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# Jinfu'an Decoction Inhibits Invasion and Metastasis in Human Lung Cancer Cells (H1650) via p120ctn-Mediated Induction and Kaiso

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Data Collection B  
Statistical Analysis C  
Data Interpretation D  
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**Background:** Previous research showed that Jin-Fu-An decoction has a significant effect on lung cancer. However, it remains unclear whether p120ctn and its transcription factor Kaiso play a role in lung cancer cell proliferation, adhesion, migration, and metastasis.





**Material/Methods:** Proliferation inhibition was detected by CCK-8 assay. The migration and invasion were detected using Transwell assay. The location and expression of p120ctn and Kaiso were monitored by immunofluorescence staining. The expression changes of p120ctn, its isoform 1A, its S288 phosphorylation, and Kaiso were measured by Western blot assay.

**Results:** The lung cancer cell line H1650 administered Jin-Fu-An decoction had significantly reduced the growth in dose-dependent and time-dependent manners. Migration and metastasis were significantly inhibited by application of Jin-Fu-An decoction in a dose-dependent manner. Additionally, Jin-Fu-An decoction decreased the expressions of p120ctn, its isoform 1A, and its S288 phosphorylation, but the protein level of Kaiso was elevated.

**Conclusions:** Jin-Fu-An decoction inhibits the proliferation, adhesion, migration, and metastasis through down-regulation of p120ctn or its isoform 1A expression, mediating the up-regulation of Kaiso. The underlying mechanism of Jin-Fu-An decoction might involve targeting the lower expression of p120ctn S288 phosphorylation, which suggests that Jin-Fu-An decoction may be a potential therapeutic measure as prevention and control of recurrence and metastasis of lung cancer.

**MeSH Keywords:** **Chinese Medicinal Formula • Invasion and Metastasis • Jinfu'an Decoction • Lung Cancer • p120ctn and Kaiso**

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## Background

Lung cancer is the leading cause of cancer deaths in women and men worldwide and is characterized by high morbidity and mortality [1–3]. To date, despite advances in diagnostic techniques and therapeutic strategies, the prognosis of lung cancer remains very poor, including poor quality of life (QOL) and survival [4]. As a result, recurrence and metastasis have been recognized as main reasons for the failure of lung cancer therapies. Therefore, the development of more potent alternative agents and therapies is still of great urgency to achieve acceptable clinical and oncological outcomes.

In recent years, traditional Chinese herbal medicine (TCHM) has become a widely-used therapeutic modality in advanced lung cancer patients. Increasing evidence proves it is effective in management of alleviating the clinical symptoms and signs and related complications, improving the QOL, and reducing adverse effects of conventional treatment [5–7].

Jinfu'an (JFA) decoction is an empirical compound formula prescribed by Dr. Deng Tietao, a traditional Chinese medicine master, based on the phlegm and blood stasis theory of lung cancer, which is an academic theory within TCM. JFA decoction has been frequently used for lung cancer patients in Guangzhou University of Chinese Medicine for over a decade and shows relief of clinical symptoms and signs, chemotherapy-related complications, and enhances QOL [8]. Previous *in vitro* and *in vivo* studies have reported that JFA decoction has inhibitory effects on human lung adenocarcinoma A549 cells and the Lewis lung cancer mouse model. Although the underlying mechanisms and active constituents are still not fully elucidated, a potential explanation might be that JFA decoction could not significantly down-regulate the protein expression of p120ctn in Lewis mouse lung cancer tissue, but also decreases the levels of phosphorylation on p120ctn in the A549 cell line [9,10]. p120ctn is also a key binding partner of transcription factor Kaiso, which is closely related with tumor metastasis. Therefore, we speculated that JFA decoction might inhibit invasion and metastasis through regulating p120ctn and transcription factor Kaiso-mediated induction in non-small cell lung cancer. In this study, we used H1650 cells as an NSCLC model to explore the potential mechanism by which JFA decoction inhibits proliferation of NSCLC cells.

## Material and Methods

### Cell lines and cell culture

The NSCLC cell line H1650 was obtained from the State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease (Guangzhou, China), and grown in

RPMI-1640 medium (Gibco, USA) containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a water-saturated atmosphere with 5% CO<sub>2</sub>.

