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Original Article

Effect of thymalfasin on myeloid-derived suppressor cells in patients with non-small cell lung cancer

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Abstract: Objective: To observe the effect of thymalfasin on myeloid-derived suppressor cells (MDSCs) subsets in peripheral blood of patients with non-small cell lung cancer (NSCLC). Methods: 50 cases of NSCLC (NSCLC group) diagnosed in Chest Hospital of Jiangxi Province were selected as the research subjects, and 50 healthy subjects who underwent physical examination in our hospital during the same period were selected as the healthy control group. The expression of HLA-DR-CD14-$CD33^+$ MDSCs in peripheral blood mononuclear cells and tumor tissue single cell suspension of NSCLC patients before and after thymalfasin treatment was explored by flow cytometry. Results: The proportion of MDSCs in peripheral blood of NSCLC group was 1.70±0.52%, which was significantly higher than that in peripheral blood (0.51±0.15%) of healthy controls ($P < 0.05$). The proportion of HLA-DR-CD14-$CD33^+$ MDSCs in the tissues of NSCLC group was 1.65±0.43% before treatment and 1.15±0.50% after treatment ($P < 0.05$). The proportion of MDSCs in peripheral blood of NSCLC patients before treatment was 1.70±0.52%, and that after treatment was 0.59±0.18% ($P < 0.05$). Conclusion: Thymalfasin can reduce the number of MDSCs in peripheral blood mononuclear cells. The application of thymalfasin in the treatment of NSCLC patients can help to enhance the anti-tumor effect.

Keywords: Thymalfasin, non-small cell lung cancer, tumor, immunosuppression, myeloid-derived suppressor cells

Introduction

Malignant tumors have emerged as a leading cause of death among Chinese residents, with Non-Small Cell Lung Cancer (NSCLC) exhibiting the highest incidence and mortality rates [1]. Usually, NSCLC is diagnosed at an advanced stage in most patients, rendering surgical intervention no longer feasible. Consequently, comprehensive treatment based on chemotherapy has become the primary treatment method [2]. Myeloid-derived suppressor cells (MDSCs) play a pivotal role in tumor immune escape and tend to accumulate in patients with tumor. Originating from bone marrow, MDSCs exert potent immunosuppressive effect [3]. Normally, the presence of MDSCs in human body is minimal. However, in individuals with illness, especially tumor patients, cytokines secreted by tumor cells can stimulate MDSC production and impede their differentiation, resulting in their accumulation. MDSCs inhibit the immune response, promote production of regulatory T cells and tumor-associated macrophages, and thus lead to immune escape of tumor cells and contribute to tumor initiation and progression [4].

Thymalfasin, also known as thymosin alpha 1, is a small peptide immunosuppressant composed of 28 amino acids, which is commonly administered via injection. Great progress has been made in its application in treating diseases. Significant advancements have been achieved in its therapeutic applications, particularly in the treatment of tumors, viral infections, immune disorders, and common infections [5]. Tumor cells evade immune surveillance, giving rise to tumor formation, which subsequently induces immune dysfunction within the body. For example, patients undergoing chemotherapy often experience myelosuppression, commonly manifested as leukopenia, leading to diminished resistance and increased susceptibility to various infections. Thymalfasin exhibits immunomodulatory properties by regulating changes in T lymphocyte subsets, rendering it effective in treating tumors, sepsis, immu-

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nodeficiency disorders, and infectious diseases [6]. Studies have reported that thymalfasin can improve the immune function of patients undergoing radiotherapy and chemotherapy, thereby prolonging the survival of patients, improving treatment efficacy and reducing the incidence of adverse reactions [7]. At present, the research on thymalfasin is primarily focusing on its effects on $CD3^+$, $CD4^+$, $CD4^+/CD8^+$ ratio, natural killer cells and dendritic cells. However, the mechanism of how thymalfasin affects the immunosuppressive activity of MDSCs to exert anti-tumor effect is not clear. Therefore, this study aims to observe the influence of thymalfasin on MDSCs in NSCLC patients, to further explore the anti-tumor immune mechanism of thymalfasin.

Materials and methods

Study subjects

NSCLC patients (NSCLC group) diagnosed by pathological examination or cytology in Chest Hospital of Jiangxi Province from January 2021 to December 2022 were selected as the research subjects. Inclusion criteria: (1) Confirmation of NSCLC diagnosis through histopathological examination of tissue or cytology; (2) Absence of prior surgical, radiotherapeutic, chemotherapeutic, biotherapeutic, or other anti-tumor interventions; (3) Absence of immune-enhancing agent usage within the last three months; (4) No history of blood transfusions within the preceding month. Exclusion criteria: (1) Patients with diabetes, coronary heart disease, chronic lung disease or other underlying diseases; (2) Patients with malignant tumors; (3) Patients with prior bone marrow transplant; (4) Patients who took immunotherapy or glucocorticoid in the past 3 months.

