Original Article
LSPb1 inhibits the proliferation of laryngeal squamous cell cancer and neonatal vessels via HMGB1/NF-B pathway

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Abstract: This study aims to explore the effect of Lepista sordida polysaccharide (LSPb) 1 on angiogenesis in nude mice tumor transplantation model of laryngeal squamous cell cancer with drug resistance, as well as investigate the mechanism of HMGB1 (high mobility group box1 protein)/NF-κB signal pathway on the regulation of invasion and metastasis of laryngeal squamous cell carcinoma. The nude mice were raised to a certain stage followed by randomly divided into LSPb1 treatment group and normal control group. The expression of VEGF among hypoxia group, hypoxia control group and normal control group was measured by ELISA. Immunohistochemical method was used to study the effect of LSPb1 on the expression of HMGB1 protein in vitro transfer of laryngeal squamous cell cancer transplantation tumor models. According to the protein expression, it was rearranged into the positive control group and negative control group. The effect of LSPb1/HMGB1/NF-κB signaling pathway on the inhibition of angiogenesis in laryngeal squamous cell carcinoma was studied. Our results showed that the expression of VEGF in hypoxia group was significantly lower than that in control group, and the expression of VEGF in LSPb1 group was remarkably lower than that in normal control group (P<0.05). The mRNA expression of VEGF in LSPb1 treated group almost disappeared. Cells with positive expression of CD34 and CD31 in the transplanted tumor with LSPb1 were fewer. The protein expression of HMGB1 in drug resistance of nude mice model was higher. The cell immunofluorescence results showed that HMGB1 were mainly expressed in the nucleus of Hep-2 cells and in the cytoplasm Hep-2/v drug resistance. The expression rate of NF-κB was significantly lower than that of the negative control group (P<0.05) after siRNA-HMGB1 interfering treatment. Cell migration and invasion ability of Hep-2 cells in the transplanted tumor model with drug resistance in nude mice were higher, which were inhibited by siRNA-HMGB1. In conclusion, LSPb1 played an important role in potential therapeutic drug for laryngeal cancer and HMGB1/NF-κB signal transduction pathway or LSPb1 post-cure could be used as a target for laryngeal cancer.

Keywords: Polysaccharide, lepista sordida, laryngeal squamous cell carcinoma, nude mice, transplanted tumor model

Introduction

Laryngeal carcinoma is the most common malignant tumor of the ophthalmology and otorhinolaryngology, and most of the patients are squamous cell carcinoma [1]. According to statistics, the incidence of laryngeal squamous cell carcinoma is less than 2% in human malignant tumors, but 40% patients have progressed to III or IV phase at the diagnosis [2]. Clinical treatment for laryngeal squamous cell carcino-

ma is surgery, radiotherapy, and chemotherapy. The treatment effect is acceptable, but 5 years survival rate is still not promising and most patients with advanced lesion even lose their voice. In recent years, clinical studies have found the risk of malignant tumors in respiratory system, digestive system among patients with laryngeal squamous cell carcinoma [3]. Therefore, early detection and treatment of laryngeal squamous cell carcinoma has important clinical significance to the prognosis and

Table 1. Levels of Vascular endothelial growth factor (VEGF) secreted by Hep-2 cells

	Normal oxygen control group	Hypoxia control group	Hypoxia+LSPb1		
			100 µg/mL	200 µg/mL	400 µg/mL
VEGF (Tpg/mL)	500	1480	520	480	450

quality of life of patients. At present, many scholars have conducted researches on the development of laryngeal squamous cell carcinoma, which is shown to be closely related with several genes, especially high mobility group box1 protein (HMGB1) gene. HMGB1 is involved in tumor development and metastasis and can be detected in many kinds of tumors [4]. Studies have shown that [5] lepista sordida may have inhibitory effect on the proliferation of cancer cells. Its fruiting bodies are not only rich in minerals, but also contain a large number of sugars and amino acids. In addition, the purified polysaccharide monomers such as LSPb1, LSPb2 and LSPc1 have strong inhibitory effect on the growth of several cancel cells, while whether LSPb1 can inhibit laryngeal cancer is unknown. The study is to analyze the role of Lepista sordida polysaccharide LSPb1 in nude mice transplantation model of laryngeal squamous cell cancer with drug resistance as well as investigate the mechanism of HMGB1/NF-κB signal pathway on the regulation of the invasion and metastasis of laryngeal squamous cell.