### Preparation of the JFA decoction extract

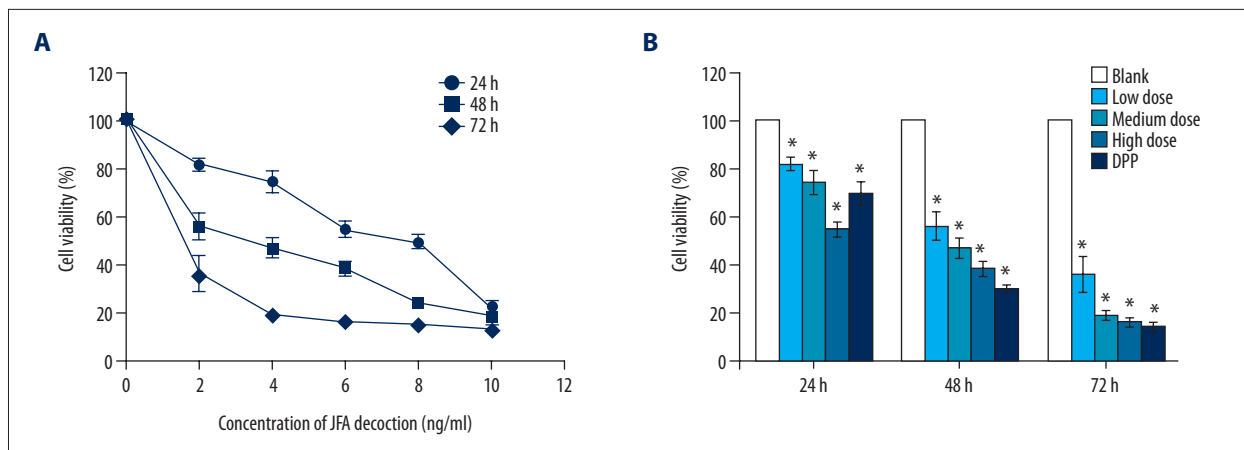
The CHM JFA decoction prescribed by the Traditional Chinese Medicine Master Dr. Tietao Deng in Guangzhou University of Chinese Medicine was composed of *Pseudostellaria heterophylla* (Miq.) 30 g, *Cremastra appendiculata* Makino 10 g, *Coix lachrymaljoli* L 30 g, *Salvia miltiorrhiza* Bunge 15 g, gecko 5 g, peach kernel 10 g, *Phragmites communis* 30 g, Thunberg fritillary bulb 15 g, raw *Pinellia ternate* 10 g, and raw *Rhizoma arisaematis* 10 g. The raw herbs for JFA decoction were purchased from the First Affiliated Hospital of Guangzhou University of Chinese Medicine. First, raw *Pinellia ternate* and raw *Rhizoma arisaematis* and the other Chinese herbal medicines were soaked in double-distilled water for 2 h, and then raw *Pinellia ternate* and raw *Rhizoma arisaematis* were boiled for 30 min. The herbal mixture was boiled for 1 h, and the cooled preparation of extracts from these Chinese medicinal herbs was filtered 3 times through 2 layers of cotton gauze and the extract was concentrated by rotary evaporation followed by freeze-drying. The total yield of the JFAT extract was 64 g lyophilized powder from water extract of 1.32 kg raw mixed herb, and the final extracts were collected and stored at –20°C until use. The preparation of herbal extract was then reconstituted to prepare 200 mg/ml sterilized stock solution, filtered through a 0.22-µm filter for *in vitro* experiments.

### Cells viability assay

H1650 cells were seeded into 96-well plates at 1×10<sup>4</sup> cells per well and incubated overnight at 37°C. Then, the cells were treated with JFA decoction at indicated concentrations for 24 h, 48 h, and 72 h. The Cell Counting Kit-8 (Dojindo, Japan) was used to measure cell viability. Briefly, the cell proliferation reagent WST-8 (10 µL) was added to each well and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Then, the optical densities (ODs) were detected at 450 nm through the use of a microplate reader. The data are presented as mean ±SD of triplicate samples from 3 repeated experiments. Cell viability was calculated using the following formula: cell viability rate (%) = (OD experiment – OD zero) / (OD control – OD zero) × 100%. Absorbance of the control group was set as 100% viability, and absorbance of cell-free wells containing medium was set as zero.

### Matrigel cell migration and invasion assay

Migration assay was performed using 24-well Transwell chambers with 8-µm pore filters (Corning, USA). Invasion assays were performed using 24-well Transwell units with 8-µm pore size



**Figure 1.** JFA decoction induced growth inhibition of lung cancer cell line H1650 following exposure at different concentrations at 24 h, 48 h, and 72 h. Cell viability was significantly decreased in the JFA decoction (A). Low dose, medium dose, and high dose of JFA decoction (2 mg/ml, 4 mg/ml, and 6 mg/ml) and DPP treatment group compared with those in the blank group at the same time-point (B) ( $p < 0.01$ ). Cell viability was inhibited by culture with JFA decoction in dose-dependent and time-dependent manners (A, B). \*  $p < 0.01$  compared with the blank control group.

polycarbonate inserts. The inserts were coated with a 100  $\mu$ l Matrigel (1: 7 dilution, Corning, USA) and cultured at 37°C for 2 h. Cells ( $1 \times 10^5$ /ml) suspended in 100  $\mu$ l of serum-free RPMI1640 medium were transferred to the upper chamber, while 500  $\mu$ l of RPMI1640 medium supplemented with 10% FBS (fetal bovine serum) was added to the lower chamber as a chemoattractant. The invasion assay was incubated for 12 h and the migration assay was cultured for 8 h. The medium was then discarded and migrated or invaded cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The invading and migrating cells were counted in 5 different randomly selected 200 $\times$  magnification fields under light microscopy. The mean was calculated from data in 5 separate fields based on 3 independent experiments.