In addition, from January 2021 to December 2022, healthy subjects undergoing physical examination, matched 1:1 with the NSCLC group in terms of gender, age, and BMI, were selected as the control group. Exclusion criteria for the control group included a family history of disease or preexisting conditions. Furthermore, thorough physical examinations and routine laboratory tests confirmed the absence of endocrine, cardiovascular, respiratory, blood, hepatorenal, or infectious diseases. Informed consent of all included individuals and their family members were obtained through signed documentation. This retrospective study was approved by the Ethics Committee of Chest Hospital of Jiangxi Province.

Collection of medical data

Medical data of participants were collected, including age, gender, BMI, hypertension, diabetes, history of smoking, type of tumor, degree of tumor differentiation, TNM staging, etc.

Specimen collection

Prior to the commencement of surgery, 4 ml of fasting peripheral venous blood was drawn from NSCLC patients. Similarly, 2 ml of peripheral venous blood was drawn from individuals of healthy control group in the early morning of physical examination day. Anticoagulant EDTA-K2 was used to prevent blood coagulation. On the day of surgery, the cancerous tissues of patients in NSCLC group were collected, immediately placed in specimen bags, preserved on ice, and sent to the laboratory for examination within 1 h.

Specimen processing

(1) Isolation of peripheral blood mononuclear cells (PBMNC): ① 2 ml of lymphocyte isolation solution was added to a 15 ml centrifuge tube, and the tube was tilted at a 45-degree angle. 4 ml of blood was then diluted with saline in a 1:1 ratio and gently added along the tube wall. ② The tube was centrifuged at a speed of 2000 r/min for 20 minutes using horizontal centrifuge machine at room temperature. ③ After centrifugation, the fluid in the tube was divided into three layers: plasma in the upper layer, lymphocyte isolation solution in the middle layer, and red blood cells and granulocytes in the lower layer. The narrow white-cloud-like band between the upper and middle layer, containing PBMCs, was gently pipetted and transferred to a centrifuge tube. ④ The cells were washed with PBS thrice to obtain PBMCs. Subsequently, PBMCs were resuspended in 10% FBS RPMII640, with the cell concentration adjusted to 1×10^7 cells/ml for later use.

(2) Cell culture: Isolated PBMNCs (5×10^5 cells/well) were inoculated into a 24-well plate. Thymalfasin injection (1.6 mg/mL, equivalent to the plasma concentration) was added to the cell culture dish (Chengdu Di'ao Jiuhong Pharmaceutical Co., Ltd., No. H20020545,

Specification: 1.6 mg×4 bottles/box). The cells were incubated in a 37°C, 5% CO_2 incubator for 4 h, following which PBMNCs were collected.

(3) Processing of tissue specimens: ① Tissues were washed with PBS to remove blood, sliced and placed in growth medium containing 0.1% collagenase tyle IV and 5% FBS. The tissue slices were incubated in an incubator at 37°C for 2 h. ② Tissue slices were grounded thoroughly, and then tissue debris was removed using 100 μm strainer. ③ The grounded tissues were centrifuged in a tube. Cell masses were removed using 30 μm strainer, yielding a single-cell suspension. ④ The single-cell suspension was centrifuged at 1500 r/min for 5 min. ⑤ After removing the supernatant, Ficoll reagent was added for cell isolation. The buffy coat was aspirated, washed, and then re-suspended in PBS containing 5% FBS (about 1×10^7/ml) for later use.

Flow cytometry

(1) 100 μl of the PBMCs were isolated from peripheral blood, co-cultured with thymalfasin, and single-cell suspension were put in tubes. (2) 20 μl of fluorescent-marked anti-HLA-DR, anti-CD14 and anti-CD33 antibodies were added to each tube, respectively. (3) The contents of the tubes were thoroughly mixed and incubated in darkness for 20 min at 4°C. (4) 2 ml of PBS was added to the tubes, followed by centrifugation at 1500 rpm for 5 min. The supernatant was removed, and the cells were washed three times. (5) 0.5 ml of PBS was added to the tubes, and Beckman Coulter flow cytometry was used for test. The expression of peripheral PBMCs of NSCLC patients, the expression of MDSCs in tumor tissues, and the proportion of PBMCs in peripheral blood and PBMCs co-cultured with thymalfasin were analyzed.

