related Factors in Oral Squamous Cancer

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ABSTRACT

Background: The relationship between Liriopes Radix nanovesicles (LRNVs), oral squamous cell carcinoma (OSCC), inflammatory and immune-related factors has not been studied.

Methods: The LRNVs were characterized and quantified. The uptake of nanovesicles was observed by laser confocal scanning microscopy. Plate cloning experiments, transwell experiments and apoptosis experiments were carried out to explore the effect of LRNVs on OSCC in vitro. The expressions of Bad, Bax, Bcl-2, MMP9 and NFκB were detected by western blot. A xenograft model of oral cancer in C57BL/6J mice was constructed and the DiR-stained LRNVs was administered to mice via intraperitoneal injection to observe the fluorescence aggregation in the abdomen of mice. Mass spectrometry flow cytometry was performed to detect changes of inflammatory and immune-related factors in tumor tissues. H&E staining of heart, liver and kidney of mice was applied to evaluate the safety of LRNVs.

Results: LRNVs were negatively charged nanoscale vesicles containing various proteins, which could be internalized into OSCC cells. It can exert anti-OSCC effect and improve the expression of inflammatory and immune-related factors such as granzyme_B, IFNγ, iNOS and TNF-α in tumor tissues.

Conclusion: LRNVs have anti-OSCC effects and they may play a role in immune regulation by increasing granzyme_B, IFNγ, iNOS and TNF-α *in vivo*.

Keywords: Liriopes Radix Nanovesicles; Oral Squamous Cell Carcinoma; Inflammation; Immune-Related Factors; Mass Spectrometry Flow Cytometry

Introduction

According to GLOBOCAN data, there were more than 300,000 new cases of oral cancer and lip cancer and more than 170,000 deaths from oral cancer and lip cancer worldwide in 2020 [1] and oral squamous cell carcinoma (OSCC) becomes a disease of great concern. At this stage, treatment is often selected based on the clinical stage of OSCC [2]. At present, multidisciplinary treatment is advocated with surgery supplemented with chemoradiotherapy and radiotherapy and some emerging treatment methods are gradually recognized by clinicians [3]. In recent years, nanotechnology and immunotherapy have shown great potential in the study of OSCC treatment modalities [4]. Inflammation and immune dysregulation are the key to the progression of OSCC and are closely related to tumor immunotherapy.

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The superficial skin or mucous membranes often break down when the tumor is too large, and immune cells and factors usually play an important role in wound healing [5]. Therefore, the search for novel therapeutic drugs that can modulate the inflammatory process and enhance the immune response is essential to improve the treatment outcome of OSCC. Nanovesicles are an emerging tissue engineering material that can modulate local immune responses and promote wound healing and tissue regeneration [6].

Many studies have reported that extracellular vesicles from various plant sources can influence the immune response process [7,8] and play an important role in modulating immune function and inflammatory response [9]. For example, ginseng-derived nanovesicles can exert anti-melanoma effects as immunomodulators [10,11]; nanovesicles derived from edible plants such as cabbage [12], citrus [13], ginger, carrot and grapefruit [14] have obvious anti-inflammatory effects. Tea-derived nanovesicles can stimulate immune system to delay breast cancer' development [15]; Novel plant nanovesicles from roses have immunostimulatory effects [16]. Therefore, it is speculated that as a plant extracellular vesicles, Liriopes Radix nanovesicles (LRNVs), may also be involved in inflammatory response or immune regulation processes and are associated with tumor suppression. However, the relationship between LRNVs and OSCC is still unclear and the expression of inflammatory and immune-related factors after LRNVs act on OSCC cells has not yet been reported. Therefore, this study related factors related to LRNVs, OSCC, inflammation and immunity were interlinked, and the changes of cell function, tumor growth, inflammatory and immune-related factors after LRNVs acting on OSCC were comprehensively explored, so as to provide a direction for more research on medicinal plants.

Materials and Methods

Extraction of LRNVs

An appropriate amount of fresh rhizomes of Liriopes Radix (LR) that was purchased from the market of fresh Chinese herbal medicines were selected and cut after washing, then added pre-cooled PBS (Servicebio, G4202-500mL) to crush them in the wall breaker. The large solid residues were filtered out and the supernatant was obtained by differential centrifugation. After centrifugation of the supernatant in the rotor of an ultra-high-speed refrigerated centrifuge, the pellet was collected by resuspension using PBS. Transferred the resuspension to a sucrose solution containing different mass fraction gradients at a speed of 150,000×g and centrifuge for 2h. Then collected the middle layer of 30%~45% sucrose solution, added an appropriate amount of PBS and centrifuged for 1h to wash off the sucrose. Finally, a small amount of PBS was used to collect the precipitate and the fresh nanovesicles solution of LR was obtained after filtration with a 0.22μm disposable vacuum filter (BIOFIL, FPV203150).

