Detection of netrin-1 as a novel biomarker for diagnosis and chemotherapeutic monitoring of lung cancer

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Abstract

Objective: Lung cancer has high morbidity and mortality. We aimed to determine the value of netrin-1 for the diagnosis and chemotherapeutic monitoring of lung cancer.

Methods: Thirty pairs of lung cancer tissues and serum were collected. Netrin-1 expression was detected by immunohistochemistry and enzyme-linked immunosorbent assay. Netrin-1 expression was downregulated in A549 cells using small interfering RNA, and the effect of netrin-1 on cisplatin-induced lung cancer cell apoptosis was determined by flow cytometry.

Results: Netrin-1-positivity was significantly higher in lung cancer tissues than in paracarcinoma tissues and high expression of netrin-1 was closely related to a poor prognosis. Serum netrin-1 levels were also significantly higher in lung cancer patients than in healthy donors, and were higher in patients with lung cancer before the beginning of chemotherapy compared with after the completion of four cycles of chemotherapy. Netrin-1 knockdown increased the rate of cisplatin-induced apoptosis in A549 cells.

Conclusions: Netrin-1 expression was increased in tissues and serum from lung cancer patients and decreased after chemotherapy, suggesting that it may be a potential diagnostic marker and indicator of chemosensitivity. Netrin-1 may participate in cisplatin resistance by reducing apoptosis, thus providing a new strategy for addressing chemoresistance in patients with lung cancer.

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Introduction

Lung cancer has the highest morbidity and mortality rates among malignant tumors worldwide.[1] Most patients have advanced lung cancer at the time of diagnosis and more than half of all lung cancer patients die within 1 year after diagnosis, with a 5-year survival rate of less than 18%.[2] Chemotherapy is the main treatment for advanced lung cancer, and platinum-based chemotherapy regimens are widely used. Cisplatin, the most commonly used chemotherapeutic agent, inhibits DNA replication, affects cell transcription and translation, and promotes tumor cell apoptosis.[3] Dysregulation of apoptosis is a sign of chemotherapy resistance,[4] and strategies aimed at overcoming chemotherapy resistance include the development of new cytotoxic drugs, regulation of cell apoptosis, and combination therapy.[5] Developing sensitive markers and effective methods to overcome chemotherapy resistance are thus important factors affecting the prognosis of patients with lung cancer.

Netrin-1, the best-characterized ligand of the netrin family, is a highly conserved, cell-secreted soluble protein that promotes axon growth and mediates neuronal development.[6] Recent studies found that netrin-1 not only induced the development and repair of the central nervous system, but was also highly expressed in a variety of human tumors[7] and was involved in glioma cell proliferation[8] and gastric cancer cell migration.[9]

Netrin-1 has also been reported to promote the invasion and metastasis of lung cancer; however, few studies have examined the biological function of netrin-1 in chemotherapy resistance. In this study, we aimed to detect tissue and serum levels of netrin-1 in patients with lung cancer before and after chemotherapy, and to analyze the relationship between netrin-1 and clinical data. We also examined the effect of netrin-1 on cisplatin-induced apoptosis in A549 cells, to provide evidence to support the use of netrin-1 as a therapeutic target.

Materials and methods

Patient sample collection

Tissue and serum samples were collected from lung cancer patients and healthy donors at the Second Affiliated Hospital of Dalian Medical University (Dalian, China) between July 2019 and January 2020, and stored in a refrigerator at $-80°C$ for later use. The healthy donors were people who were admitted to the Physical Examination Department of the Second Affiliated Hospital of Dalian Medical University, with no abnormal findings on physical examination. The inclusion criteria were: (1) all patients with lung space-occupying lesions detected by high-resolution computed tomography and confirmed by pathology or cytology as lung cancer; (2) serum samples collected from patients before receiving any radiotherapy or chemotherapy drugs; and (3) further serum samples collected after four cycles of chemotherapy with platinum-based chemotherapy drugs. The exclusion criteria were: (1) patients with diabetes, severe heart disease, or mental diseases; (2) patients who had received previous radiotherapy or chemotherapy; and (3) patients with insufficient pathological or clinical data. The present study was approved by the Institutional Review Board of the Second Affiliated Hospital of Dalian Medical University, Dalian, China (approval number: 201934; date of approval: 3 July 2019). All procedures were performed according to the principles outlined in the Declaration of Helsinki. All participants provided written informed consent prior to enrollment for the use of their tissue and/or serum samples for the purposes of the present study and for publication of the

data. The clinical characteristics of all participants, including age, sex, smoking history, and pathological results, were recorded.

