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Cinobufotalin effect and mechanism on serum MMP-2, MMP-9, Beclin1, LC3-II in advanced NSCLC patients

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ABSTRACT

This study was conducted to explore cinobufotalin's effects and related mechanisms on serum MMP-2, MMP-9, Beclin1, and LC3-II in advanced non-small-cell lung cancer (NSCLC) patients. For this purpose, 150 patients with advanced NSCLC in our hospital from Jan. 2020 to Feb. 2022 were chosen as participants in the research study. Using a random number table method, the 150 patients were divided evenly into two groups - a control group (C) and an observation group (O). Group C received conventional NP regimen chemotherapy, while Group O received cinobufotalin capsules based on the control group. The follow-up was conducted for 4 months, and the differences in serum MMP-2, MMP-9, Beclin1, LC3-II and chemotherapy resistance rates were compared. Results showed that There was no statistically significant difference in MMP-2, MMP-9, Beclin1, and LC3-II levels between the two before treatment ($P>0.05$); 4 months later, Group O's MMP-2, MMP-9, Beclin1, and LC3-II levels were lower than those before treatment and Group C during the same period, with a statistically significant difference ($P<0.05$); At 4 months after treatment, the clinical efficacy of Group O was better and its ORR was higher, with a statistically significant difference ($P<0.05$); Using Pearson correlation analysis, a weak positive correlation was identified between MMP-2, Beclin1, LC3-II, and chemotherapy resistance (r=0.167, 0.197, 0.273, $P<0.05$), a positive correlation between MMP-2 and MMP-9, Beclin1, LC3-II (r=0.592, 0.852, 0.665, $P<0.01$), a positive correlation between MMP-9 and Beclin1, LC3-II (r=0.552, 0.472, $P<0.01$), and a positive correlation between Beclin1 and LC3-II (r=0.647, $P<0.01$). It was concluded that cinobufotalin has an inhibitory effect on the serum MMP-2, MMP-9, Beclin1, and LC3-II levels in advanced NSCLC patients, which can promote clinical efficacy improvement and reduce the risk of chemotherapy resistance by downregulating MMP-2, Beclin1, and LC3-II levels.

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Introduction

The majority of patients diagnosed with non-small cell lung cancer (NSCLC) are already in the middle to late stages of the disease (1). At present, NSCLC patients often receive intravenous chemotherapy drugs, which can promote the improvement of quality of life and physical condition within 6 months after radical surgery (2). Meanwhile, intravenous chemotherapy is also a treatment option for NSCLC patients who cannot be treated surgically (3). A study on platinum paclitaxel chemotherapy for NSCLC reported that in terms of safety, 5 out of the 11 patients (45%) experienced adverse events associated with neoadjuvant therapy, while only 1 patient (9%) had level 3 or more severe treatment-related adverse events (4). Cisplatin chemotherapy is a commonly used intravenous chemotherapy method. When NSCLC is diagnosed, the tumor is more advanced and cancer cells are more likely to develop acquired drug resistance (5). Research suggests that Cisplatin, a type of platinum-based drug, is frequently used in combination chemotherapy for NSCLC because of its high rate of clinical response. Nevertheless, the development of acquired resistance to cisplatin is ultimately an inevitability. (6). Chinese scholars have found that cinobufotalin has multiple active ingredients and has a significant inhibitory effect on up to 278 genes in liver cancer, participating in the P53 signaling pathway (7). Pu et al. (8) used cinobufotalin, etoposide, and cisplatin to treat elderly NSCLC patients, and found that the combination group had a decrease in inflammatory factors and serum tumor markers. The main factors that impact inflammation and tumor growth are Nuclear factor-κB and Signal Transducer and Activator of Transcription 3 (STAT3). When these factors are activated, they promote the proliferation of lung cancer cells. Downregulation of Matrix Metalloproteinase-2 (MMP-2) and Matrix Metalloproteinase-9 (MMP-9), coupled with upregulation of Tissue Inhibitor of Metalloproteinase-2 (TIMP-2), inhibits tumor cell migration. Moreover, tumor cells resistant to cisplatin exhibit higher autophagic characteristics, with microtubule-associated protein 1 light chain 3-II (LC3-II) and autophagy-associated protein 1 (Beclin1) levels increasing (9). Prospective research is implemented to evaluate cinobufalin effects on serum MMP-2, MMP-9, Beclin1, and LC3-II in advanced

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NSCLC patients by platinum-based intravenous chemotherapy, and to determine the mechanism of its impact on platinum-based drug resistance gene expression.