Characterization and Identification of LRNVs and Analysis of Protein Fractions

100 μL of the isolated LRNVs sample were resuspended in an appropriate amount of PBS, and their surface charge was detected by a nanoparticle size analyzer (Zetasizer Nano Zs90, Malvern); 10μL sample were added dropwise to a 300-mesh copper mesh with a support membrane, allowed to stand for 15 min, and then counterstained with 2% phosphotungstic acid dropwise for 1 min and the excess liquids were sucked off from the edge of the droplet before imaging with HT-7800 transmission electron microscope; Protein fractionation identification (RIGOL L-3000 HPLC system) of LRNVs were performed using a data-independent scanning mode and GO analysis and KEGG analysis were performed on the identified proteins.

Cell Culture

The mouse oral carcinoma (MOC2) cell lines (obtained from National Infrastructure of Cell Line Resource) grew with culture medium prepared in a DMEM basal medium: fetal bovine serum: penictomycin solution volume ratio of 89:10:1.

Cell Uptake Assay and IC50 Assay

An appropriate amount of 5μM DiO dye (Beyotime, C1038) was co-incubated with the LRNVs for 30 min, centrifuged and resuspended with PBS to obtain the LRNVs labeled with DiO fluorescent probe (DiO-LRNVs). DiO-LRNVs were added to the medium containing cell crawlers with DiO-PBS as a negative control. The cells were fixed after 3h, 6h, and 12h of co-incubation, the nuclei were clean up with PBS and stained with DAPI staining solution and finally covered with a coverslip. The uptake of LRNVs by MOC2 cells under different incubation times was obtained under laser scanning confocal microscopy (LSCM, OLYMPUS DP72). The CCK8 cell proliferation and activity detection kit (Mei5 Biotechnology Co.,Ltd) was used to detect cytotoxicity and calculate the IC50 and the experimental groups were determined.

Colony Formation Experiments

500 MOC2 cells were seeded into a six-well plate per well, the original medium was removed the next day and complete medium containing 0 μg/uL, 15 μg/uL, and 30 μg/uL LRNVs was added according to the group. The cells were evenly dispersed by gently patting the edge of the well plate to compare whether there was any difference in the clone formation rate of cells between the groups.

Transwell Assays

Cell suspensions were seeded in Transwell chambers (Corning Life Sciences, United States) and different concentrations of LRNVs were added to test its effect on MOC2 cells' ability of migration and invasion. Invasion experiments require prior glue spreading. After 24 hours of incubation, the original culture medium in the 24-well

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plate was removed, then the unworn cells in the upper chamber were carefully wiped off with a wet cotton swab after methanol fixation, 1% crystal violet staining and PBS washing. Finally, the data were observed and recorded under the microscope after waiting for the chamber to dry.

Flow Cytometry

Using a 12-well plate to seed 1×105 cells/(well·mL). Samples were prepared according to the AnnwxinV Alexa Fluor 647/PI Apoptosis detection Kit (Beijing 4A Biotech Co., Ltd) and apoptosis situation was analyzed by using Flowjo_V10 software.

Western Blot

The proteins of MOC2 cells co-incubated with different concentrations of LRNVs were extracted. Next, the protein samples were electrophoresised, transferred, blocked and incubated with antibodies of Bad, Bax, Bcl-2, MMP9 and NFκB which were purchased from Uping Bio. Finally, the gray values of bands were analyzed by imageJ software to analyze the changes in protein expression.