### Immunofluorescence

When reaching 60% confluence, cells treated with indicated concentrations were fixed with 4% methanol, permeabilized with 0.1% Triton-X-100, and blocked with 5% BSA for 1 h, before being incubated at 4°C overnight with anti-p120ctn mAb (BD Transduction Laboratories) and anti-Kaiso mAb (Santa Cruz Biotechnology, INC), followed by incubation with a secondary antibody conjugated to TRITC/FITC-labeled antibody. The nuclei were counter-stained with DAPI and laser scanning confocal microscopy was performed at 488 nm to take pictures in 200 $\times$  magnification.

### Western blot analysis

All proteins in the H1650 cell culture supernatant were analyzed directly for p120ctn and Kaiso and prepared in ice-cold RIPA lysis buffer for 30 min, followed by centrifugation at

12 000 rpm for 30 min at 4°C. Total protein concentrations were determined by BCA assay (Beyotime, Jiangsu, China), loaded onto SDS-PAGE gels for electrophoresis at a constant voltage, and electrotransferred onto PVDF membranes (Millipore, USA) for Western blot analysis, which were blocked with 5% BSA. Afterwards, the membranes were probed with the primary antibody (BD Transduction Laboratories, Santa Cruz Biotechnology, INC) at 4°C overnight, followed by washing 3 times with TBS-T buffer and incubated with secondary antibody (Santa Cruz Biotechnology, USA) at room temperature for 1 h. Then, the membranes were washed 3 times, visualized with an BeyoECL Plus kit (Beyotime Biotechnology, China), exposed, and processed with the Bio-Rad ChemiDoc XRS+ Chemiluminescence imaging system (Bio-Rad, USA), with GAPDH as a control for each sample. All experiments were repeated 3 times.