Immunohistochemistry (IHC)

Netrin-1 expression in the tissue samples was examined by IHC. Briefly, tissue sections (6 μm) were deparaffinized, rehydrated, incubated with 3% H_2O_2 in methanol, and subjected to antigen retrieval in citrate buffer. The sections were blocked with 3% goat serum working fluid (ZSGB-BIO, Beijing, China) and probed with anti-netrin-1 (Proteintech Group, Wuhan, China) at 4°C overnight. The sections were reacted with biotinylated secondary antibodies and detected using a Streptavidin-Peroxidase IHC assay kit and 3,3′-diaminobenzidine (ZSGB-BIO). Immunostaining was evaluated by two certified pathologists in a blinded manner and scored according to the staining intensity (negative staining: 0 points; weak staining: 1 point; moderate staining: 2 points; and strong staining: 3 points), multiplied by the percentage of stained cells (positive cells <30% of cells: 0 points; 30%–70% of cells: 1 point; >70% of cells: 2 points). Scores of 0–1 were considered negative (−), 2–3 were weakly positive (+), and scores ≥4 were strongly positive (++).

Kaplan–Meier Plotter database

We accessed the Kaplan–Meier Plotter (KM Plotter) database (http://www.kmplot.com) using mRNA lung cancer as the cancer type and the gene symbol NTN1. We set the data-analysis parameters, selected lung adenocarcinoma as the histological type, and chose the median value as the cutoff value. Tumors with netrin-1 mRNA expression levels lower than or equal to the median were designated as having "low expression" and those with mRNA expression levels above the median were designated as having "high expression". We then carried out Kaplan–Meier analysis using the "draw Kaplan–Meier plot" instruction in the database.

Enzyme-linked immunosorbent assay (ELISA)

Netrin-1 concentrations in human serum samples were detected using a Netrin-1 ELISA kit (Cusabio, Houston, TX, USA) following the manufacturer's instructions.

Cell culture

The human lung adenocarcinoma cell line A549 was obtained from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2.

Cell transfection with small interfering (si) RNA

A549 cells were seeded into six-well plates (2×10^5/well) and incubated overnight. The cells were transfected beginning the following day using 50 nmol/L siRNA or siRNA-Mate transfection reagent (GenePharma, Jiangsu, China) as a negative control for 48 hours. The target sequences for si-Netrin-1 #1 and si-Netrin-1#2 were 5′-GCAAGAAGUUCGAAGUGACTT-3′ and 5′-UCCAGCAGCGUGAGAAGAATT-3′, respectively. Finally, si-Netrin-1 #1 showed a better silencing effect and was therefore selected for subsequent experiments.

Western blot analysis

Total protein was extracted from the cells using radioimmunoprecipitation assay buffer (Cell Signaling Technology, Danvers, MA, USA), and the concentration of total protein was determined using a BCA protein assay kit (Abcam,

Cambridge, UK). Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gels and transferred onto polyvinylidene fluoride membranes. After blocking with a 5% milk solution in $1\times$ Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature, the membranes were incubated with primary rabbit monoclonal antibodies to netrin-1 (dilution 1:1000, cat. no. BA1671-1; Proteintech Group) or β-actin (dilution 1:5000, cat. no. 20536-1-AP; Proteintech Group) overnight at 4°C. After three washes with TBST buffer, the membranes were incubated with the corresponding secondary antibodies for 1 hour at room temperature. Antibody-binding signals were visualized using a Super Signal West Pico kit (Thermo Fisher Scientific, Inc., Anthem, AZ, USA), and quantitative densitometric analysis was performed using Eagle Eye II software (Eagle Eye Technology, Ltd., London, UK).