Animal Experiments

In order to determine whether LRNVs can be absorbed into body after intraperitoneal injection, we co-incubated DiR with LRNVs and washed off excess DiR using an ultracentrifuge and resuspended the pellet with PBS to obtain DiR-labeled LRNVs. After the mouse tumor volume was close to 100 mm^3, 100 μL of resuspension were injected intraperitoneally. DiR-PBS were used as the control group and the fluorescence distribution of DiR-LRNVs in mice for different times (0 min, 5 min, 15 min, 0.5 h, 1 h, 2 h, 6 h, 12 h, 24 h) after injection was observed by using a small animal in vivo imager (AniView-680, Guangzhou Boluteng Biotechnology Co., Ltd.). At the same time, after the last in vivo imaging, the tumors, hearts, livers, spleens, lungs and kidneys of mice were dissected, the fluorescence expression on all the organs was also observed. In addition, we employed hemolytic activity assays for qualitative and quantitative studies of hemolytic phenomena to assess the biocompatibility of LRNVs in vivo. A total of 12 healthy mice (♂6, ♀6, 6~8w, 15~20g) were used to construct an ectopic xenograft model of mouse oral squamous cancer. After tumorigenesis, the mice were randomly assigned to two groups -- treatment group (LRNVs) and control group (NC).

All the mice were administered by intraperitoneal injection. The administration concentration of LRNVs in the treatment group were 6.0 mg/kg and the control group were injected with PBS, and the dosing frequency was set to once a day. The tumor size, weight and weight of the mice were recorded during the operation. At the end of the experiment, mouse heart, liver, kidney and tumor tissues were dissected, the heart, liver and kidney were stained by H&E staining, mouse tumor tissues were dissociated into single-cell suspension and then stained with protein. At last, the expressions of inflammatory and immune-related factors were detected by mass spectrometry flow cytometry (Fluidigm Helios CyTOF). All mice were housed in SPF grade animal houses.

Statistical Analysis

The variance homogeneity was tested by the analysis of variance method of complete random design, the differences between multiple groups were compared using one-way ANOVA and the LSD-t or Dunnett's method was applied to analyse the differences between two groups. Where appropriate, Student's t-test was used. The results of all experiments were replicated independently for multiple times (n ≥ 3).

Results

LRNVs were Negatively Charged Nanoscale Particles with Many Proteins that can Enter MOC2 Cells

The Zeta potential detection of LRNVs showed that their charge was around -7mV, which was relatively stable (Figure 1a). Transmission electron microscopy (TEM) can be seen as a bilayer membrane vesicle structure with a diameter of about 50-150 nm in the shape of a "cup holder" (Figure 1b). More than 2000 proteins (Figure 1c, supplementary files 1) were detected in LRNVs, including argininosuccinate lyase, glutamine synthetase, ATP citrate synthase, etc. GO analysis suggested that most of the detected proteins were concentrated in cytoplasm to perform functions, closely related to phosphorylation and had ATP-binding transporter activity (Figure 1d). In addition, among the enriched KEGG pathways, the glycolysis/gluconeogenesis, proteasome and the pyruvate metabolism pathway were the top three (Figure 1e). The experimental results showed that the viability of MOC2 cells decreased with the increase of LRNVs concentration (Figure 1f). According to the calculated IC50 of LRNVs for 24 hours, 15 μg/μL of LRNVs were added to the low concentration group of MOC2 cells and 30 μg/μL of LRNVs were added to the high concentration group for follow-up experiments. LSCM showed that the content of LRNVs in MOC2 cells increased with the increase of co-incubation time (Figure 2).

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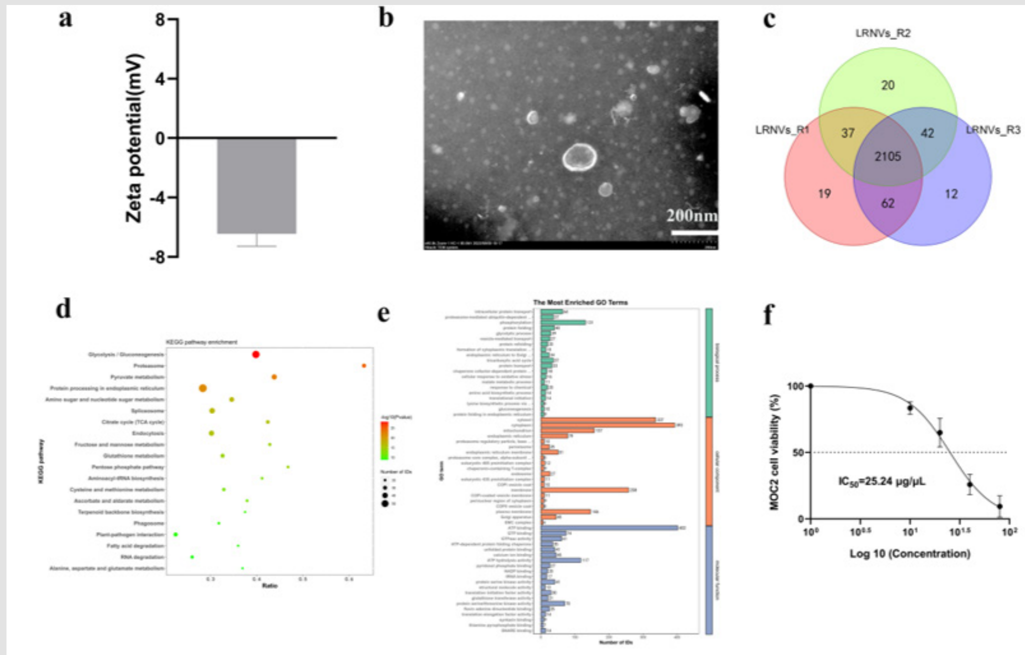