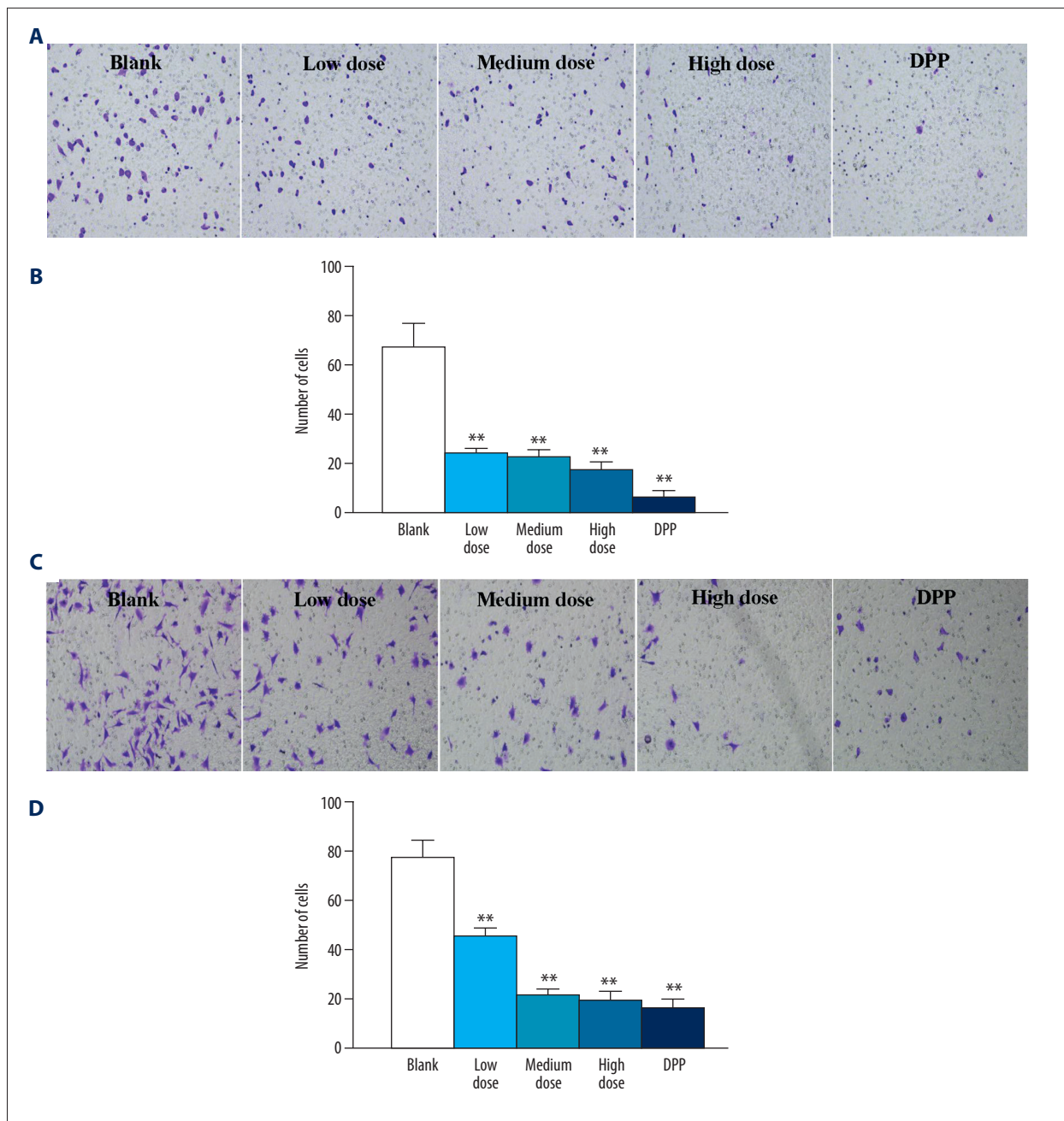
### Statistical analysis

All data are presented as either mean  $\pm$  SD or a percentage, relative to the blank group. Using SPSS 22.0 software, the significance of differences between groups was evaluated by using one-way ANOVA followed by Dunnett's test. A P-value of less than 0.05 was regarded as statistically significant.

## Results

### Suppression of H1650 cell proliferation by JFA decoction

The effect of JFA decoction on cell growth in human NSCLC cells was detected by CCK-8 assay. After treatment with JFA decoction varying from 2 to 10 mg/mL after 24, 48, and 72 h, the results revealed that cell viability was inhibited by JFA