Materials and Methods

General materials

150 patients with advanced NSCLC in our hospital from Jan. 2020 to Feb. 2022 were chosen for research. Utilizing a random number table method, the patients were randomly allocated into Group C and Group O with 75 in each group. A research team was formed. This study was reviewed by the medical ethics committee of our hospital and granted approval.

Inclusion criteria

(A) Patients comply with the relevant diagnostic criteria of the NCCN Clinical Practice Guidelines: Non-Small Cell Lung Cancer (2020. V8) (10); (B) Age>18; (C) The clinical staging is between stage IIIa and stage IIIb; (D)All patients and their families were provided with information about the study and have given their informed consent by signing a consent form.

Exclusion criteria

(A) Patients with combined immune deficiency or abnormal coagulation function; (B) Expected survival time<6 months, KPS score<60 points; (C) Patients who are allergic to chemotherapy drugs or cinobufalin; (D) Patients in other drug-related trials before enrollment or those who withdrew midway; (E) Patients with a clear history of platinum drug resistance.

Methods

Both groups were treated with NP regimen chemotherapy. Vinorelbine (produced by Yunnan Botanical Pharmaceutical Co., Ltd.; Product name: vinorelbine tartrate for injection; Specification: 10mg) was given intravenously on the first and eighth days, the injection amount was 25mg/(kg·m^2), and 21 days of continuous treatment was a cycle; Intravenous infusion of cisplatin (produced by Yunnan Plant Pharmaceutical Co., Ltd.; Product name: cisplatin injection; Specification: 2ml: 10mg), with a dosage of 75mg/(kg·m^2), combined with conventional treatment methods such as hydration, was conducted for the first to fifth days. One cycle lasts for 28 days and lasts for 4 cycles. Group O was treated with cinobufalin injection (produced by Anhui Huarun Jinchan Pharmaceutical Co., Ltd.; Product name: Huachansu injection; Specification: 5ml/piece) on the basis of NP regimen chemotherapy. The dosage was 20ml dissolved in 500ml of 5% glucose injection and slowly infused. The medication period was 7 days, and the next cycle was carried out after an interval of 2 days. The four cycles were one course of treatment, and after one course of treatment, it was changed to cinobufalin tablets (produced by Anhui Huarun Jinchan Pharmaceutical Co., Ltd., product name: Huachansu tablets, specification: 0.3g/tablet) for oral use, 0.6g/dose, bid, for 3 months. And the serum MMP-2, MMP-9, Beclin1, and LC3-II levels, and the expression of platinum resistance genes in patients were monitored before and 4 months after treatment. Among them, serum MMP-2, MMP-9, Beclin1, and LC3-II level detection samples were collected from the fasting elbow venous blood 5ml of patients in both groups in the morning, centrifuged 15min at 3000 r/min. Enzyme-linked immunosorbent assay (ELISA) extracted and tested supernatant. The reagents were provided by Shanghai Yiyan Biotechnology Co., Ltd. and tested using the FC/K3 tabletop enzyme marker produced by Thermofly in the United States. The complete remission (CR, complete disappearance of the target lesion, no new lesion, and normal tumor markers), partial remission (PR, maximum diameters sum of the target lesion decreases by more than 30% and lasts for 4 weeks or more), disease stability (PD, the sum of the maximum diameters of the target lesion decreases by PR or does not increase to PD), disease progression (SD, the sum of the maximum diameters of the target lesion increases by more than 20% or new lesions appear) were collected and recorded. The CR PR patients were defined as chemotherapy-sensitive, while PD and SD patients were defined as chemotherapy-resistant. The clinical objective response rate (ORR=chemotherapy sensitive rate) was compared.