Material and methods

Materials

Lepista sordida fruitbody was purchased from our hospital pharmacy in April 2014. Laryngeal squamous cell carcinoma in drug resistance nude mice: BALB/C (nu/nu) rats, 9-12 weeks old, weighed 18-24 g, were from the experimental animal center of Affiliated Sixth People's Hospital of Shanghai Jiao Tong University. Human laryngeal carcinoma cell line Hep-2 and Hep-2/V cancer resistant cells were purchased from tumor research Chinese Medical Science Institute. VEGF ELISA kit was from Shanghai kordsa Biological Products Co. Ltd. HMGB1 Rabbit anti human polyclonal antibody, Goat anti rabbit polyclonal antibody: Beijing Boisynthesis Company. TAKARA: reverse transcription kit was purchased from Dalian Bao Biological Engineering Co. Ltd. Flow cytometry was from the United States BD Company. The other chemicals and reagents were purchased

from Shanghai Qisheng Biological Preparation Co.

Study method

The separation and purification of Lepista sordida fruiting bodies: the separation and purification of Lepista sordida polysaccharides were extracted. The polysaccharide LSPa1 was used in this study.

Establishment of transplantation tumor model in nude mice: firstly, prepare cell suspension (in RPMI-1640 culture solution) and 0.2 mL cell suspension, and then establish the model of transplantation tumor in nude mice. Model was established successfully in 15 days after the drug treatment. When the tumor volume reached 100-150 m^3, the animal experiment in accordance with the principle of completely random was divided into LSPb1 treatment group (injection 400 mg/kg of LSPb1) and normal control group (injected with PBS).

Human vascular endothelial growth factor (VEGF) enzyme linked immunosorbent assay [6]: according to the VEGF concentration in the sample, they were divided into hypoxia group, hypoxia control group, normal oxygen group, and each group were treated with LSPb1 100 g/mL, 200 g/mL, 400 g/mL respectively. ELISA was performed to detect the expression of vascular endothelial growth factor in laryngeal squamous cell Hep-2 cells which was cultured in vitro. The expression of VEGF protein and mRNA in transplanted tumor was detected by ELISA and RT-PCR.

Immunohistochemistry quantitative analysis of the number of angiogenesis in transplanted tumor tissues: using immunohistochemical method, endothelial cells were labeled with CD31 and CD34. The number of angiogenesis was analyzed. The Lepista sordida polysaccharide LSPb1 on laryngeal carcinoma angiogenesis inhibition was determined.

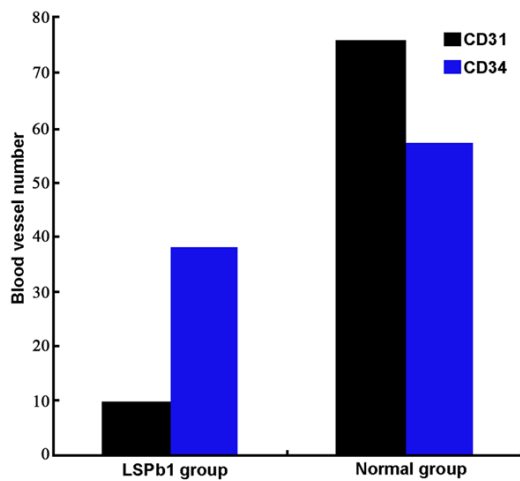Lepista sordida polysaccharide LSPb1 by HMGB1/NF-κB signal pathway in laryngeal
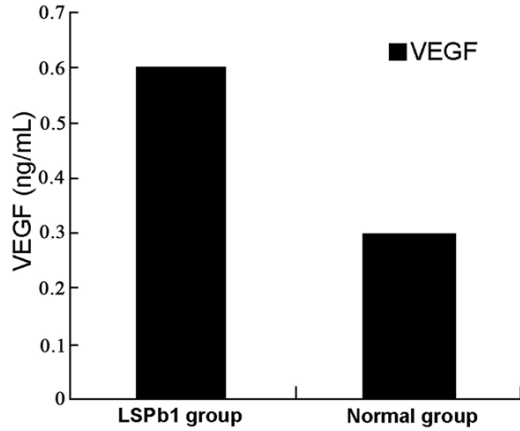
Figure 1. ELISA determination of VEGF protein level in transplanted tumor.
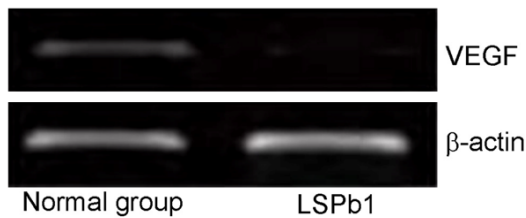
Figure 2. mRNA Expression of VEGF in the transplanted tumor.