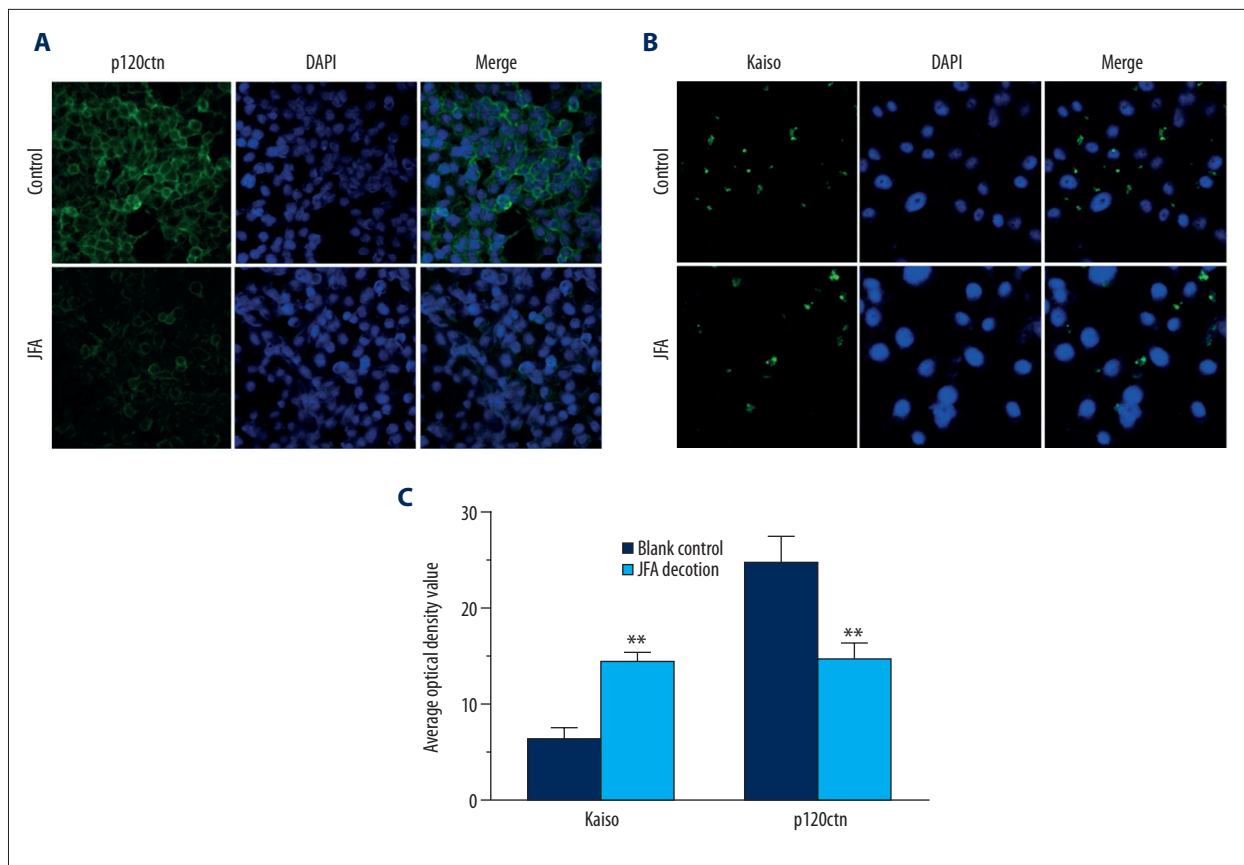


**Figure 2.** The average number of cells that migrated through the pores during the Matrigel and non-Matrigel experiment was blocked with the treatment of different dose of JFA decoction and DPP, in contrast with the blank group, and was dose-dependent (A–D). JFA decoction reduced the H1650 lung cancer cell adhesion, migration, and invasion. \*\*  $p < 0.05$  compared with the blank control group.

decoction in dose-dependent and time-dependent manners (Figure 1A). Similar findings in the Cisplatin group were also observed that JFA exerted its antiproliferative functions primarily by inhibiting the rapid growth of cells with no massive cell death (Figure 1B).

#### Inhibition of migration and invasion in H1650 cells by JFA decoction

Transwell assays were performed to determine the effects of JFA decoction on migration and invasion of human lung adenocarcinoma cell line H1650. Migration of H1650 cells treated with JFA decoction extract of different concentration was



**Figure 3.** P120ctn in lung cancer cell H1650 in the blank control group was mainly located and significantly overexpressed in the cytoplasm and cytomembrane. Moreover, cultured with JFA decoction, its expression level was decreased (A). In contrast, Kaiso in the blank control group was more often located in cytoplasm and in the JFA decoction group its level was increased (B). Immunofluorescence staining revealed that expression of p120ctn with the JFA decoction was downregulated, but the protein level of Kaiso was upregulated (C). The scale is 50  $\mu$ m in each image. \*\*  $p < 0.05$  compared with the blank control group.

blocked dose-dependently compared with the blank control group (Figure 2A, 2B). In a consistent manner, JFA decoction extract treatment suppressed invasion of human lung cancer cells in a dose-dependent manner. These data indicate that JFA decoction inhibits the migration and invasion of human lung adenocarcinoma cells (Figure 2C, 2D).

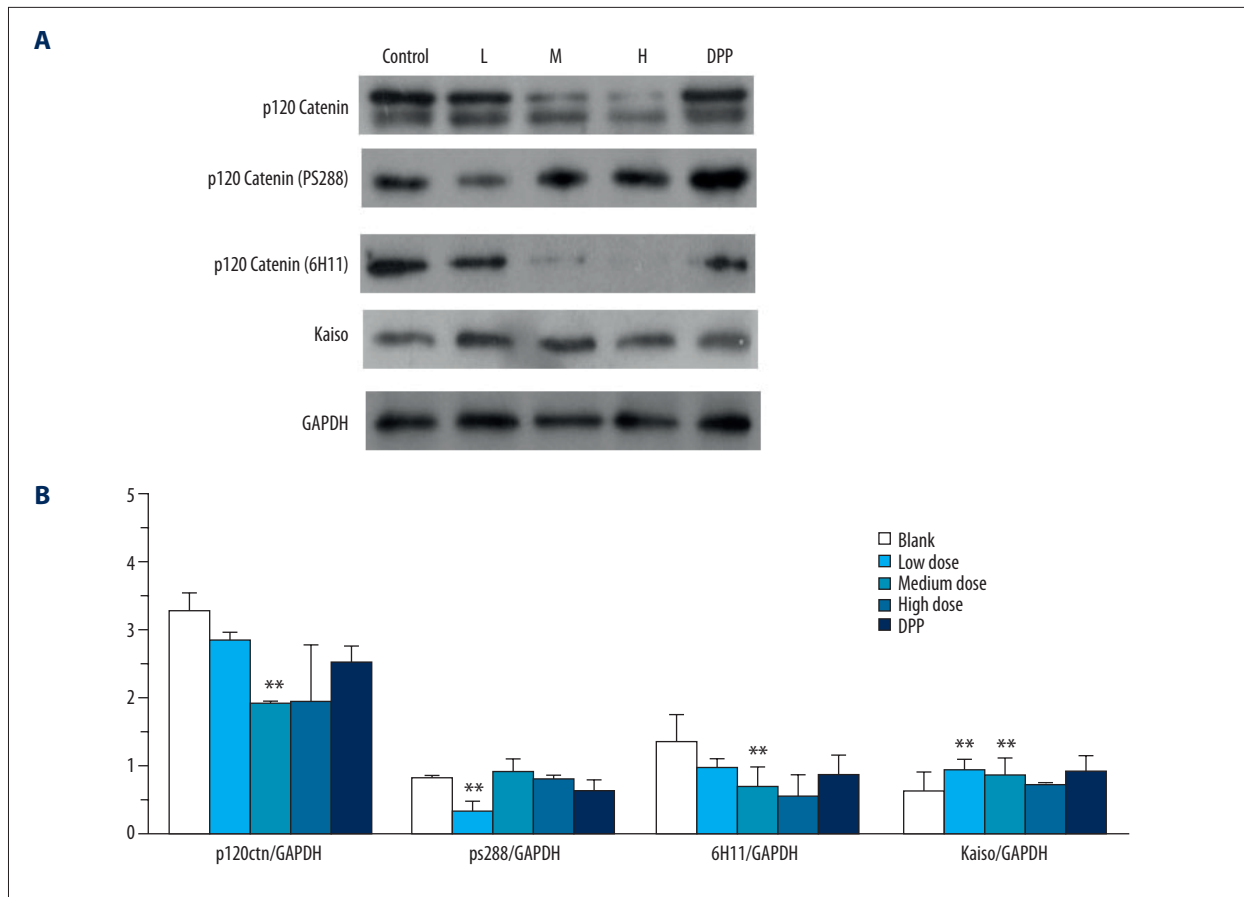
#### Induce the level of p120ctn and Kaiso by JFA decoction