Flow cytometric analysis

Flow cytometric analysis was performed using a flow cytometer (BD FACS Accuri C6; BD Biosciences, Franklin Lakes, NJ, USA). Briefly, the different groups of cells were stained using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences) in the dark at room temperature for 15 minutes and the fractions of apoptotic cells were then determined using the FACS analysis system.

Statistical analysis

Data were expressed as the mean ± standard deviation. Differences among the data were analyzed by Student's *t*-test, analysis of variance with least significant difference, or χ^2 test using SPSS v. 23 statistical software package (IBM Corp., Armonk, NY, USA). A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of netrin-1 in lung cancer tissues

We began with 30 lung cancer tissue samples; however, some samples were stripped during the experimental procedures, resulting in the final results shown in Table 1. Positive staining of netrin-1 protein in lung cancer tissues was mainly observed in the cytoplasm, as brown-yellow granules (Figure 1). The overall netrin-1-positivity rate in lung cancer tissues was 52%, including weak-positive expression (+) in 28% and strong-positive (++) expression in 24%. The overall netrin-1-positivity rate in paracarcinoma tissues was 7.7%, with all tissues showing weak-positive (+) staining. The netrin-1-positivity rate was significantly higher in lung cancer tissues than in the adjacent paracarcinoma tissues (P < 0.01) (Table 1).
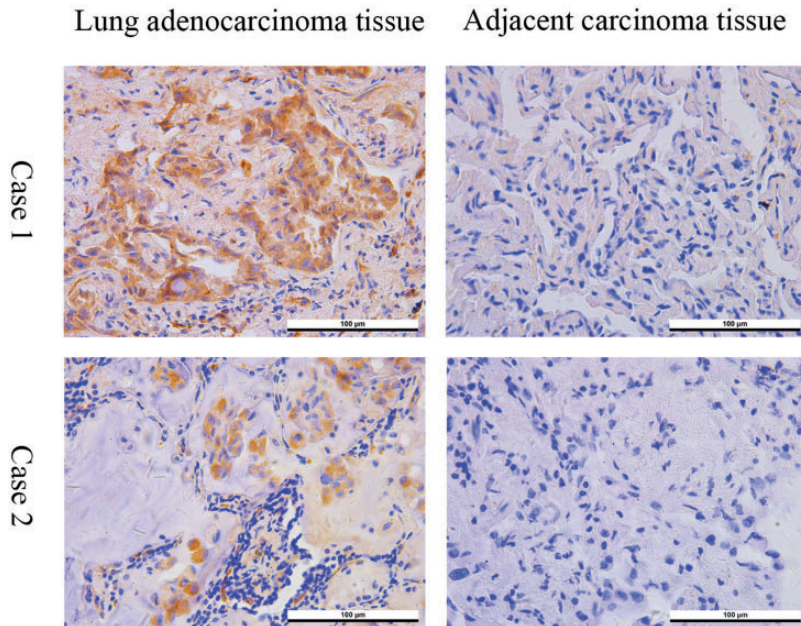
Expression of netrin-1 was closely related to a poor prognosis in patients with lung cancer

The association between netrin-1 expression and overall survival in 672 patients with lung adenocarcinoma was further evaluated by Kaplan–Meier analysis using the KM Plotter database (http://www.kmplot.com).

Table 1. Differential expression of netrin-1 in cancerous and paracancerous lung tissues.

	Netrin-1 expression		
	+	−	P-value
Lung cancer tissues (n = 25*)	13	12	<0.01
Paracarcinoma tissues (n = 26#)	2	24	

*Five samples not included; #four samples not included; this was because of dropping off during contact with boiling citric acid buffer and during swing-drying.

Lung adenocarcinoma tissue   Adjacent carcinoma tissue

Figure 1. Expression of netrin-1 in lung adenocarcinoma (stain: diaminobenzidine (DAB)).

The median survival time of patients with high netrin-1 expression was 91 months, which was significantly lower than the median survival time of patients with low netrin-1 expression (125.77 months; $P = 0.02$) (Figure 2a). We also evaluated the association between netrin-1 expression and overall survival in a cohort of 1144 patients with lung cancer, and found that the median survival time in patients with high netrin-1 expression was 63.03 months, which was significantly lower than the median survival time in patients with low netrin-1 expression (91 months; $P = 0.005$) (Figure 2b). These results suggest that high expression of netrin-1 predicts a poor prognosis in patients with lung cancer.