Observation indicators

Primary outcome measures: The expression of MDSCs in peripheral blood of NSCLC patients and healthy controls was compared; the expression of MDSCs in tumor tissues and peripheral blood of NSCLC patients was compared; the contents of HLA-DR-CD14-$CD33^+$ MDSCs in peripheral blood and tumor tissues of the NSCLC patients were analyzed before and after thymalfasin treatment.

Secondary outcome measures: The basic characteristics of both the NSCLC group and healthy control group were analyzed.

Statistical analysis

SPSS 24.0 was used for statistical analysis. Normally distributed data were expressed as mean ± standard deviation. Independent sample t test was used to compare the differences between two groups. ANOVA followed with LSD-t test was used to compare the differences of means among multiple groups. Qualitative data were expressed as frequency and percentage (n%) and tested by chi-square test. $P < 0.05$ indicated statistically significant difference.

Results

Analysis of basic characteristics

There were no significant differences between NSCLC group and healthy control group in age, gender, BMI, hypertension, diabetes, history of smoking, and history of alcohol drinking, etc. (all $P > 0.05$, **Table 1**).

The expression of MDSCs in peripheral blood of NSCLC patients and healthy controls

As shown in **Figure 1**, the proportion of MDSCs in peripheral blood of patients in NSCLC group was 1.70±0.52%, which was significantly higher than 0.51±0.15% of healthy control group ($P < 0.05$).

Comparison of HLA-DR-CD14-$CD33^+$ MDSCs in tumor tissue samples before and after thymalfasin treatment

As shown in **Table 2**, the expression of HLA-DR-CD14-$CD33^+$ MDSCs in tumor tissues of NSCLC patients before and after thymalfasin treatment was analyzed. The results showed that the proportion of HLA-DR-CD14-$CD33^+$ MDSCs in tumor tissues of NSCLC patients after treatment was significantly lower than that before treatment ($P < 0.05$).

Change in the proportion of MDSCs in peripheral blood of NSCLC patients before and after thymalfasin therapy

As shown in **Table 3** and **Figure 2**, the proportion of MDSCs in peripheral blood of NSCLC patients before thymalfasin therapy was 1.70±0.52%, and thymalfasin therapy substantially

Table 1. Comparison of basic characteristics between the two groups [n%, (x ± sd)]

Characteristics	NSCLC group (n = 50)	Healthy control group (n = 50)	χ^2/t	P
Age	51.20±7.56	50.68±7.20	0.352	0.725
Gender			0.040	0.841
Male	27 (54.00)	26 (52.00)		
Female	23 (46.00)	24 (48.00)		
BMI (kg/m^2)	23.08±1.22	23.22±1.24	0.569	0.571
Hypertension			0.641	0.423
Yes	28 (56.00)	24 (48.00)		
No	22 (44.00)	26 (52.00)		
Diabetes			0.040	0.841
Yes	26 (52.00)	25 (50.00)		
No	24 (48.00)	25 (50.00)		
History of smoking			0.164	0.685
Yes	22 (44.00)	20 (40.00)		
No	28 (56.00)	30 (60.00)		
History of alcohol drinking			0.167	0.683
Yes	21 (42.00)	19 (38.00)		
No	29 (58.00)	31 (62.00)		

Note: BMI: body mass index; NSCLC: non-small cell lung cancer.

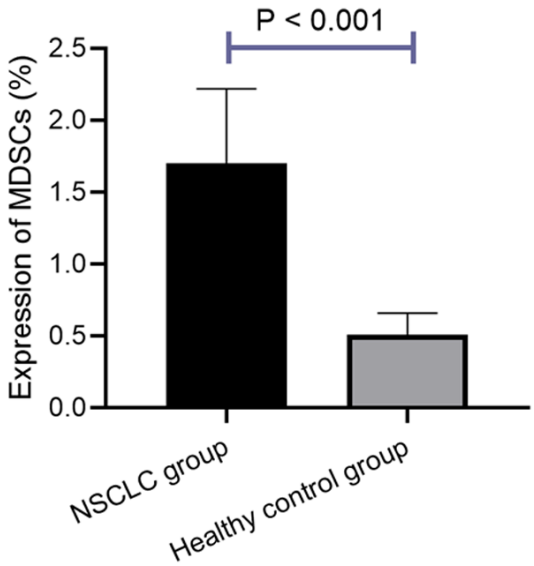

Figure 1. Expression of MDSCs in peripheral blood of the two groups. Note: MDSCs: myeloid-derived suppressor cells; NSCLC: non-small cell lung cancer.

decreased the contents of MDSCs to 0.59± 0.18% in NSCLC patients ($P < 0.05$).