We explored the possible explanation for its suppression of lung cancer cell proliferation, migration, and invasion. P120ctn, its related transcription factor Kaiso, and the phosphorylation of serine 288 on p120ctn, which were closely associated with invasion and metastasis of lung cancer, were chosen for use in the experiments. As shown in Figure 3A and 3B, immunofluorescence showed that p120ctn in lung adenocarcinoma cell lines H1650 with blank control was mainly located and significantly upregulated in the cytoplasm and cytomembrane, but Kaiso in the lung cancer cell line was often located

in cytoplasm. After JFA decoction treatment, p120ctn significantly decreased and the cytoplasmic Kaiso staining was increased (Figure 3C).

#### The expression of proteins closely related with invasion and metastasis of human lung cancer cells by JFA decoction

The results in Figure 4 show that protein expression of p120ctn and its isoform 1A were obviously reduced in the JFA decoction group compared with the blank control group. JFA decoction treatment was also effective in decreasing the level of the phosphorylation of serine 288 on p120ctn. Western blot analysis and immunofluorescence tests showed that the expression of Kaiso was upregulated by the JFA decoction and the same results of p120ctn, its isoform 1A, and transcription factor Kaiso were found in the Cisplatin group. Nevertheless, the phosphorylation of serine 288 in p120ctn was upregulated by treatment with Cisplatin.



**Figure 4.** JFA decoction decreased the expression of p120ctn, its isoform 1A, and p120ctn S288 phosphorylation compared with the blank control group but enhanced the protein expression of Kaiso. \*\*  $p < 0.05$  compared with the blank control group.

## Discussion

Lung cancer was the leading cause of cancer mortality among males worldwide in 2012, being responsible for 8.2 million cancer deaths [1]. Lung cancer was also the most common cancer and the leading cause of cancer mortality in China, based on data of the National Central Cancer Registry of China [2] and the 5-year relative survival rate is currently only 18%; progress has been slow, in contrast to the steady progress in survival for most cancers [3]. Recurrence and metastasis are the key reasons for the low survival rates and are the malignant biological characteristics of lung cancer. Chinese herbal medicines (CHM), which are the most important therapeutic modality of TCM, have proven advantages in preventing and treating lung cancer because they have shown promising results in protecting cancer patients against complications and have relatively few adverse effects compared to conventional treatment, and they minimize the adverse effects of traditional remedies.

However, most studies on the molecular mechanisms have focused on the effects of a single component, but not the prescribed multiple herb formula. In this study, we used the model

of human lung adenocarcinoma cell line H1650 to further explore the effects of lyophilized powder of JFA decoction on the adhesion, migration, and invasion and its underlying mechanism of action. JFA decoction, which has the function of dissipating phlegm and removing blood stasis, is a traditional Chinese medicine that is an extract comprising 10 species of medicinal herbs. Several components have recently been reported to have some potential in treating cancers [5–7]. The formula has been frequently used for lung cancer patients in Guangzhou University of Chinese Medicine for more than 10 years [8]. Our clinical practice experience shows that the JFA decoction can improve the quality of life of the elderly patients with medium- or late-stage non-small cell lung cancer, especially for physical status [9,10]. JFA decoction also alleviates advanced non-small cell lung cancer-related symptoms, improving tumor stability and control of distant metastasis among patients with late-stage non-small cell lung cancer [11]. JFA decoction is an effective and safe treatment for advanced-stage non-small cell lung cancer patients who are not suitable for or are unwilling to accept radiotherapy and chemotherapy, as well as those who cannot afford these therapies or reject them for other reasons.