Serum levels of netrin-1 were elevated in patients with lung cancer

We detected netrin-1 expression levels in serum samples from 22 healthy donors and 30 lung cancer patients (22 non-small cell lung cancer (NSCLC), 8 small cell lung cancer (SCLC)) before the beginning of chemotherapy using ELISAs. The patients' clinical data are summarized in Supplementary Table 1. There was no significant difference in age, sex, or smoking history between the lung cancer patients and healthy control participants ($P > 0.05$) (Supplementary Table 1). The mean netrin-1 serum level was significantly higher in the lung cancer patients (1098.31 ± 605.90 pg/mL) compared with the healthy control group (609.53 ± 365.37 pg/mL) ($P < 0.01$). Serum netrin-1 levels in NSCLC (935.24 ± 503.80 pg/mL) and SCLC patients (1546.75 ± 667.87 pg/mL) were both significantly higher than in the healthy control group ($P < 0.05$) (Figure 3), but levels were significantly higher in the SCLC group compared with the NSCLC group ($P < 0.05$). There were no significant differences in serum netrin-1 levels in relation to age, sex, smoking history, lymph node metastasis, and clinical stage (Table 2).
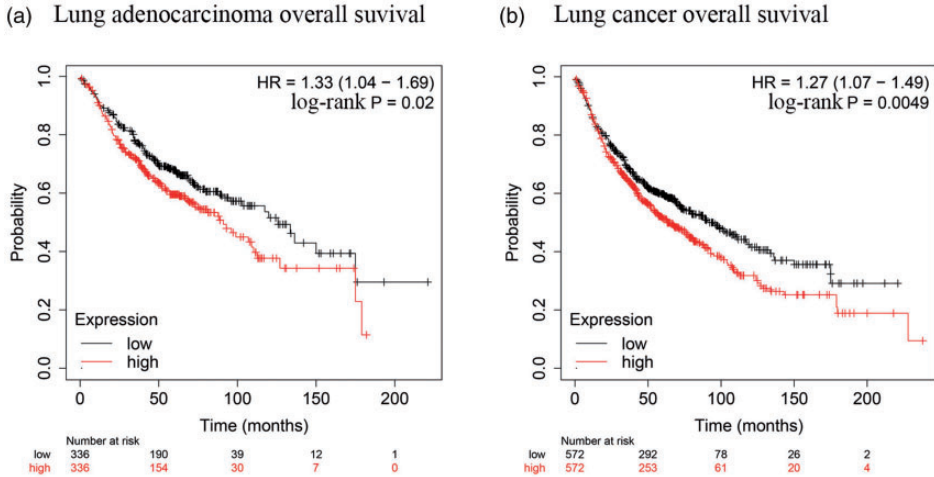
Figure 2. Kaplan–Meier overall survival curves in patients with (a) lung adenocarcinoma and (b) lung cancer with low and high netrin-1 expression levels.
HR, hazard ratio.
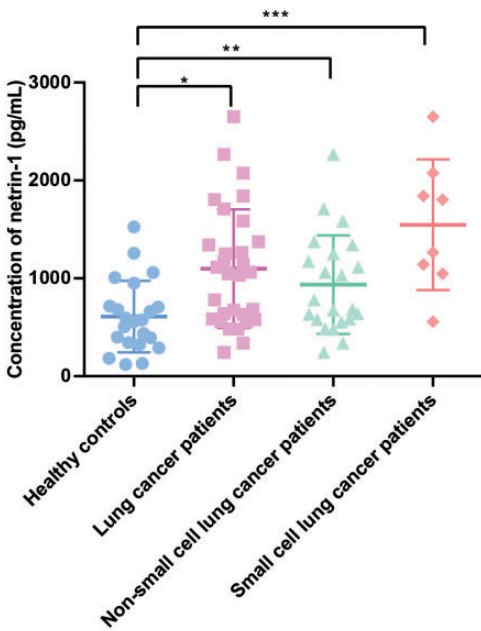
Figure 3. Serum netrin-1 levels in lung cancer patients and healthy controls. $*P < 0.01$, $**P < 0.05$, $***P < 0.01$ vs healthy controls.