Statistical methods

In data processing, SPSS 26.0 software was used to test the normal distribution and homogeneity of variance of the data obtained from the study. Categorical data were presented as percentages (%), and the chi-square test was utilized. Normally distributed continuous data were expressed as mean ± standard deviation (s), while independent sample t-tests and paired-sample t-tests were carried out to identify any statistical differences in the data points between groups and within groups at different time points, and Mann Whitney test was used for hierarchical data. Pearson correlation analysis can clarify the correlation between serum sample data and chemotherapy resistance. The level is corrected as α=0.05.

Results

General materials comparison between two groups

There was no statistically significant difference in age, gender, clinical stage, tumor type, and KPS score (P>0.05) shown in Table 1.

Changes comparison in MMP-2 and MMP-9 before and after treatment

Prior to treatment, there was no significant difference in MMP-2 and MMP-9 (P>0.05). 4 months later, there was a significant reduction in MMP-2 and MMP-9 in Group O compared to both pre-treatment levels and the same period in Group C (P<0.05) in Table 2 and Figure 1.

Changes comparison in Beclin1 and LC3-II before and after treatment

There was no statistically significant difference in Beclin1 and LC3-II before treatment (P>0.05); Beclin1 and LC3-II in Group O after 4 months of treatment were lower than those before treatment and in Group C during the same period, with statistical significance (P<0.05), as shown in Table 3 and Figure 1.

Clinical efficacy comparison after treatment

After 4 months of treatment, the clinical efficacy of Group O was better, and the ORR was higher, with statistical significance (P<0.05) as shown in Table 4 and Figure 1.

Table 1. General materials comparison.

Group		Observe group (n=75)	Control group (n=75)	Statistics	*P*
Age		67.01±4.87	66.84±4.81	-0.219	0.827
Gender	Male	44(58.67)	46(61.33)	0.111	0.739
	Female	31(41.33)	29(38.67)		
Clinical stage	IIIa	37(49.33)	42(56.00)	0.669	0.414
	IIIb	38(50.67)	33(44.00)		
Tumor type	Adenocarcinoma	26(34.67)	27(36.00)	0.409	0.815
	Squamous cell carcinoma	34(45.33)	36(48.00)		
	Large cell carcinoma	15(20.00)	12(16.00)		
KPS score		71.12±4.92	71.71±4.11	0.792	0.429

Table 2. MMP-2 and MMP-9 comparison before and after treatment.

Group	Time	Group O (n=75)	Group C (n=75)	Statistics	*P*
MMP-2 (μg/L)	Before	148.55±14.01	147.28±15.65	0.523	0.602
	After	23.68±4.90*	61.91±7.69*	36.272	<0.001
MMP-9 (ng/L)	Before	436.16±22.47	434.62±22.43	-0.421	0.674
	After	331.29±29.49*	369.58±15.94*	9.892	<0.001

Note: Compared with before treatment, *P<0.05.

Table 3. Beclin1 and LC3-II comparison before and after treatment.

Group	Time	Group O (n=75)	Group C (n=75)	Statistics	*P*
Beclin1 (ng/mL)	Before	4.79±0.94	4.66±0.83	0.947	0.345
	After	1.47±0.42*	3.07±0.41*	23.638	<0.001
LC3-II (ng/mL)	Before	87.72±9.40	88.45±11.87	0.416	0.678
	After	57.38±4.47*	67.99±6.70*	11.403	<0.001

Note: Compared with before treatment, *P<0.05.

Table 4. Clinical efficacy comparison before and after treatment.

Group	Group O (n=75)	Group C (n=75)	Statistics	*P*
CR	16(14.67)	11(14.67)		
PR	29(38.67)	20(26.67)	2.193*	0.028
SD	20(26.67)	27(36.00)		
PD	10(13.33)	17(22.67)		
ORR	45(60.00)	31(41.33)	5.228#	0.022

Note: * is tested by Mann-Whitney, and # is tested by chi-square.

