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RESEARCH ARTICLE

THE STUDY ON THE MECHANISM OF LENTINAN IN IMPROVING IMMUNE FUNCTION IN PATIENTS WITH ADVANCED NSCLC

[1]Xi-Xian Lou, [1]Meng-Chu Zhu, [1]He-Ping Lin and [2,*]Chang Cai

[1]Department of Respiratory Medicine, The Third Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, 325200, China
[2]Department of Respiratory Medicine, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, 325000, China

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**Corresponding author:*
Chang Cai

ABSTRACT

Objective: To explore the possible mechanism of lentinan in improving the immune function of patients with advanced NSCLC through a variety of pathways related to lung cancer immunity, and to provide a theoretical basis for the improvement of immunity of lentinan and its anti-tumor effect. ***Methods:*** The subjects were the same as the Part □. The fasting venous blood was taken before and after treatment. The PBMNCs were obtained after PBMNC separation, centrifugation and washing. The expression levels of PD-1 protein, PD-L1 protein, Jak2 protein, Jak3 protein, Stat3 protein, P13K protein, p-AKT protein in PBMNCs were detected by Western blot. The expression levels of PD-1 mRNA, PD-L1 mRNA, Jak2 mRNA, Jak3 mRNA, Stat3 mRNA, P13K mRNA, p-AKT mRNA in PBMNCs were detected by Real-time quantitative PCR. ***Results:*** In the PD-1/PD-L1 signaling pathway, the levels of PD-1 protein, PD-1 mRNA, PD-L1 protein and PD-L1 mRNA in PBMNC were significantly lower after treatment ($P<0.05$). The levels of PD-1 protein, PD-1 mRNA, PD-L1 protein and PD-L1 mRNA in the treatment group were lower than those in the control group. The difference of PD-1 protein, PD-1 mRNA, PD-L1 protein and PD-L1 mRNA before and after treatment was higher than that of the control group. ($P<0.05$).In JAK/STAT signaling pathway, the levels of Jak2 protein, Jak2 mRNA, Jak3 protein, Jak3 mRNA, Stat3 protein and Stat3 mRNA in PBMNC were significantly lower than those before treatment ($P<0.05$). Comparison between groups showed combination therapy. The levels of Jak2 protein, Jak2 mRNA, Jak3 protein, Jak3 mRNA, Stat3 protein and Stat3 mRNA in PBMNC group were lower than those in control group. The difference between Jak2 protein, Jak2 mRNA, Jak3 protein, Jak3 mRNA, Stat3 protein and Stat3 mRNA before and after treatment was higher than that of control. Group ($P < 0.05$).In the PI3K-AKT signaling pathway, the levels of P13K protein, P13K mRNA, p-AKT protein, p-AKT mRNA in PBMNC were significantly lower than those before treatment ($P<0.05$). The comparison between groups showed that the levels of P13K protein, P13K mRNA, p-AKT protein, p-AKT mRNA in PBMNC of the combined treatment group were lower than those of the control group, P13K protein, P13K mRNA, p-AKT protein, p- The difference of AKT mRNA levels before and after treatment was higher than that of the control group ($P<0.05$). ***Conclusion:*** Lentinan may improve the immunity of NSCLC patients through PD-1/PD-L1 signaling pathway, JAK/STAT signaling pathway and PI3K-AKT signaling pathway.

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INTRODUCTION

There are complex interactions between tumor and immune system, which are shown as follows: immune system inhibits tumor growth and progression through immune response; The immune system promotes tumor growth, survival and angiogenesis by inducing inflammatory responses. Tumor USES immune regulatory mechanism to form immunosuppressive microenvironment, which can not only suppress host immune response, but also forms a barrier to block anti-tumor immunotherapy (Zhou *et al.,* 2018).

Therefore, in-depth exploration of the molecular mechanism between tumor and immune system will lay a foundation for theoretical research on tumor immunotherapy and provide more new strategies for tumor immunotherapy. At present, Many signal pathways that are related to the occurrence and development of malignant tumors have been found in clinic, such as programmed death-1 (pd-1)/programmed death-ligands (pd-ls) signal pathways (Bersanelli and Buti, 2017) and just another kinase. Signal pathways of protein/Signal transduction and activator of transcription 3 (STAT3) (Wang *et al.,* 2017), phosatidylinositol 3-hydroxy kinase (PI3K)/serine/threonine

kinase (Akt) (Nandini *et al.,* 2017), etc. All have been proved to be involved in malignant tumor immunosuppression and immune escape. This study based on previous research to choose 114 cases of patients with locally advanced NSCLC inRuian people's hospital during August 2015 to October 2017 treated respectively for NP chemotherapy lentinan alone and in combination, to the tumor immune related signaling pathways as observation index, analysis of lentinan improve NSCLC and its possible mechanism of immune function.