Figure 3. The distribution of blood vessels in the transplanted tumor.

squamous cell carcinoma xenograft model with the inhibitory effect [7]: first, Lepista sordida polysaccharides LSPb1 was used to treat experimental mice. Immunohistochemistry method was applied to observe the effect of Lepista

sordida polysaccharides LSPb1 on the expression of HMGB1 protein in transplanted tumor model. Immunofluorescence analysis was used to measure the expression pattern of HMGB1 in laryngeal carcinoma cells Hep-2 and Hep-2/V resistant laryngeal carcinoma. siRNA sequence of HMGB1 gene expression vector was synthesized. Liposome was transferred into human laryngeal carcinoma Hep-2 cells and laryngeal carcinoma Hep-2/V resistant drug cell line. Transient transfection of Hep-2 cells for 24 h followed by measurement of the mRNA and protein expression of HMGB1 by RT-PCR and Western Blotting respectively. In addition, western Blotting was used to analyze the expression of NF- κB protein and RAGE protein in laryngeal carcinoma cells Hep-2 after transfection. The changes of MMP-2 and MMP-9 protein in laryngeal carcinoma Hep-2 cells before and after transfection were detected by gelatin zymography. Migration changes of laryngeal carcinoma Hep-2 cells assessed by scratch assay and the changes of Hep-2 cell migration evaluated by transwell assay before and after siRNA interference were observed.

Observed index

To observe the expression of vascular endothelial growth factor (VEGF) secreted by Hep-2 cells in laryngeal squamous cell carcinoma, the expression of VEGF protein and mRNA in tumor tissues, and the number of new vessels in tumor tissues.

To observe the protein expression of HMGB1 in nude mice model, the migration and invasion ability of Hep-2 cells.

Statistic method

All data were processed by SPSS 17 statistical software, and the data were expressed by x^2 test, the measurement data represented as mean ± SD were analyzed by t-test, $P < 0.05$ was considered as statistically significant.

Result

In vitro, the amount of vascular endothelial growth factor (VEGF) secreted by Hep-2 cells in three groups of laryngeal squamous cells

Normal oxygen control group was 500 Tp g/mL, hypoxia control group was 1480 Tp g/mL, hypoxia with addition of LSPb1 100 µg/mL,
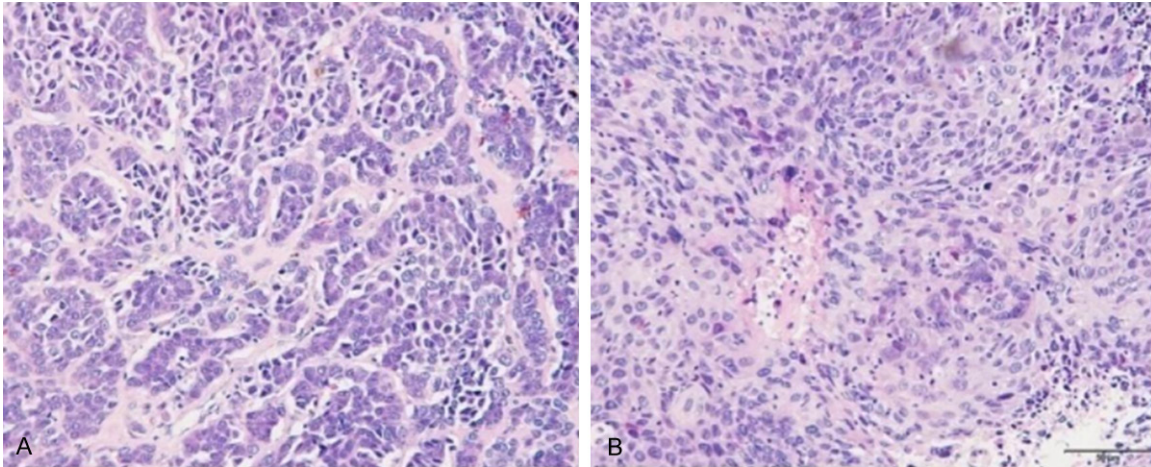
Figure 4. HE staining results. A: With lymph node metastasis of laryngeal cancer tissues, B: Not associated with lymph node metastasis of laryngeal cancer tissues.