Discussion

Immunosuppression and its mechanism are the hot topics in tumor immunity research. MDSCs, Treg, and NK T cells play key roles in immunosuppression, of which MDSCs are thought to be probably the most important group of immunosuppressive cells [8]. MDSCs, originating from bone marrow, represent immature cell populations that serve as precursors to mature macrophages and granulocytes. They exert potent anti-tumor immune response, can proliferate and become activated in abundance within peripheral blood, bone marrow, or lesions. After being activated in peripheral blood, MDSCs participate in immune escape, immune tolerance, and inflammatory response [9]. The common characteristics of MDSCs include their myeloid origin, immature phenotype, significant inhibition of T cell response, and negative immune regulation in tumors and other diseases. Although the phenotype of MDSCs in cancer patients remains unclear, CD11b$^+$, CD33$^+$, and HLA-DRneg/low MDSCs are commonly considered as characteristic markers. To date, MDSCs have been shown to be highly expressed in patients with liver cancer, stomach cancer, breast cancer, multiple myeloma, etc. [10-13]. Ongoing research aims to elucidate the expression of MDSCs in patients with different tumor types, with emerging evidence suggesting a potential correlation between MDSC levels and tumor stage.

With the continuous deepening of modern medical research, it has been found that MDSCs possess immunosuppressive effects in a variety of animal tumor models and tumor patients, promoting tumor progression. The immunosuppressive functions of MDSCs can be antigen-specific or antigen-non-specific, which is determined by the local microenvironment and the nature of the tumor [14]. For example, MDSCs can promote tumor angiogenesis by releasing pro-angiogenesis factors. In addition, they can inhibit the function of T cells by producing arginine, reactive oxygen species and nitric oxide. Natural killer (NK) cells represent a crucial component of the body's frontline defense in immune protection, as they possess the ability to

Table 2. Expression of HLA-DR-CD14-CD33$^+$ MDSCs in tumor tissues of NSCLC patients before and after thymalfasin therapy (x ± sd)

Group	n	Proportion of MDSCs (%)	t-value	*P*-value
Before treatment	50	1.65±0.43	5.357	< 0.001
After treatment	50	1.15±0.50		

Table 3. Change of the proportion of MDSCs in peripheral blood of NSCLC patients before and after thymalfasin therapy

Group	n	Proportion of MDSCs (%)	t-value	*P*-value
Before treatment	50	1.70±0.52	12.260	< 0.001
After treatment	50	0.59±0.18		

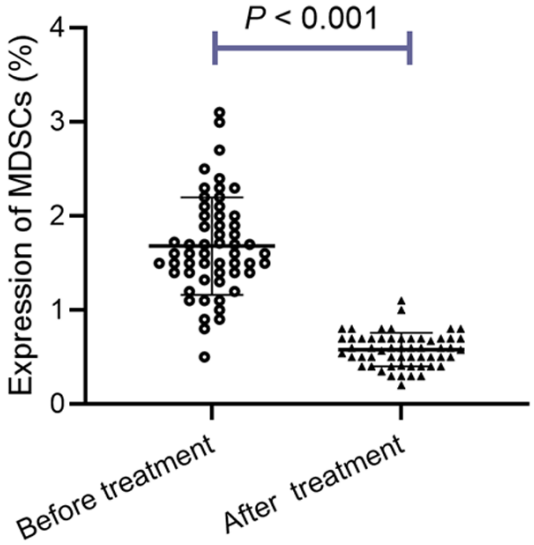

Figure 2. Changes in the proportion of MDSCs in peripheral blood of NSCLC patients before and after thymalfasin therapy.

indiscriminately kill tumor cells. In addition to their cytotoxic activity, NK cells can also regulate the immune function of the body [15]. MDSCs can inhibit NK cell activity by membrane-bounding TGF-β1, thereby diminishing the expression of NKG2D and IFN-γ in NK cells. Moreover, MDSCs can also differentiate into regulatory dendritic cells at the tumor site, resulting in a decreased ability of T cells to secrete IFN-γ. A series of immune dysfunction in tumor patients can result in abnormal changes in the body's response to microbes and their toxins. Immune abnormalities have a direct or indirect effect on the planting, adhesion and proliferation of tumor cells, thereby inducing the continuous progression and deterioration of cancer [16].