Figure 1: Characterization, protein components, functional analysis and IC50 testing of LRNVs.

a) Potentiogram,
b) TEM
c) Scale bar at 200 nm), Venn plot
d) GO analysis
e) And KEGG
f) Analysis. With the increase of LRNVs concentration, their ability to inhibit MOC2 cell activity is also enhanced.

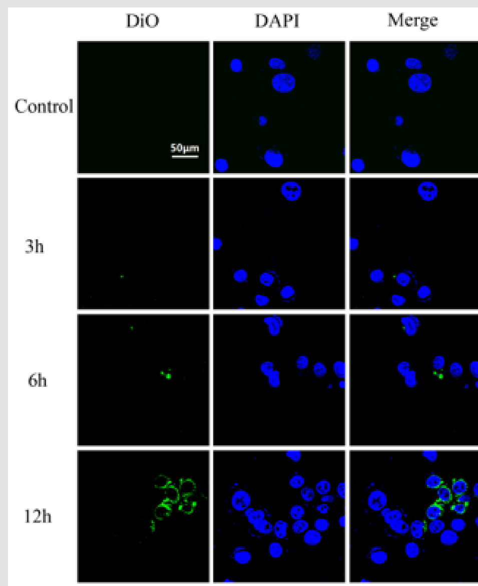

Figure 2: LSCM results of LRNVs.

Effect of LRVNs on MOC2 Cell activity

LRNVs Can Inhibit Colony Formation, Migration and Invasion of MOC2 Cells: After 24h of LRNVs, the colony formation level (Figure 3a), migration (Figure 3b) and invasion (Figure 3c) ability of MOC2 cells in 15 μg/μL and 30 μg/μL of LRNVs were significantly lower than those in the control group and the inhibitory effect of LRNVs on MOC2 cell function increased with increasing concentration. The difference was statistically significant.

LRNVs can affect the apoptosis of MOC2 cells as well as Protein Expressions of Bad, Bax, Bcl-2, MMP9 and NFκB: Compared with the control group, the apoptosis of MOC2 cells in 15 μg/μL and 30 μg/μL of LRNVs increased when LRNVs were co-incubated with MOC2 cells for 24h and the apoptosis rate was statistically significant (Figure 3d). The results of WB showed that the relative expressions of Bad and Bax protein increased in 30 μg/μL of LRNVs while Bcl-2, MMP9 and NFκB protein expressions decreased in both 15 μg/μL and 30 μg/μL of LRNVs (Figure 3e).