MATERIALS AND METHODS

Patients: This study was approved by the medical ethics committee of Ruian people's hospital, and written informed consent was obtained from the patients and their families. In this study, 114 patients with advanced NSCLC admitted to our hospital from August 2015 to October 2017 were selected. All the enrolled patients were unsuitable or refused to receive surgery and targeted therapy due to various reasons.

Treatment methods: All enrolled patients received NP chemotherapy regimen, vinorelbine 25 mg/m2, intravenous infusion on the first day and the eighth day of treatment. Cisplatin 30 mg/m2 was given by intravenous drip on the 1st to 5th day of treatment. The chemotherapy regimen continues until the patient refuses, the disease progresses, or the toxicity is intolerable.The combined treatment group was given intravenous infusion of lentinan for injection on NP chemotherapy regimen. Chinese medicine approval number: H20067183), 2 times /w, 1mg/ time. Lentinan was treated with 21 days as one course of treatment, and patients in both groups were given continuous medication for at least 4 courses of treatment.Before and after treatment, the patients underwent weekly blood routine examination, liver function, renal function, periodic electrocardiogram, chest X-ray, section and chest CT examination. Chemotherapy doses and lentinan doses were adjusted for the toxicity effects measured on days 1 and 8 of each cycle. After treatment, patients were followed up regularly through outpatient service, telephone service, inpatient medical record system and other means. The end of follow-up was the death of patients or the day of the end of this study. Overall survival (OS) was the day from the first treatment to the death of the patients or the end of this study, and the overall survival (PFS) was the day from the first treatment to the recurrence of tumor or the end of this study. The duration of follow-up was 6 to 36 months, and the mean follow-up time was (26.30±7.11) weeks.

Isolation and culture of PBMNCs: Peripheral anticoagulant blood was collected on an empty stomach in the morning before and after treatment in the two groups. After treatment with PBMNC separation solution, the cells in the middle layer were centrifuged at 2000 r/min for 20 min. The middle layer cells were washed with 5x volume normal saline and centrifuged at 2000 r/min for 10 min×2 times to obtain PBMNCs. Then cell culture bottles were added and the concentration of PBMNCs cells was adjusted to 2×106/ml with RPMI 1640/10% FCS culture medium. The cells were cultured at 37□ with 5% CO2.

Western blot: The PBMCs cells of the patients were inoculated in cell culture bottles, placed in the incubator and cultured for 48 h, and the cells were collected. The same amount of samples were taken for 12% gel electrophoresis, and transferred to PVDF membrane. 5% skim milk powder was used overnight to seal and block antibodies. The diluted solution of primary antibody was 0.5%BSA solution. The imprinted membrane was incubated at room temperature for 2h and removed. The membrane was washed with TBST for 10 min×3 times. The imprinted membrane was incubated at room temperature for 2h and removed. The membrane was washed with TBST for 15 min×3 times. With the addition of ECL reagent, gel imager observation and statistical analysis.

Real-time fluorescence quantitative PCR detection: PBMCs cells were taken from the patients, total RNA was extracted by Trizol method, and cDNA was synthesized according to the instructions of reverse transcription kit. The reaction systems were SYBR Premix Ex Taq TM5 ul, PCR Forward primer 0.2 ul, PCR Reverse primer 0.2 ul, and cDNA dilution solution 2 ul, adding RNase Free H2O to reaction system 10 ul. After Total RNA was added into the reaction system, it was placed in the gradient PCR instrument, and reverse transcription conditions were set: 37 □ for 15 min, 85 □ for 5 SEC, to complete the reverse transcription reaction and obtain cDNA. CDNA fluorescence quantitative PCR instrument was used, and the reaction conditions were pre-denaturated at 95□×3min. 95 □ by 5 s degeneration; Annealing/extension 60□×30s, 40 cycles in total. Beta-actin was used as the internal reference gene and repeated 3 times in each sample. After the end of amplification, the system software automatically generated the Ct value and drew the amplification curve fusion curve. The relative expression level of the target gene was calculated by the $2^{-\Delta\Delta Ct}$ method: $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{internal reference gene}}$.