Table 2. Expression of proteins in the model of drug resistance in nude mice

		RAGE protein	NF-κB	MMP-9	MMP-2
The expression of the two groups after transient transfection (%)	24 h	50.91%	32.71%	91.24%	82.19%
	48 h	--	--	55.37%	40.03%

200 µg/mL, 400 µg/mL, was 520, 480, 450 Tp g/mL respectively (**Table 1**). The amount of VEGF in hypoxia group was significantly lower than that in control group (P<0.05), suggesting LSPb1 has the effect of inhibiting the secretion of VEGF cells in Hep-2 cells with hypoxic conditions.

The expression of VEGF protein and mRNA in transplanted tumor was significantly lower than that in normal control group (LSPb1), as shown in **Figure 1**, and VEGF mRNA in LSPb1 treated group was almost not expressed, as shown in **Figure 2**.

The number of new blood vessels in the transplanted tumor model

The blood vessel density of LSPb1 treated group was significantly lower than that of the normal control group (P<0.05). Cells with positive expression of CD34 and CD31 in the transplanted tumor with LSPb1 treatment were very few, and the blood vessel distribution density was also lower, as shown in **Figure 3**.

The expression of HMGB1 in the model of drug resistance of nude mice model

The positive expression rate of HMGB1 protein was 93.14%. The positive expression rate of HMGB1 protein in nude mice model with lymph node metastasis was 95.27%. The positive expression rate of HMGB1 protein in nude mice model with non-lymph node metastasis was 76.41%. The difference between the two groups has statistically significant (X^2=13.91, P<0.05) (**Figure 4**). Cell immunofluorescence revealed that HMGB1 was expressed in the nucleus of Hep-2 cells and in the cytoplasm of Hep-2/v resistant drug cell line. The inhibition rate of HMGB1 mRNA on Hep-2 cells in siRNA-HMGB1 cells was 59.21%, and the inhibition rate of HMGB1 was 45.16%. The expression of RAGE protein in the positive control group was significantly lower than that in the negative control group (24 h) (P<0.05). NF-κB protein expression decreased 32.71% against negative control group (P<0.05). Hep-2 cells were transiently transfected with 24 h, 48 h, and MMP-9 protein expression in the positive control group was significantly lower (91.24%, 82.19%) than that in the negative control group (P<0.05). MMP-2 protein activity was also decreased significantly (P<0.05), which were 55.37%, 40.03% respectively, **Table 2**.

Hep-2 cell migration rate drug resistance in nude mice of laryngeal cancer

Transiently transfected Hep-2 cells on the second day, 6 h, 12 h, 24 h, and the relative mobil-

Table 3. Hep-2 cell migration rate drug resistance in nude mice of laryngeal cancer (mean ± SD)

	Transiently transfected Hep-2 cells		
	6 h	12 h	24 h
Positive control	42.36 ± 3.19	51.9 ± 1.82	95.7 ± 0.91
Negative control	22.19 ± 4.93	57.38 ± 1.25	84.18 ± 2.9

ity of the positive control group were (42.36 ± 3.19), (51.9 ± 1.82), (95.7 ± 0.91), respectively. The relative mobility of the negative control group were (22.19 ± 4.93), (57.38 ± 1.25), (84.18 ± 2.9), respectively, the relative mobility decreased greatly in 12 h-24 h against with that in 6 h-12 h (P<0.05), **Table 3**.