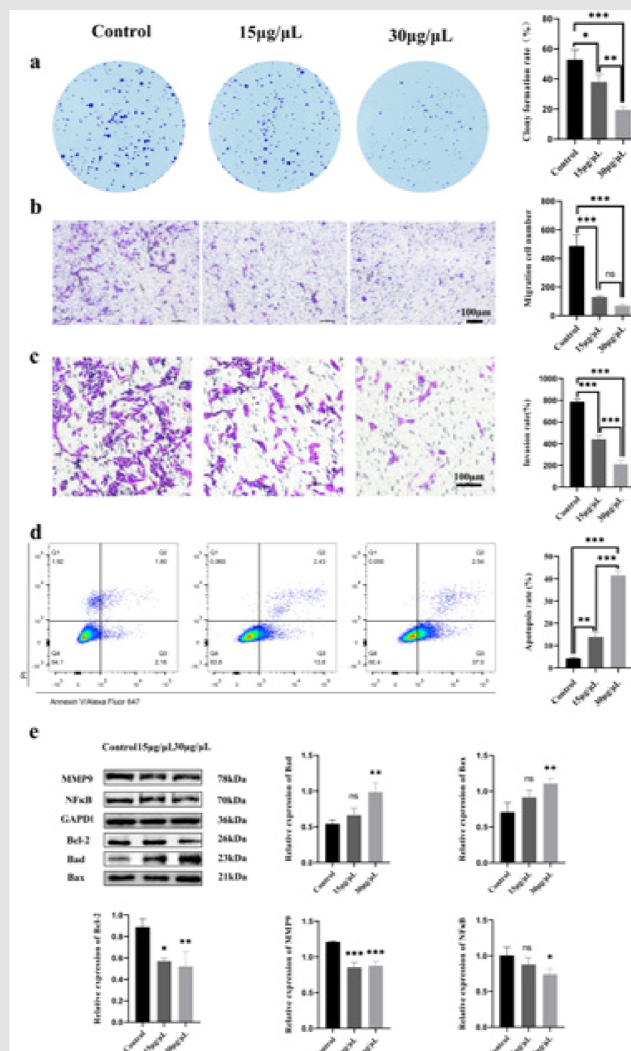

Figure 3: LRNVs can affect the activity of MOC2 cells.

a) LRNVs can inhibit colony formation,
b) Migration
c) invasion
d) Of MOC2 cells, and can promote apoptosis
e) Apoptosis-related factors Bax and Bad, at the same time, inhibit the expression of Bcl-2
f) The high concentration group had a stronger inhibitory effect on cell viability than the low concentration group. *P<0.05, **P<0.01, ***P<0.001

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Distribution, Stability and Security Analysis of LRNVs in C57BL/6J

In vivo imaging of small animals showed strong fluorescence aggregation in the abdomen of C57BL/6J mice and the fluorescence expression range increased over time (Figure 4a). In addition, compared with the DiR-PBS group, the DiR-LRNVs group had different degrees of fluorescence aggregation in other organs except the heart (Figures 4b & 4c). The results of hemolysis test showed that the hemolysis rate of each concentration was less than 5%, which met the national standard (Figure 4d). It had been shown that the use of LRNVs in vivo were relatively safe.

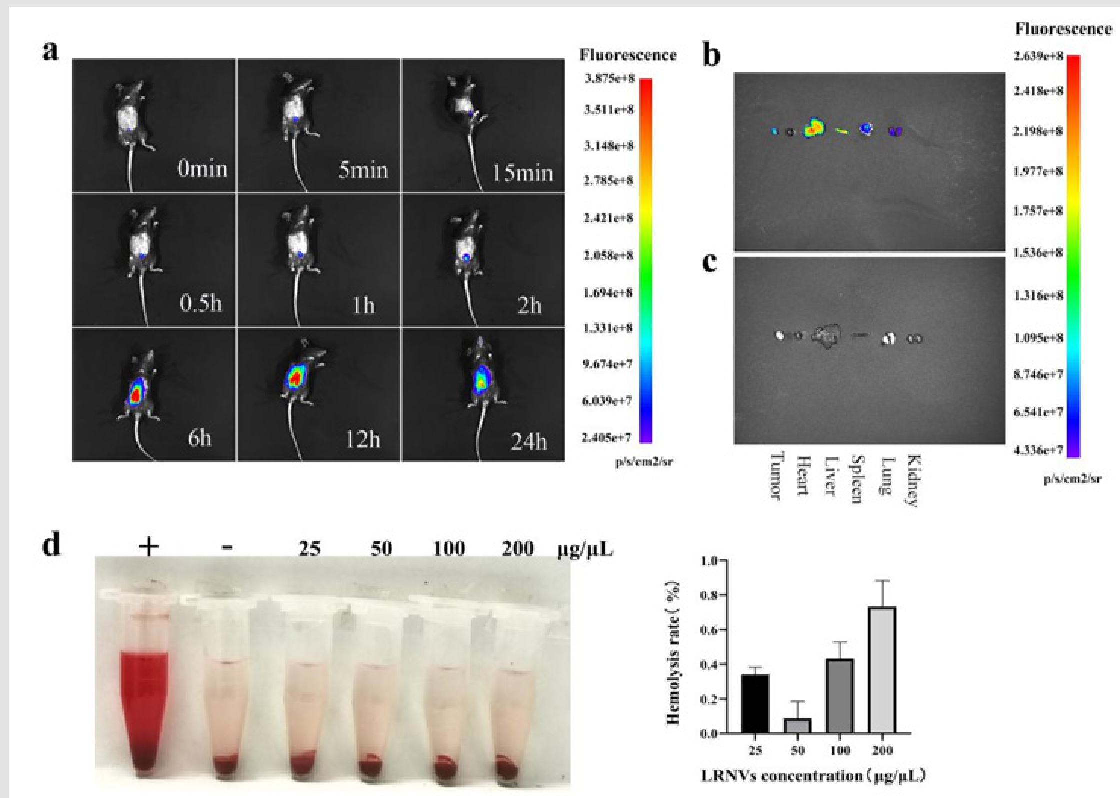

Figure 4: Fluorescence aggregation of tumors and some organs in mice and hemolysys rate.