RESULTS

Comparison of mrna levels of pd-1, pd-1, pd-l1 and pd-l1 in PBMNC before and after treatment in the two groups: PD-1, PD-1mRNA, PD-L1, PD-1mRNA levels of the two groups were not statistically significant before treatment (P>0.05). After treatment, the levels of PD-1, PD-1mRNA, PD-L1, PD-1mRNA in the two groups were significantly reduced, with statistically significant differences (P<0.05). Comparison between the two groups showed that the expressions of PD-1, PD-1mRNA, PD-L1, PD-1mRNA in the combined treatment group were lower than those in the control group (P<0.05). See Table 1.

Comparison of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3, Stat3 mRNA, and Stat3 in PBMNC before and after treatment in the two groups: The levels of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3 mRNA, Stat3 mRNA and Stat3 on PBMNC before and after treatment were compared between the two groups. There was no statistical significance in the levels of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3 mRNA, Jak3, Jak3 mRNA and Stat3 on PBMNC before treatment (P>0.05). The levels of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3 mRNA, Stat3 mRNA and Stat3 in PBMNC of the two groups were significantly lower after treatment than before treatment, with statistically significant differences (P<0.05). The inter-group comparison showed that the levels of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3 mRNA, Stat3 mRNA and Stat3 on PBMNC of the combined treatment group were lower than those of the control group, and the differences of Jak2, Jak2 mRNA, Jak3, Jak3 mRNA, Stat3 and Stat3 mRNA on PBMNC were higher than those of the control group, with statistically significant differences (P<0.05). See Tables 2.

Table 1 Comparison of mrna levels of pd-1, pd-1, pd-l1 and pd-l1 in PBMNC before and after treatment in the two groups

Group	Patients	Time	PD-1	PD-1mRNA	PD-L1	PD-L1mRNA
Combined treatment group	54	Before treatment	0.64±0.13	0.006±0.002	7.42±0.62	0.003±0.001
		After treatment	$0.42\pm0.10^{*\Delta}$	$0.003\pm0.001^{*\Delta}$	$5.16\pm0.10^{*\Delta}$	$0.001\pm0.001^{\Delta}$
		Difference between two groups	$0.22\pm0.09^{\Delta}$	$0.004\pm0.002^{\Delta}$	$2.26\pm0.23^{\Delta}$	$0.002\pm0.001^{\Delta}$
Control group	60	Before treatment	0.67±0.15	0.006±0.001	7.50±0.71	0.003±0.001
		After treatment	$0.51\pm0.13^{*}$	$0.005\pm0.001^{*}$	$6.16\pm0.26^{*}$	$0.002\pm0.001^{*}$
		Difference between two groups	0.16±0.06	0.001±0.001	1.34±0.36	0.001±0.001

Table 2 Comparison of Jak2, Jak2 mRNA, Jak3 mRNA, Jak3, Stat3 mRNA, and Stat3 in PBMNC before and after treatment in the two groups

Group	Patients	Time	Jak2	Jak3	Stat3	Jak2mRNA	Jak3mRNA	Stat3 mRNA
Combined	54	Before treatment	12.62±2.16	1.16±0.42	0.65 ±0.10	12.63±1.32	16.32±0.62	4.11±0.62
treatment		After treatment	$7.21\pm1.63^{*\Delta}$	$0.82\pm0.16^{*\Delta}$	$0.39\pm0.09^{*\Delta}$	$4.98\pm0.94^{*\Delta}$	$10.63\pm1.15^{*\Delta}$	$2.16\pm0.29^{*\Delta}$
group		Difference between two groups	$5.41\pm0.96^{\Delta}$	$0.34\pm0.06^{\Delta}$	$0.26\pm0.08^{\Delta}$	$7.65\pm0.35^{\Delta}$	$5.69\pm0.46^{\Delta}$	$1.95\pm0.56^{\Delta}$
Control group	60	Before treatment	12.43±2.10	1.12±0.20	0.61±0.13	12.49±1.40	16.50±0.70	4.13±0.53
		After treatment	$9.65\pm1.34^{*}$	$0.96\pm0.15^{*}$	$0.45\pm0.11^{*}$	$6.16\pm0.84^{*}$	$12.73\pm1.09^{*}$	$3.36\pm0.30^{*}$
		Difference between two groups	2.78±0.98	0.16±0.08	0.16±0.15	6.33±0.48	3.77±0.53	0.77±0.19