Discussion

Squamous cell carcinoma of laryngeal cancer is the most common cancer in clinic. Surgical treatment and radiotherapy can effectively inhibit the rapid proliferation and metastasis of laryngeal cancer cells. However, there are still some side effects, which affect the quality of life. Therefore, it is urgent to find effective treatment for laryngeal cancer. Moreover, molecular and gene research could explore potential therapy target.

In this study, we focused on the effect of LSPb1 and HMGB1/NF-κB signal transduction pathway in nude mice transplantation model of laryngeal squamous cell cancer with drug resistance. Then we explored its anti-tumor mechanism and inhibition of angiogenesis. Lepista sordida is a rare wild edible and medicinal fungi. Its fruiting body contains 18 kinds of amino acids, including 8 human essential amino acids, zinc, iron and other essential trace elements. It also has the oxidation resistance effect with enhanced immunity and anti-aging [8]. Research showed that the trace elements have good tonic effect on five internal organs of the body. It can also improve blood supply to tranquilize the nerves, so as to improve the amnesiacs, fatigue, anemia and other symptoms. Lepista sordida also can inhibit the proliferation of cancer cells [9]. Malignant tumor containing abundant angiogenesis, cancer cells around a lot of vascular network, so LSPb1 was chosen to investigate its role on angiogenesis in nude mice transplantation model of laryngeal squamous cell cancer with drug resistance. The results

showed that the density of blood vessels in LSPb1 treated group was significantly lower than that in normal control group (P<0.05). Cells with positive expression of CD34 and CD31 in nude mice treated by LSPb1 were few. Therefore, LSPb1 could inhibit the formation of new blood vessels.

In recent years, studies have found that the formation of new blood vessels plays an important role in cancer, including the process of metastasis, occurrence, development, and progression of malignant tumor [10]. Angiogenesis is consisted of endothelial activation, proliferation, migration, basement membrane disruption, neovascularization and vascular network formation. All processes above are regulated by multiple growth factors, such as VEGF, granulocyte macrophage colony stimulating factor and G-CSF (granulocyte colony-stimulating factor) [11]. The main inducing factors of angiogenesis in tumor are hypoxia and associated proteins, especially VEGF. The expression of the hypoxia inducible factor and the expression of target genes downstream regulation are closely related with the formation of new blood vessels [12]. Therefore, it is important to control the content of VEGF in the treatment of laryngeal carcinoma. The results of this study showed that the level of VEGF in normal control group was 500 Tp g/mL, hypoxia group 1480 Tp g/mL, hypoxia+LSPb1 100 μg/mL, 200 μg/mL, 400 μg/mL, was 520, 480 and 450 Tp g/mL, respectively. VEGF in hypoxia group was lower than that in hypoxia control group (P<0.05), suggesting LSPb1 has the effect of inhibiting VEGF secretion of Hep-2 cells in cells under hypoxic conditions. In addition, the value of VEGF in LSPb1 treated group was significantly lower than that in normal control group (P<0.05). RT-PCR results showed that the mRNA expression of VEGF was almost disappeared. Therefore, LSPb1 not only inhibited the secretion of VEGF, but also inhibited the expression of mRNA.

HMGB1 is the most abundant protein in the cell, and has many biological characteristics, which is involved in the maintenance of the structure and dynamics of the structure. It can participate in the regulation of gene, gene recombination, telomere stability and regulation of steroid hormone receptor activity [13]. HMGB1 binds to cell surface receptor and acti-

vates downstream signaling pathways immediately, such as NF-κB signaling pathway and phosphatidylinositol 3 muscle enzyme pathway, interferon regulatory factor-3 (IRF3) signaling pathway. Further, HMGB1 stimulates cell adhesion and migration. Moreover, after macrophage phagocytic function was inhibited, cell proliferation and angiogenesis increased and finally induced autophagy formation [14]. In the tumor cell microenvironment, tumor cells and infiltrating leukocytes can secrete HMGB1. Therefore, HMGB1 plays a role in the occurrence and development of many kinds of tumor cells.