Netrin-1 serum levels decreased after chemotherapy

Netrin-1 serum levels in patients with lung cancer were significantly higher before the beginning of platinum-based chemotherapy than after the completion of four cycles of chemotherapy ($P < 0.05$). Netrin-1 serum levels were significantly higher before compared with after chemotherapy in patients with SCLC (1546.75 ± 667.87 pg/mL versus 728.33 ± 321.85 pg/mL, respectively; $P < 0.05$), but not in patients with NSCLC (Figure 4).

Netrin levels were measured in 30 patients with lung cancer before chemotherapy and after four cycles of platinum-based chemotherapy. Among the 22 lung cancer patients with decreased netrin-1 indexes, the efficacy evaluations after four cycles of chemotherapy were stable disease or partial response. After a review of the clinical data of the eight patients with elevated

Table 2. Correlations between serum netrin-1 levels and clinicopathological characteristics.

Characteristic	n	Netrin-1 (mean ± SD)	P-value
Age, years			0.30
≤60	17	1010.46 ± 623.70	
>60	13	1213.18 ± 585.91	
Sex			0.122
Male	18	1238.82 ± 656.49	
Female	12	887.53 ± 470.12	
Smoking history			0.888
No	14	1081.24 ± 657.11	
Yes	16	1113.24 ± 578.82	
Lymph node metastasis			0.698
No	7	1178.18 ± 822.34	
Yes	23	1074.00 ± 544.84	
Stage			0.525
I + II	5	1259.06 ± 847.32	
III + IV	25	1066.16 ± 563.45	
Histological type			0.012
NSCLC	22	935.24 ± 503.80	
SCLC	8	1546.75 ± 667.87	

SD, standard deviation; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer.

netrin-1 indexes after chemotherapy, six had no disease progression and two had progressive disease, and their chemotherapy drugs were changed.

Effect of netrin-1 on cisplatin-induced apoptosis in lung adenocarcinoma cells

A549 cells were transfected with netrin-1 siRNAs to effectively knockdown netrin-1 expression (Supplementary Figure 1). The effects of netrin-1 on the apoptosis rates of lung adenocarcinoma A549 cells with and without cisplatin (20 μmol/L) were detected by flow cytometry. The apoptosis rates of A549 cells without cisplatin were 6.5% in the blank control group, 7.2% in
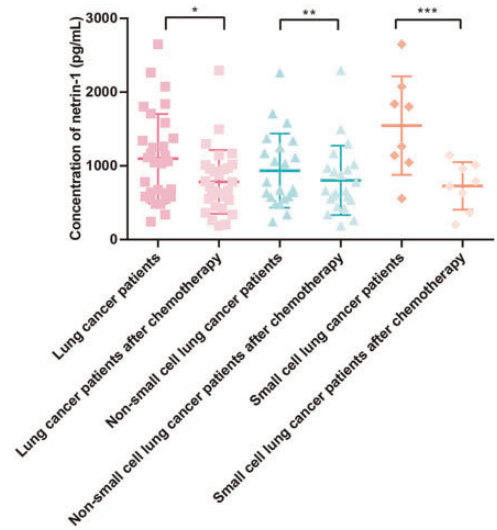

Figure 4. Serum netrin-1 levels before and after administration of chemotherapeutic drugs. *P < 0.05, **P>0.05, ***P < 0.01.

the negative control group, and 14.8% in the netrin-1-knockdown group. After the addition of cisplatin, the apoptosis rates increased to 20.2%, 26.8%, and 75.6%, respectively. The cisplatin-induced apoptosis rate in lung cancer cells was higher (but not statistically significant) in the netrin-1-knockdown group than that in the control group (Figure 5).