Table 3. Comparison of the mRNA levels of P13K, P13K, p-Akt and p-Akt on PBMNC before and after treatment in the two groups

Group	Patients	Time	P13K	P13K mRNA	p-AKT	p-AKT mRNA
Combined treatment group	54	Before treatment	98.62±4.26	1.29±0.12	140.63±5.60	1.38±0.20
		After treatment	$80.14\pm4.59^{*\Delta}$	$1.20\pm0.09^{*\Delta}$	$123.95\pm6.31^{*\Delta}$	$1.15\pm0.19^{*\Delta}$
		Difference between two groups	$18.48\pm3.10^{\Delta}$	$0.09\pm0.05^{\Delta}$	$16.68\pm2.63^{\Delta}$	$0.23\pm0.09^{\Delta}$
Control group	60	Before treatment	96.17±5.03	1.30±0.10	141.39±4.98	1.34±0.16
		After treatment	$90.14\pm4.34^{*}$	$1.24\pm0.06^{*}$	$133.01\pm5.45^{*}$	$1.26\pm0.20^{*}$
		Difference between two groups	6.03±2.17	0.06±0.03	8.38±2.69	0.08±0.03

Comparison of the mRNA levels of P13K, P13K, p-Akt and p-Akt on PBMNC before and after treatment in the two groups: The levels of P13K, P13K mRNA, p-Akt and p-Akt mRNA on PBMNC before and after treatment were compared between the two groups, and the levels of P13K, P13K mRNA, p-Akt and p-Akt mRNA on PBMNC before and after treatment were not statistically significant ($p > 0.05$). After treatment, the mRNA levels of P13K, P13K, p-Akt and p-Akt in PBMNC of the two groups were significantly lower than those before treatment, with statistically significant differences ($p < 0.05$). The intergroup comparison showed that the mRNA levels of P13K, P13K, p-Akt and p-Akt in PBMNC of the combined treatment group were lower than those of the control group, and the differences of the mRNA levels of P13K, P13K, p-Akt and p-Akt before and after treatment were greater than those of the control group, with statistically significant differences ($p < 0.05$). See Table 3.

DISCUSSION

PD-1 /PD-Ls is a signal pathway of immune mechanism in malignant tumors that has been extensively studied in clinical studies, and it belongs to negative immune regulatory molecules. PD-1 is composed of extracellular Ig variable domain like structure domain, hydrophobic transmembrane domain and cytoplasmic domain, which is related to promoting programmed cell death. The cytoplasmic region of PD-1 retains the tyrosine suppression motif and tyrosine transformation motif of the immune receptor, which are mainly expressed in activated immune cells and some tumor cell lines or tumor cell surfaces (Taube, 2014; Dong *et al.,* 2017). Gene knockout experiment (Chun *et al.,* 2011) showed that PD-1 plays an important role in maintaining lymphocyte activation and peripheral immune tolerance. PD-L1 and PD-L2 are the main ligands of PD-L1. However, PD-L1 is less expressed in tumor cells and plays a smaller role in inhibiting the body's anti-tumor immunity.

The negative regulatory effect of pd-1 / pd-ls in tumor cells is mainly mediated by PD-1 / PD-L1. PD-L1, a member of the B7 superfamily, is found to have abnormally high expression in a variety of malignant tumor tissues, and is widely expressed in a variety of tumor-related antigen presenting cells and some organ tissues, with dual functions of co-simulation and co-inhibition. Previous studies (He *et al.,* 2015) have shown that the activation of PD-1 / PD-L1 signaling pathway is involved in autoimmune diseases, tumor immune escape, and the promotion of tumor cell growth. Expression of PD- L1 tumor cells and T cell surface receptor molecules PD 1, the specific binding to cytoplasm area immune receptor tyrosine phosphorylation PD- 1 inhibit tyrosine motif structure domain, raise with Src homology domain 2 protein tyrosine phosphatase 2, impels PI3K, and Akt protein phosphorylation, thereby to tumor cells, and inhibitory signals are activated to restrain activity of anti-tumor immune killer T cells and tumor cells apoptosis (Wang *et al.,* 2015). In addition, PD-L1 expressed on tumor cells combined with PD1 on the surface of tumor infiltrating lymphocytes can inhibit the killing effect of lymphocytes on tumor cells, induce lymphocyte apoptosis, weaken the anti-tumor immune response of the body, and eventually lead to the occurrence of tumor immune escape (Gasser *et al.,* 2016). Therefore, PD-L1 on tumor surface is generally considered as a molecular barrier to block immune effector cells and other immune killing tumor cells. In addition to the above mechanisms, recent studies (He *et al.,* 2015) also found that the PD-1 / PD-L1 signaling pathway can also promote the development of iTreg cells and the expression of Foxp3 on their surface, thus inducing the differentiation of CD4+CD25+ Foxp3+Treg and maintaining its immunosuppression function, indirectly inhibiting the proliferation of T lymphocytes and promoting the immune escape of tumor cells. The JAK/STAT signaling pathway starts with cytokine receptors. The basic STAT signaling pathway can promote tumor cell growth, proto-oncogene expression, mediating tumor immune escape, and tumor cell apoptosis