a) Fluorescence distribution at 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 6 h, 12 h, and 24 h after injection,

b) The fluorescence aggregation in tumors and organs of mice in treatment group,

c) Control group.

d) 24 hours later. With the increase of injection time, the fluorescence range became larger but the intensity decreased after 24 h. Compared with the control mice, the tumor, liver, spleen, lung and kidney of mice in treatment group were expressed in different degrees of fluorescence. The hemolysis rate of each concentration.

LRNVs' Anti-Tumor Properties, Security Testing and CyTOF Analysis of Immune Invasion

The general outlook of the mice at the endpoint of the experiment were similar (Figure 5a). With the increase of dosing time, the body weight of mice in both control group and treatment group remained in the range of 16~20g (Figure 5b). The tumor volume growth curve showed that the tumor volume of mice in both control group and treatment group gradually increased over time, but the tumor volume growth speed of mice in treatment group was significantly slower than that in control group after the 4th day of administration (Figure 5c). After the end of administration, the tumors of the mice were dissected and weighed. It was found that compared with the control group (0.99±0.05) g, the tumor weight of the mice in the treatment group (0.69±0.05) g significantly decreased (Figures 5d & 5e). In H&E staining, the pathological morphology of heart, liver and kidney tissues did not show much difference between the two groups (Figure 5f). The results of CyTOF analysis indicated that changing circumstances of inflammatory and immune-related factors such as granzyme_B, IFNγ, iNOS or TNF-α in tumor tissues in treatment group increased compared with control group (Figures 5g & 5h).

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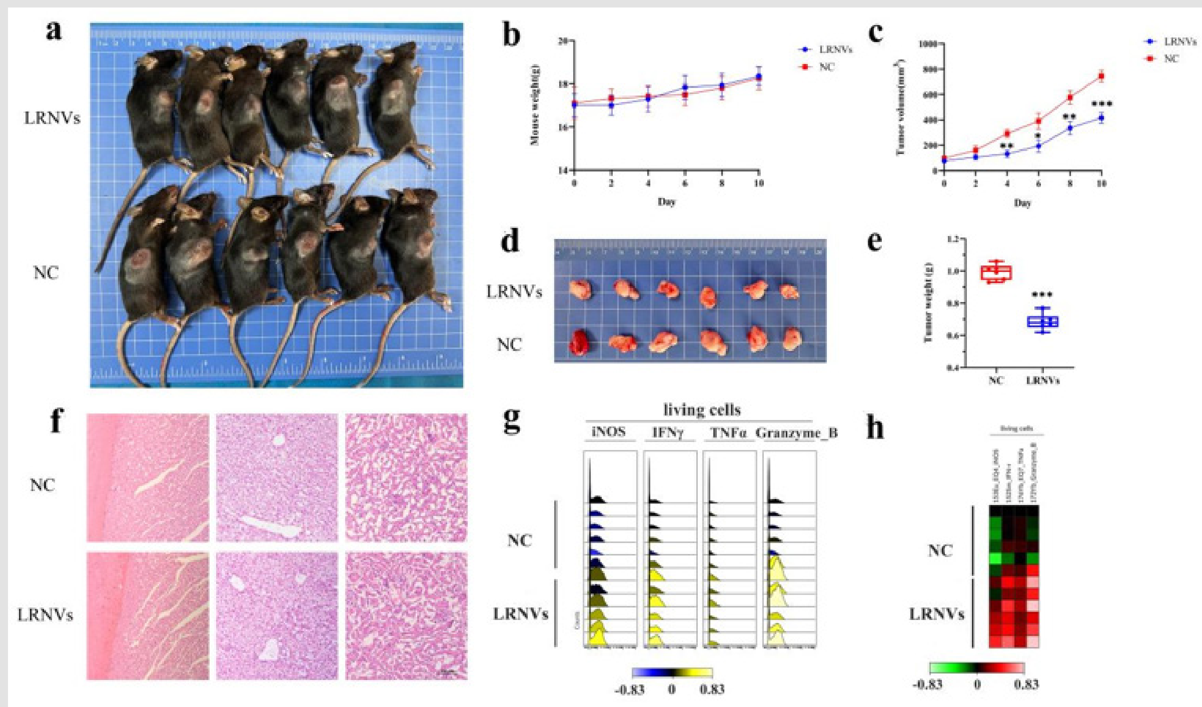