Although clinical studies have confirmed the importance of HMGB1 in cancer patients, there are still some questions warranted investigations, including whether LSPb1 intervention is effective in patients with laryngeal carcinoma, whether the expression of HMGB1 is inhibited or not, and whether multiple signaling pathways are mediated by HMGB1 (especially HMGB1/NF-κB pathway). HMGB1/NF-κB signaling pathway was also investigated in nude mice transplantation model of laryngeal squamous cell cancer with drug resistance after treated with LSPb1 and results showed that HMGB1 protein expression rate was 93.14% of all the nude mice model, lymph node metastasis, the positive expression of HMGB1 protein in nude mice model of rate was 95.27%, no positive expression of HMGB1 protein in nude mice model of lymph node metastasis rate was 76.41%, (X^2=13.91, P<0.05). Cell immunofluorescence revealed that HMGB1 was expressed in the nucleus of Hep-2 cells and expressed in the cytoplasm of Hep-2/v cells.

The inhibition rate of HMGB1mRNA on Hep-2 cells in siRNA-HMGB1 cells was 59.21%, and the inhibition rate of HMGB1 protein was 45.16%. The results showed that HMGB1 participates in tumorigenesis and had associations with tumor cell proliferation and metastasis. With the intervention of Lepista sordida polysaccharide LSPb1, HMGB1 protein expression decreased. Therefore, we speculated that the intervention attenuated the effect of HMGB1 on the tumor process so as to improve prognosis.

Yang et al. [15] found that the expression of HMGB1 was associated with cell proliferation

in leukemia, which was consistent with the results of this study. NF-κB is a fast response transcription factor and is a key point of multiple signaling pathways. The changes may affect the downstream signaling pathway. Increase of NF-κB expression is involved in many pathological processes, such as inflammation, immune response, tumor and cell cycle regulation, cell differentiation [16]. Our study showed that compared with negative control group, positive control group had a 50.91% decrease in protein expression of RAGE at 24 h after Hep-2 transfection (P<0.05), and a 32.71% decrease in NF-κB (P<0.05).

The results showed that the LSPb1 attenuated squamous cell carcinoma of the larynx via HMGB1/NF-κB signaling pathway. Moreover, RAGE was the main receptor for ATP and depletion of RAGE could influence ATP production. Thus, when the expression of HMGB1 and HMGB decreased, the expression level of NF-κB decreased [17], which was consistent with Chen RC et al. [18]. MMPs are zinc ion dependent proteolytic enzymes, which can degrade the extracellular matrix. MMP-9 and MMP-2 can be overexpressed in laryngeal carcinoma tissues, which lead to tumor angiogenesis, cell proliferation, tumor invasion and metastasis [19, 20]. Studies demonstrated a positive correlation between MMP-9 and HMGB1 expression, and the expression of MMP-9 depended on NK-KB [21]. Therefore, if the HMGB1/NK-KB signal pathway was blocked, the expression of MMP-9 could be inhibited, which finally attenuated the growth and metastasis of tumor cells. Our study also showed that the expression of MMP-9 in Hep-2 cells decreased significantly compared with the negative control group at 24 h and 48 h instantaneous transfection of Hep-2 cell. In addition, the results showed that the migration and invasion abilities of Hep-2 cells in the positive control group were significantly lower than those in the negative control group. (P<0.05).

In summary, as a potential therapy for laryngeal cancer, LSPb1 is of promising clinical significance. HMGB1 can inhibit HEp-2 cells migration, invasion and proliferation. HMGB1 also can reduce drug resistance of laryngeal squamous cell carcinoma. Therefore, HMGB1/NF-κB signaling pathway is a potential target for

LSPb1 treatment on laryngeal carcinoma. LSPb1 is expected to become a new direction of research and development for clinical treatment of laryngeal carcinoma, so it warrants further exploration.

Disclosure of conflict of interest

None.

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