resistance, thus participating in the occurrence and development of malignant tumors (Lee *et al.,* 2016). JAK pathway as the key part of connected cells inside and outside the signal pathway, exogenous cytokines and receptor can be converted into intracellular signal activate JAK, conservative Tyr phosphorylation STAT protein C end, and the formation of dimer into the momentum effect element area with specific DNA in the nuclei, regulate gene expression (Li *et al.,* 2015). At the same time, studies (Asakawa *et al.,* 2015) found that the JAK/STAT signaling pathway itself can also serve as an important pathway of inflammatory response, through which many cytokines complete the signal transduction from the cell to the nucleus. In addition, studies (Akla, 2012) also found that STAT protein can regulate inflammatory/immune response by regulating the expression of Gene of phosphate and ignhomology deleted on chromsome ten (PTEN), Cyclooxygenase 2 (cox-2) and Indoleamine 2, 3-dioxygenase (IDO). It is a key factor to determine whether the immune response in tumor microenvironment develops towards tumor formation or inhibition. PI3K/ AKT has been shown to be involved in a variety of cellular biological processes and is one of the signaling pathways closely associated with tumorigenesis (Zhang *et al.,* 2017). According to clinical statistics, almost all malignant tumors have at least one gene mutation that affects the PI3K/AKT signaling pathway, including PI3R1, PIK3R2 and PTEN gene. Changes in any of the above genes can affect the expression of the whole signaling pathway, leading to imbalance of cell differentiation, proliferation and apoptosis, and inducing the occurrence of malignant tumors. Activation of P13K phosphorylates phosphatidylinositol-4, 5-biophosphate (PIP2), and phosphatidylinositol-4, 5-biophosphate, phosphorylates AKT/PKB protein onto the cell membrane, activating AKT's biological activity and regulating tumor cell growth, proliferation, and apoptosis (Thapa *et al.,* 2015; Amancio *et al.,* 2014). At the same time, studies also found that in the PI3K/AKT signaling pathway, AKT regulates the expression of cox-2 through nf-kappab pathway, and can mediate the immune escape of tumor, but the specific mechanism is not yet clear.

The anti-tumor mechanism of polysaccharides can be divided into two types: direct anticancer and indirect anticancer. The direct anti-cancer method mainly plays an anti-tumor role by changing the biological activity of tumor cell membrane, inhibiting the production of new blood vessels and signal transduction, inhibiting the growth and proliferation of tumor cells and reducing the degree of tumor deterioration. In the indirect anti-tumor mode, the anti-tumor effect is mainly exerted by improving the immunity of the body. Studies showed that lentinan is a stimulant of T cells, which can promote the proliferation of T cell subpopulations through host mediation and enhance the killing activity of CTL. Moreover, IDO can also effectively promote lymphocyte activators, promote mature differentiation of various immature precursor T cells, and improve the synthesis and secretion of il-22 and its reactive activity. In humoral immunity, lentinan is helpful to enhance the body's specific response to antigen, promote the transformation of B lymphocytes into plasma cells, and improve the synthesis and secretion of antibodies in the body. Secondly, lentinan can also help improve the biological activity of helper T cells, increase the antibody dependent cytotoxic effect, and promote the formation of B lymphocytes and antibodies. Thus, lentinan is involved in the immune network composed of T cells, B lymphocytes and other immune cells, and mediates the synthesis and secretion of various immune factors. In this study, based on the past research choice above signaling pathways related indicators as observation object, found in the two groups after treatment PD - 1 / PD - Ls signaling pathways, JAK/STAT signal pathway, PI3K/AKT signaling pathway related indexes such as expression levels were significantly lower, compare the difference between groups was statistically.

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