Figure 5: Effects of LRNVs on body weight, tumor volume, tumor weight, pathological morphology of heart, liver and kidney, and immune infiltration of tumor tissues in mice.

a) LRNVs had no significant effect on the shape,
b) Body weight
c) Tumor volume,
d) Tumor weight
e) Tumor weight
f) Heart, liver and kidney pathological morphology
g) Of mice. The expression of granzyme_B, IFNγ, iNOS and TNF-α in the tumor tissues of mice in the treatment group increased.
h) Of mice. The expression of granzyme_B, IFNγ, iNOS and TNF-α in the tumor tissues of mice in the treatment group increased.

Discussion

Oral cancer is one of the most common cancers in humans, with a high incidence worldwide, with a five-year survival rate of only about fifty percent [17]. At present, immunotherapy has played a great advantage in treatment of OSCC [18] and the rise of nanotechnology has also provided a new way to treat cancer through the use of nanovesicles [19]. The occurrence and development of oral cancer is closely related to the immune system and the search for new nanoscale therapeutic drugs that can regulate inflammation and enhance immune response can provides a new direction for thinking about improving the efficacy of OSCC. Liriope spicata is a traditional Chinese medicine recorded in the Chinese Pharmacopoeia (2020 edition) and its active ingredients have pharmacological effects in different medical fields such as cardiovascular, inflammatory regulation, immunomodulatory, and antitumor [20-22], but the role of nanovesicles extracted from its rhizomes in OSCC has not been reported. In this study, we firstly extracted from the Chinese herbal medicine plant Liriope spicata and then identified and analyzed their protein components.

It was found to be similar in structure to nanovesicles from many other plant sources such as ginger, ginseng as well as arabidopsis, LRNVs are double-layer membrane vesicle-like nanoparticles with a diameter of about 50~150nm and negatively charged. In contrast, LRNVs have uniquely shown effects on OSCC. At present, there are few studies on the effect of plant extracellular vesicles on OSCC and only bitter melon nanovesicles have been reported to reduce the resistance of OSCC cells to 5-FU treatment [23]. LRNVs also contain a large number of proteins involved in amino acid and glucose metabolism. Among them, amino acid proteins play an important role in tumor immunity. For instance, glutamine is an essential molecule for T cell activation and T cells cultured without glutamine cannot produce IFN-γ [24]. Amino acids can also improve immunity by promoting glycolysis, tricarboxylic acid cycle, etc., which echoes the first three signaling pathways in the analysis results of KEGG enrichment pathway. Arginine is produced through the urea cycle, and ornithine is used to synthesize polyamines, which are important for T cell growth [25]. Secondly, the biological role of LRNVs in vitro was explored through

many cell function experiments. The results showed that LRNVs could inhibit the colony formation, migration and invasion ability of MOC2 cells and increase their degree of apoptosis, suggesting that LRNVs have certain anti-OSCC effects.

In addition, the WB results showed that the protein relative expressions of Bad and Bax increased while Bcl-2, MMP9 and NFκB protein expressions decreased, which further indicated that LRNVs could regulate the apoptosis process of OSCC cells and participate in invasion and inflammatory processes. Thirdly, we used a small animal in vivo imager to observe that with the increase of LRNVs injection time, the range of red fluorescence aggregation in the abdomen of mice expanded. The dissection found that other organs and tumors except the heart had different degrees of fluorescence aggregation, indicating that the DiR-labeled LRNVs entering the abdominal cavity could spread over time and be absorbed by some organs, which had a targeted tumor effect. In addition, the hemolysis rate of LRNVs met the national standard. Finally, we also found that LRNVs could inhibit tumor growth in mice without affecting body weight and pathological morphology of the heart, liver and kidney. It's a guess that this anti-tumor effect may be related to the increase of granzyme_B, IFNγ, iNOS or TNF-α, so it is speculated that LRNVs can induce the up-regulation of granzyme_B, IFNγ, iNOS or TNF-α in OSCC tissues.