Discussion

Chemotherapy is currently one of the most effective methods for the treatment of cancer,[10] with platinum-based chemotherapy often being the first-line treatment for SCLC and advanced NSCLC.[11] However, multidrug resistance, especially resistance to cisplatin regimens, is common in patients with advanced lung cancer and is the major cause of chemotherapy failure in patients with aggressive NSCLC.[12] The mechanisms of tumor resistance to cisplatin
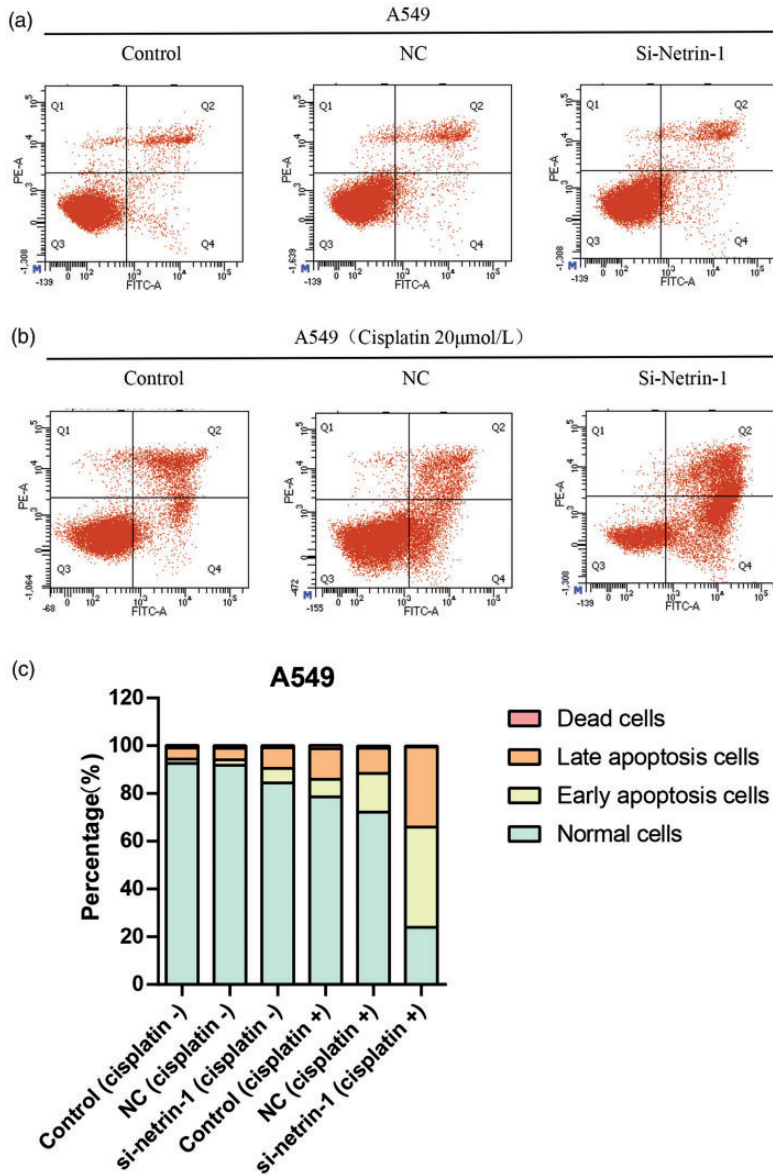
Figure 5. Effect of netrin-1 on cisplatin-induced apoptosis of A549 cells. Effect of netrin-1 on (a) apoptosis of A549 cells detected by flow cytometry and (b) apoptosis of A549 cells induced by cisplatin (20 μM, 24 h) and (c) Graph showing effects of netrin-1 and cisplatin on apoptosis.
NC, negative control.

include reduced membrane transport of drugs, increased cytoplasmic detoxification, enhanced DNA repair, and resistance to apoptosis.[13] Dysregulation of apoptotic pathways may not only promote tumor occurrence, but may also lead to drug resistance.[14,15] It is therefore important to understand the molecular mechanisms

responsible for chemotherapy resistance and to identify biomarkers of drug resistance.[16]