In previous research, we demonstrated that JFA decoction suppressed biological behavior of human lung adenocarcinoma A549 cells and reduced the numbers of metastatic foci in a Lewis mouse model of lung cancer, likely via decreasing the levels of p120ctn tyrosine phosphorylation and lowering the abnormal expression of p120ctn, RhoA, and Cdc42 [12,13]. The potential molecular mechanisms and active constituents by which JFA decoction prevents invasion and metastasis and treats the lung malignancies remain unclear and need further study.

In the present study, we confirmed that JFA decoction has an obvious tumor-suppressing effect, especially in prolonging survival time, which was consistent with the findings from clinical practice. Our results also indicated that growth of lung cancer cells was dramatically inhibited by treatment with JFA decoction in both a dose-dependent and time-dependent manner *in vitro*. This could be a result of either inhibiting proliferation or increasing cell death. Moreover, we have shown that the migration and invasion of human lung adenocarcinoma cell line H1650 were blocked by JFA decoction and it dose-dependently inhibited the migration and invasion of tumors. Similar findings were reported in our previous clinical research, which may show that metastasis and recurrence ratios of lung cancer were reduced by treatment using JFA decoction [8].

Furthermore, to elucidate the underlying mechanism of action, we explored the protein expression levels of regulators involved in the process of lung cancer cell adhesion, mobility, invasion, and metastasis. We found that p120ctn is mainly located in cytoplasm and cytomembrane, with weak nuclear expression, and Kaiso is frequently found in the cytoplasm in the lung cancer cell line H1650. The expression of p120ctn and its isoform 1A was downregulated by JFA decoction in contrast to the control group, but JFA decoction increased the expression of Kaiso.

p120ctn, an Armadillo repeat protein, originally known as a Src kinase substrate and prominent tyrosine kinases, binds to the juxta membrane domain (JMD) of E-cadherin [14,15], where it functions in cadherin-mediated adhesion and cell migration, and stabilizes E-cadherin involved in cell migration and invasion [16,17]. It also has some effects on Rho-family GTPases such as Rac1, Cdc42, and RhoA in some cell types [17,18], which modulates cell-cell adhesion, cell migration, and invasion by regulating cadherin [19]. However, p120ctn how inhibits or promotes tumor growth, migration, and invasion is not yet known.

There is mounting evidence that p120ctn, as a tumor suppressor, may play an important role in tumor progression and metastasis, and its down-regulation is associated with poorly differentiated tumors and a metastatic phenotype in diseases such as

prostate and lung cancers and adenocarcinoma of the gastroesophageal junction [20–22]. However, in our experiments we revealed that JFA decoction reduced the expression of p120ctn to induce poor invasion and migration, which is not consistent with these previous studies. Thus, we speculated that p120ctn functions as a tumor-promoting gene in type H1650 lung cancer cell. Previous studies have shown that p120ctn may play opposing roles depending on whether E-cadherin is located on the membrane or in the cytoplasm of cells [23,24]. Studies have also shown that p120ctn may promote or inhibit tumor growth and invasiveness, depending on whether E-cadherin is expressed or not [25,26] and can affect its localization by subcellular localization and function of p120ctn. The expression and location of E-cadherin in the lung cancer cell line H1650 was not assessed detected in our research, and further studies are needed to elucidate the exact roles.

In addition, p120ctn has bidirectional regulation of lung cancer cell adhesion, mobility, invasion, and metastasis because p120ctn has a variety of subtypes with different biological functions, and they have tissue specificity. In theory, the p120ctn isoforms (1 to 4) as the result of 4 transcriptional start sites all share a full central Armadillo repeat domain that can bind E-cadherin to regulate cell adhesion on the cell membrane, although they have divergent N- and C-terminal ends [27]. Each isoform which arises from alternative splicing events may have tissue- or cell-specific functions [28–30]. In previous research, some scholars have reported that only isoforms 1A and 3A are expressed in normal and cancerous lung tissues [31] and they play somewhat different roles in tumor cell migration, invasion, and metastasis [32].