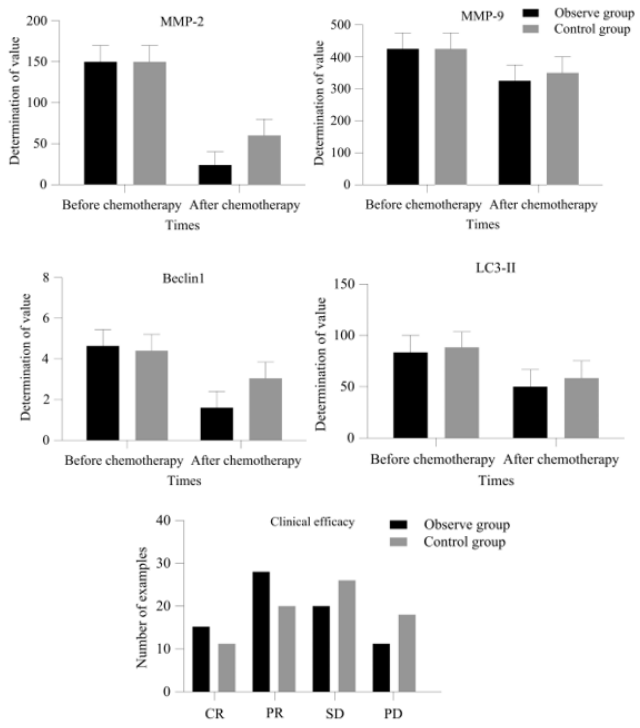

Figure 1. Clinical efficacy results of MMP-2, MMP-9, Beclin1, LC3-II before and after treatment.

Correlation between MMP-2, MMP-9, Beclin1, LC3-II and chemotherapy resistance in patients with advanced NSCLC

Pearson correlation analysis reveals a weak positive correlation among MMP-2, Beclin1, LC3-II, and chemotherapy resistance (r=0.167, 0.197, 0.273, P<0.05), among MMP-2, MMP-9, Beclin1, and LC3-II (r=0.592, 0.852, 0.665, P<0.01), among MMP-9, Beclin1, and LC3-II (r=0.552, 0.472, P<0.01, and between Beclin1 and LC3-II (r=0.647, P<0.01) shown in Table 5.

Discussion

The treatment of mid to late-stage NSCLC mainly focuses on inhibiting the growth of tumor cells at the lesion site, reducing the risk of tumor invasion and metastasis. The main obstacle of platinum-based chemotherapy for advanced NSCLC is chemotherapy resistance. Zhou et al. (10) showed higher basal autophagy levels in cisplatin-resistant A549/DDP cells treated with cisplatin, and increased autophagy proteins' expression levels in Beclin 1 and LC3. Cisplatin-based chemotherapy is a preferred treatment option for NSCLC (11). However, long-term exposure to hypoxia in NSCLC patients can easily lead to

Table 5. Correlation between MMP-2, MMP-9, Beclin1, LC3-II levels and chemotherapy resistance in NSCLC patients.

Indicators	MMP-2		MMP-9		Beclin1		LC3-II		Chemotherapy resistance	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
MMP-2	0.000	1.000								
MMP-9	0.592	0.000	0.000	1.000						
Beclin1	0.852	0.000	0.552	0.000	0.000	1.000				
LC3-II	0.665	0.000	0.427	0.000	0.647	0.000	0.000	1.000		
Chemotherapy resistance	0.167	0.041	0.065	0.428	0.197	0.016	0.273	0.001	0.000	1.000

cisplatin resistance (12). The occurrence and development of tumors are significantly influenced by autophagy. In the early stage, autophagy can eliminate surgical organelle and oncogenic proteins, but in the late stage of tumors, the degradation of organelle and proteins in cells is often used to provide energy and nutrients for tumor cells. Their growth is sustained by systemic autophagy, which facilitates immune evasion and sustains the biosynthesis and energy generation necessary for cancer cell metabolism. In the absence of autophagy, the tumor becomes susceptible to damage and cannot recover. This provides further conceptual evidence that inhibiting autophagy is an effective pathway for cancer treatment (13). The regulation of the autophagy mechanism in the body helps to form an inhibitory effect on tumor cell growth.