Among them, TNF-α, granzyme_B and IFNγ are three main mediators of cytotoxicity that all produced by T cells [26]. TNF-α is an inflammatory factor produced mainly by monocytes macrophages in the body and can inhibits the growth of tumors [27]. In gastric tumors [28] and colorectal tumors [29], TNF-α can induce and kill tumor cells. The use of special drug carriers to transport TNF-α to the tumor site can not only induce apoptosis of tumor cells, but also avoid adverse reactions caused by systemic drugs. Granzyme_B is an important factor for immune cells to exert anti-tumor effects and participate in wound healing injuries [30]. Both granzyme B and TNF-α are closely connected with chimeric antigen receptor T cell immunotherapy (CAR-T therapy) [31]. IFNγ exerts anti-tumor effects in multiple steps: IFNγ enhances the secretion of FAS and its ligands and TNF-related apoptosis-induced ligands by regulating the cellular apoptosis process; IFNγ is also involved in the tumoricidal effect of macrophages. Many scholars believe that the increase of iNOS indicates that macrophages differentiate more to M1 that M1 macrophages are mainly involved in the tumor suppression process. The arginine succinate lyase contained in LRNVs can promote the production of arginine.

iNOS is inseparable from arginine and the catabolism of arginine can be mediated by iNOS, which in turn catalyzes the conversion of arginine into citrulline and nitric oxide, which has a certain effect on energy production [32,33]. Most of these factors are related to inflammatory processes and immune regulation and can produce anti-OSCC effects in various channels in vivo and LRNVs maybe have the potential to achieve a similar effect in OSCC. All of the results suggest that LRNVs have anti-OSCC effects in vivo or in vitro and their anti-tumor properties may be related to inflammatory and immune-related pathways, which fully illustrates that there is a very close connection with LRNVs, OSCC, inflammatory and immune-related factors. Nanovesicles are hollow on the inside and often act as drug carriers for cancer immunotherapy [34]. Phospholipid membrane-based nanovesicles are currently being transformed in the direction of tumor vaccines [35]. Zhang Y, et al. discovered that isolated cell-bound membrane vesicles can be used as a novel class of drug nanocarriers to encapsulate doxorubicin for tumor treatment [36].

In addition to exosomes derived from animal cells, nanovesicles derived from plant cells [37] and bacteria [38] can also act as drug delivery systems in a variety of diseases. In addition, cell-bound membrane vesicles contain antioxidant proteins that may have antioxidant functions in cells or have therapeutic potential [39]. Actually, both nanovesicles from organic agriculture and engineered materials with artificial nanoparticles have the ability to deliver drugs [40]. Like many other nanovesicles, LRNVs not only play a role in anti-inflammatory and anti-OSCC, but may also have the potential for drug delivery systems. The limitation lies in the fact that the proteins contained in LRNVs have not been studied in a targeted manner and the specific mechanisms affecting the function of OSCC cells and the related processes of inflammation and immunity have not been deeply explored, which is also one of the directions of further experiments. In summary, this study lays a theoretical basis for LRNVs as a new type of drug carrier for OSCC's treatment and offers a novel idea for the individualized treatment strategy of LRNVs for specific tumor-related inflammation.

Conclusion

In conclusion, this study preliminarily demonstrated that LRVNs, which contain multiple proteins, are plant-derived nanovesicles that can cause MOC2 cells to be more susceptible to apoptosis and also inhibit colony-forming, migration, proliferation of MOC2 cells as well as the growth of ectopic xenografts in C57BL/6J mice, which may be related to the elevation of granzyme_B, IFNγ, iNOS or TNF-α in OSCC tissues.

Statement of Usage of Artificial Intelligence

We declare that the paper was written without the help of any Artificial Intelligence (ChatGPT for example).

Data Availability

The protein data detected in this study have been uploaded to the figshare website (doi:10.6084/m9.figshare.26768200). Additional raw data can be requested from the first author.

Author Contributions

Data curation, Zhenxia Wei; Funding acquisition, Xuanping Huang; Methodology, Shuangyu Hu; Project administration, Xiaoping Su; Software, Zhenxia Wei; Supervision, Xiaoping Su; Validation, Shuangyu Hu; Writing – original draft, Shuangyu Hu; Writing – review & editing, Xuanping Huang.

Conflict of Interest

None of the authors have any conflicts of interest.

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Appendix

The study has been approved by the Laboratory Animal Ethics Committee of Guangxi Medical University (No. 202407001) with the consent of all participants.

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