Here, we demonstrated that JFA decoction could decrease the level of isoform 1A, which reduce the migration, invasion, and metastasis of H1650 lung cancer cell. This finding is consistent with what has been previously described in the literature. It has been reported that both p120ctn isoforms 1A and 3A promote cell proliferation and cell cycle progression. Nonetheless, p120ctn isoform 1A up-regulates cyclin D1 and cyclin E via mediating the expression of  $\beta$ -catenin at the protein level, and isoform 3A accomplishes this function via binding to Kaiso, which is a negative transcriptional factor of cyclin D1 [33]. Others also found different effects of p120ctn isoform 1A and 3A on proliferation and invasion in tumor cells exhibiting different localizations of E-cadherin [34]. When in tumor cells with cytoplasmic E-cadherin, p120ctn isoform 1A promoted EMT and increased cell invasiveness, while p120ctn isoform 3A had the opposite effects [35]. Thus, whether JFA decoction also decrease the growth, migration, invasion, and metastasis in tumor cells with E-cadherin at different locations by regulating p120ctn isoforms 1A remains unknown. Based on the above analysis, we speculated that JFA decoction may suppress invasion and metastasis in lung cancer cells H1650 by regulating

the expression and location of E-cadherin via down-regulating p120ctn and isoforms 1A. To address the aforementioned issues, this hypothesis requires further study.

Herein, we also aimed to determine if Kaiso expression events affected by JFA decoction are involved in the progression and poor prognosis of lung cancer. In the current research, we revealed that Kaiso, a new member of the BTB/POZ zinc finger family of transcription factors originally described as p120ctn-binding partner [36,37], was weakly expressed in H1650 cells and was frequently found in the cytoplasm. p120ctn is mainly located in cytoplasm and cytomembrane, with weak nuclear expression.

There exists mounting evidence that Kaiso is a nuclear protein that plays a role in transcription repression [38–41]. It is predominantly localized in the nuclei of mammalian cell lines and is sometimes observed in tissues. Interestingly, it is usually cytoplasmic or even absent *in vivo*. From the above, it is obvious that not all findings on Kaiso subcellular location can be readily explained. Our results were similar to those of some previous studies reporting that Kaiso's cytoplasmic localization almost always coincided with cytoplasmic p120ctn [42]. Cytoplasmic p120ctn may also promote migration and metastasis though modulating the activity of Rho GTPases [43], aside from p120ctn binding. Besides, p120ctn and transcription factor Kaiso might co-participate in the progression and lymph node metastasis of lung cancer tissues and cell lines. Also, the expression and localization of Kaiso in lung cancer cells were regulated by p120ctn. Thus, we suspect that the down-regulation of p120ctn and its isoform 1A by JFA decoction might modulate the expression of Kaiso, and thereby contribute to reducing malignancy and invasiveness. Further research may determine the exact underlying factors, which are likely to differ on a case-by-case basis.

On the other hand, our data have described that the lung cancer cell line expressed lower levels of the serine 288 phosphorylated form with the treatment of JFA decoction. The previous experiment established that S288 phosphorylation level was elevated in lung tumor tissue and cell lines, enhancing the binding affinity with Kaiso [44]. Meanwhile, p120ctn S/T phosphorylation promoted the invasion of lung cancer cells and S288 phosphorylation contributes to lung cancer progression [45]. Collectively, we believe that JFA decoction can regulate the level of p120ctn S288 phosphorylation though relieving Kaiso-mediated transcription repression and can prevent

invasion in lung cancer cells via a phosphorylation-dependent mechanism.

The present study is the first to demonstrate the therapeutic and preventive potential of JFA decoction in human lung cancer, showing that JFA decoction may down-regulate p120ctn and its isoform 1A though relieving Kaiso-mediated transcription repression. Another possibility is that JFA decoction prevents the lung cancer progression via decreasing the level of p120ctn S288 phosphorylation. Because medicinal herbs or the prescription drugs derived from them are originally based on multi-target/multi-component strategies, JFA decoction can be promising source materials in the development of anticancer drugs; however, it is mainly used as complementary Chinese traditional patent medicine in the treatment of lung cancer and it has not been generalized to be applied to the lung cancer. Furthermore, we believe that JFA decoction may contribute to the improvement of patients' quality of life. Further studies are required to understand its exact mechanism.

## Conclusions

This study confirmed that JFA decoction treatment of H1650 lung cancer cells significantly inhibited the proliferation, adhesion, migration, and invasion, suggesting that JFA decoction can regulate the adhesion, migration, and invasion of lung cancer cell line H1650. We also found that the expression of p120ctn and its isoform 1A were suppressed at the protein level and the transcription factor Kaiso as a key binding partner of p120ctn was elevated. In light of these findings, it is reasonable to speculate that JFA decoction affects the proliferation, adhesion, migration, and metastasis though suppression of p120ctn or its isoform 1A expression mediating Kaiso. We also showed that JFA decoction inhibited proliferation, migration, and invasion and decreased the expression of p120ctn S288 phosphorylation. However, the exact functional mechanism of JFA decoction needs to be further elucidated because the prescription drugs derived from them are originally based on multi-target/multi-component strategies. In conclusion, the present study lays a scientific foundation for research on the detailed mechanisms underlying the anti-lung cancer effect of JFA decoction.

## Conflict of interest

None.



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