As a neurological guiding factor, netrin-1 has attracted increasing attention in relation to tumor development. Netrin-1 expression has been found to be upregulated in some tumors, and may be a potential diagnostic biomarker for human cancers. Yin et al.[17] collected gastric cancer tissues and paracarcinoma samples from 86 gastric cancer patients and found that netrin-1 expression was upregulated in gastric cancer tissues. Gong et al.[18] found that netrin-1 levels were correlated with tumor stage and 5-year survival, based on tumor and paracarcinoma specimens from 72 cases of renal clear cell carcinoma, suggesting that netrin-1 plays an important role in the occurrence and development of renal clear cell carcinoma. In the current study, expression levels of netrin-1 were higher in lung adenocarcinoma tissues than in paracarcinoma tissues, and high netrin-1 expression was closely related to a poor prognosis among patients with lung cancer. Moreover, serum levels of netrin-1 were also significantly higher in lung cancer patients than in healthy donors, and differed among pathological types of lung cancer, being higher in patients with SCLC than NSCLC. These results suggest that netrin-1 may be a potential diagnostic biomarker for lung cancer. Further studies are planned to improve the detection of netrin-1 in the serum of patients with benign lung diseases, and to expand the sample sizes of the different groups to obtain more comprehensive results.

We also detected serum netrin-1 levels in lung cancer patients before and after four cycles of chemotherapy, and showed that netrin-1 levels decreased after chemotherapy. Among the 22 lung cancer patients with decreased netrin-1 indexes, the efficacy evaluations after four cycles of chemotherapy were stable disease or partial response.

We considered that the decrease in netrin-1 index was related to the efficacy of the chemotherapy. Among the eight patients with elevated netrin-1 indexes after chemotherapy, six had no disease progression in the efficacy evaluation (equivalent to 5 months of follow-up) temporarily, and the other two were evaluated with progressive disease after a review of the clinical data, and their chemotherapy drugs were changed. We therefore hypothesized that netrin-1 may be related to chemotherapeutic resistance, and may play an important role in evaluating chemosensitivity in lung cancer patients.

Our previous study showed that netrin-1 promoted lung cancer invasion and metastasis. However, few studies have examined the role of netrin-1 in drug resistance in patients with lung cancer. Apoptosis resistance is closely related to the occurrence, development, and drug resistance of tumors. Arakawa[19] found that netrin-1 bound to its receptor and inhibited p53-dependent apoptosis. p53 is directly involved in the transcriptional regulation of netrin-1 and its receptor, indicating that the netrin-1 receptor pathway may play an important role in tumorigenesis. Paradisi et al.[20] found that interfering with the netrin-1/netrin-1 receptor interaction *in vitro* enhanced doxorubicin-, cisplatin-, or 5-fluorouracil-induced cancer cell death. In the current study, knockdown of netrin-1 in A549 cells increased cell apoptosis, suggesting that netrin-1 has an anti-apoptotic effect in lung cancer cells. Targeted therapy against netrin-1 may provide a new treatment approach for lung cancer. Liu et al.[21] found that cisplatin induced the expression of netrin-1 protein in bladder cancer cells, which in turn inhibited the apoptosis induced by cisplatin. Our present results showed that netrin-1-knockdown increased the cisplatin-induced apoptosis rate of A549 cells, suggesting that netrin-1 may promote

cisplatin resistance by decreasing apoptotic activity in lung cancer cells.

There are limitations to this study. Owing to the difficulty collected lung cancer tissue samples after chemotherapy, we were unable to measure netrin-1 levels post-chemotherapy. Additionally, larger sample sizes will be valuable, and we are planning a similar study with a larger sample size to address this.

Conclusions

Netrin-1 expression was increased in tissues and serum in patients with lung cancer but decreased after chemotherapy, suggesting that it may be a potential diagnostic marker and indicator of chemosensitivity. Moreover, netrin-1 had an anti-apoptotic effect and may thus promote cisplatin resistance by inhibiting apoptotic activity in lung cancer cells, further supporting its use as a therapeutic target. Further studies will be conducted to clarify the molecular mechanism of netrin-1-induced cisplatin resistance in drug-resistant lung cancer cell lines, to provide new ideas for identifying and overcoming drug resistance in patients with lung cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article.

Author contributions

Study concept and design: Lihua Cao and Yuanyuan Zhao; acquisition of data: Yuanyuan Zhao, Xuejiao Ding, and Yu Hao; analysis and interpretation of data: Yuanyuan Zhao and Jing Song; draft of the manuscript: Jing Song and Yuanyuan Zhao; critical revision of the manuscript for important intellectual content: Lihua Cao and Jing Song.

Declaration of conflicting interest

The authors declare that they have no competing interests.

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Supplemental material

Supplemental material for this article is available online.

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