In this study, the expression of MDSCs was markedly elevated in NSCLC group compared with healthy control group. In the tumor microenvironment, tumor cells can induce the production and amplification of MDSCs [17]. In addition, MDSCs can inhibit the immune function of host cells. For NSCLC patients, high expression of MDSCs may contribute to the suppression of the anti-tumor immune response, particularly by inhibiting the immune function of other cells, thereby leading to immune escape of tumor cells [18]. Studies have shown that the expression of MDSCs is closely related to the TNM staging and lymph node metastasis in NSCLC patients, while no significant associations were observed with patients' gender, age and pathological type [19]. However, in terms of TNM staging, the proportion of patients with T3 and T4 cancer was more than that of patients with T1 and T2 cancer. Previous studies have indicated that MDSC level is tied to tumor progression and disease severity, making it a key indicator to access NSCLC progression [20]. Therefore, it is imperative to closely monitor the levels of MDSCs in NSCLC patients in clinical practice. Timely feedback should be provided, and reasonable interventions should be taken, so as to improve patients' prognosis.

Flow cytometry revealed that the percentage of MDSCs in peripheral blood of NSCLC group before treatment was 1.70±0.52%, substantially higher than 0.59±0.18% after treatment. This indicates elevated contents of MDSCs in NSCLC patients, likely due to tumor-induced mobilization of MDSC precursor cells into the bloodstream, where they subsequently proliferate and differentiate. Additionally, the expression of HLA-DR-CD14-CD33$^+$ MDSCs in tumor tissues of NSCLC patients was analyzed. The results showed that the proportion of HLA-DR-CD14-CD33$^+$ MDSCs in tumor tissues of NSCLC patients after treatment was significantly lower than that before treatment. It indicates different recruitment patterns influenced by cytokine profiles in the tumor microenvironment. Notably, the isolation of MDSCs from tumor tissue requires mechanical and enzymatic treatment, resulting in incomplete recovery of MDSCs [21, 22]. Prior research has

revealed a lower proportion of MDSCs in tumor tissues compared to peripheral blood in head and neck tumors [23]; MDSCs showed high expression in the tumor tissue microenvironment of lung cancer, stomach cancer, and bladder cancer [24]; compared with tumor tissue-derived MDSCs, only MDSCs isolated from peripheral blood can inhibit T cell proliferation in melanoma patients [25].

In the context of immunotherapy, thymosin, particularly synthetic thymosin like thymalfasin, has garnered attention for its immunomodulatory properties. Thymalfasin enhances T-cell growth and differentiation, playing a crucial role in immune function improvement [26, 27]. Clinical application of thymalfasin alongside chemotherapy in NSCLC treatment demonstrates decreased MDSC levels in peripheral blood, akin to healthy controls, suggesting enhanced immune function and diminished myeloid-derived suppression. Besides, most patients showed good tolerance and high safety after use, and no relevant adverse reactions have been reported so far [28]. In this study, after the treatment with thymalfasin, the percentage of MDSCs in peripheral blood of NSCLC patients was significantly decreased, indicating that thymalfasin can substantially improve the immune function of patients and reduce myeloid-derived suppression to a certain extent. The reasons may be that thymalfasin can enhance B cell-mediated humoral immunity, enhance NK cell activity, increase cytokine secretion, thereby enhancing killing activity of lymphocyte and improving immune function. It has been reported that the application of thymalfasin in patients with lung cancer can promote the maturation of T lymphocytes, enhance the expression of IL-2 receptors on the surface of lymphocytes, promote the dynamic balance of Th1/Th2, activate $CD4^+$ cells, enhance the mixed lymphocyte reaction, improve the aggregation and killing ability of NK cells, and thus enhance immune function [29]. Thymalfasin combined with chemotherapy can directly inhibit tumor cell growth and promote immune function recovery by directly inhibiting the growth, promotion and apoptosis of lung cancer A549 cells [30].

In conclusion, MDSCs play a pivotal role in tumor immune evasion and disease progression. Thymalfasin treatment in NSCLC management holds promise in bolstering anti-tumor efficacy, with implications for novel tumor therapy targets involving MDSC modulation. The presence of MDSCs in NSCLC patients' peripheral blood may serve as a valuable clinical indicator, potentially indicative of immunosuppression. Targeting MDSCs holds potential for inhibiting NSCLC growth and metastasis, albeit necessitating further elucidation of specific mechanisms.

Disclosure of conflict of interest

None.

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