Cinobufalin, processed as a dried toad skin extract, is rich in toad bufadienolides, cinobufagin, as well as alkaloids, reducing sugars, amino acids, as well as toad venom, toad tryptamine, and other components. It is suitable for various tumors (14). Cinobufalin has a clear cytotoxic effect, which can directly kill tumor cells, inhibit tumor growth, alleviate the toxic side effects of chemotherapy during tumor treatment, and improve the body's immunity, and the objective response rate to the treatment (15). A meta-analysis found that the application of cinobufalin on the basis of conventional platinum chemotherapy can promote chemotherapy efficacy, improve patient life quality, and reduce the side effects of platinum chemotherapy drugs (16). At the same time, some scholars believe that cinobufalin has the functions of inhibiting cell proliferation, inducing cell differentiation, inhibiting angiogenesis, reversing multiple drug resistance and immune regulation (17). The application of cinobufalin on the basis of conventional chemotherapy can help inhibit the tumor cells growth and improve the body autophagy function.

The results showed that cinobufalin by chemotherapy can inhibit the MMP-2, MMP-9, Beclin1, and LC3-II levels in patients with advanced NSCLC. The reason for this is that cinobufalin is rich in a large amount of bufalin, which can act on bone marrow red blood cells, alleviate bone marrow suppression, and promote immune function improvement (18). MMP-2 can promote a decrease in intercellular adhesion and is the main cofactor for tumor infiltration and metastasis. The decrease in MMP-2 levels marks a decrease in the activity status of tumor cells (19). MMP-9 is mainly used to degrade the extracellular matrix and is an important influencing factor for tumor cell metastasis and growth. The decrease in its level marks a decrease in tumor cell metastasis ability (20) Cellular autophagy activity is significantly associated with Beclin1, an essential factor responsible for inducing cellular stress, decreasing gene stability, and promoting tumor formation. When the level of Beclin1 decreases, the autophagy activity of cells also decreases (21). LC3-II is the gold standard for evaluating autophagy activity (22). At the same time, the clinical efficacy of Group O was better at 4 months after treatment, and the ORR was higher, with a statistically significant difference (P<0.05). Cinobufalin with chemotherapy can improve clinical efficacy. Based on the fact that cinobufalin is rich in alkaloids, amino acids, and other substances, it can assist in producing anti-tumor effects, inhibit DNA and RNA related to cancer cells in tumor cell cycle S-phase, induce cancer cell apoptosis, and inhibit cancer cell growth.

Through Pearson correlation analysis, this study found a weak positive correlation among MMP-2, Beclin1, LC3-II, and chemotherapy resistance (r=0.167, 0.197, 0.273, P<0.05). The chemotherapy resistance of mid to late stage NSCLC is mainly related to MMP-2, Beclin1, and LC3-II. Cinobufalin can inhibit MMP-2, Beclin1, and LC3-II levels. It is considered that cinobufalin mainly reduces the risk of chemotherapy resistance by downregulating MMP-2, Beclin1, and LC3-II levels. There is a positive correlation among MMP-2, MMP-9, Beclin1, and LC3-II. The reason for this is that there is a direct or indirect relationship between MMP-2, MMP-9, Beclin1, and LC3-II, all of which are related to cell apoptosis, cell proliferation, and immune response. This result is similar to the research results of Wang et al. (23) and Guan et al. (24).

In summary, cinobufalin has an inhibitory effect on the serumMMP-2, MMP-9, Beclin1, and LC3-II levels in patients with advanced NSCLC, which can promote clinical efficacy improvement and reduce the risk of chemotherapy resistance by downregulating MMP-2, Beclin1, and LC3-II levels.

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