Inhibition effects of Yuxiao San combined with cisplatin on transplanted tumor growths via upregulation of nm-23 and downregulation of K-ras in Lewis lung cancer mice

MO TANG, SHUMEI WANG, YULIN WEI and JIANTI FU

College of Traditional Chinese Medicine, Chongqing Medical University, Chongqing 400016, P.R. China

Received August 5, 2017; Accepted November 29, 2017

DOI: 10.3892/ol.2018.9673

Abstract. Traditional Chinese medicine applied to cancer therapy has a long history. This study aimed to investigate the effect and underlying mechanism of the traditional Chinese medicine, Yuxiao San, in combination with cisplatin, on Lewis lung cancer mice. Lewis lung cancer mouse models were established in mice, and were randomly divided into model (normal saline + egg white), cisplatin (cisplatin injection + egg white), traditional Chinese medicine (Yuxiao San + egg white) and combined medication (Yuxiao San + cisplatin injection + egg white) groups. The weight and tumor size of mice was measured every 3 days, and the number of lung surface metastasis nodules were calculated after 14 days. Nucleoside diphosphate kinase (nm-23) and K-ras expression levels in tumor tissues were determined by immunohistochemical methods and reverse transcription-quantitative polymerase chain reaction. The results suggested that compared with the model group, cisplatin, traditional Chinese medicine and combined medication groups significantly inhibited growth of transplanted tumors, reduced the number of lung metastasis nodules, increased the inhibition rate of lung metastasis (P<0.05 or P<0.01), significantly increased tumor suppressor gene nm-23 expression and significantly downregulated oncogene K-ras expression. Among all groups, the effect of the combined medication group for inhibiting tumor growth was most favorable (P<0.01). Compared with the cisplatin group, nm-23 expression in the traditional Chinese medicine group and combined medication group was significantly increased, and K-ras expression in the traditional Chinese medicine and combined medication groups was significantly reduced (P<0.01). To conclude, Yuxiao San and cisplatin injection effectively inhibited growth of the transplanted tumor. The underlying mechanism may be associate with nm-23 upregulation and K-ras downregulation. Therefore, the Yuxiao San and cisplatin injection may become a potential therapeutic strategy for tumor treatment.

Introduction

Conventional modern medical treatments of malignant tumors include surgery, radiation therapy, chemotherapy and molecular targeted therapy; however, these methods frequently induce trauma and unwanted side effects, and are poorly tolerated in patients with advanced disease (1,2). Cisplatin is a widely used anti-cancer drug that may induce serious toxicity in the organs and tissues (2). Until now, cisplatin has been extensively used to treat many tumors, including breast, gastric and pancreatic cancer, and cervical carcinoma. A high dose of cisplatin may induce side-effects for patients or in animal models, and the dosage of cisplatin must be reduced. Traditional Chinese medicine applied to cancer therapy has a long history, and has attracted attention (3). In recent years, in the field of cancer therapy, especially for breast cancer and cervical carcinoma, traditional Chinese medicine drugs have alleviated symptoms of tumors (2,3). The traditional Chinese medicine drugs prevented chemotherapy side effects and complications, treatment of cancer pain, severe pleural effusion, severe ascites, cancerous mass, cancer complications and constipation (3). Yang et al (3) reported that traditional Chinese medicines potentially target the tumor cellular proteasome and NF-kB pathway whose activation is dependent on the proteasome activity. Therefore, in order to reduce the side-effects of cisplatin, the present study firstly used traditional Chinese medicine and cisplatin combination chemotherapy to treat tumors in a mouse model of lung cancer, as this particular model may reflect best the therapeutic effects of the aforementioned combination.

Yuxiao San is composed of Curcuma aromatica, mint, rhubarb, pubescent holly root, dandelion and Glauber’s salt. In traditional Chinese medicine Yuxiao San is believed to treat qi stagnation and blood stasis, eliminate phlegm and remove toxins, to prevent swelling and relieve pain. The present study used the established Lewis lung cancer mouse model (4) and studied the effects and underlying mechanism of Yuxiao San and cisplatin injection in Lewis lung cancer mice. To the best of our knowledge, there are currently no studies published evaluating the effects of Yuxiao San on tumor cell growth or development. The...
objective of the present study is to provide basic data regarding the use of Yuxiao San in the clinical treatment of lung cancer.

Materials and methods

Animals and cell lines. A total of 45 male C57BL/6 mice weighing 18-22 g, aged from 6 to 8 weeks were purchased from Chongqing Medical University Experimental Animal Center (production license: SCXK-Chongqing-2012-0001). All of the mice were raised in the standard cleaning environment as the following: Temperature 23±2°C, humidity 55±10%, 12 h illumination, noise ≤60 dB and free access to food and water. A Lewis lung cancer cell line, C57BL, was purchased from the West China University Cancer Center (Chengdu, China). The present study was approved by the ethics committee of College of Traditional Chinese Medicine of Chongqing Medical University, Chongqing, China (approval no. 2014052).

Experimental drugs, reagents and instruments. Yuxiao San was purchased from Chongqing Tong Junge Large Pharmacy (Chongqing, China). According to high performance liquid chromatography, Yuxiao San primarily consisted of hexadecenoic acid, β-sitosterol, burdock oligosaccharide, kikyo saponin, litchi saponin, curcumin and American ginseng total saponin. Cisplatin (cat. no. 2A1A1401002A) was purchased from QiuPharmaceutical Co., Ltd. (Hainan, China). Nm-23 and K-ras antibody (cat. nos. bs-0688R and bs-1033R, respectively) were purchased from the Beijing Boosen Biotechnology Co., Ltd. (Beijing, China). 3,3’-diaminobenzidine color developing reagent kit (cat. no. K155922C) and Biotin-Streptavidin horseradish peroxidase Detection System (SP test kit; cat. no. 15155A08) were purchased from Beijing Zhongshans Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Formaldehyde (cat. no. 2015050501), glacial acetic acid (cat. no. 2015040201) were purchased from Chengdu Kelong Chemical Co., Ltd. (Chengdu, China). Picric acid (cat. no. P9330) was purchased from the Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China).

Establishment of the Lewis lung cancer mouse model. Exponentially growing cells were resuspended to 1x10^7 cells/ml, ready for subcutaneous injection into C57BL/6j mice (n=5). On day 14, the mice were sacrificed. A cancer cell suspension was prepared by stripping tumor tissue using radioimmunoprecipitation assay (RIPA) buffer at final concentration of 1 M (cat. no. P0013B; Biyotime Biotech. Shanghai, China) and dissolved in HuMEC Basal serum-free medium (cat. no., 12753018, Gibco; Thermo Fisher Scientific, Inc. Waltham, MA, USA). A total of 1x10^7 cells/ml (total volume 0.2 ml) were subcutaneously injected into the dorsal region of the remaining 40 C57BL/6j mice.

Grouping and administration. After 6 days of growing subcutaneous tumors, the mice were randomly divided into four groups (n=10 per group): Model group, cisplatin group, traditional Chinese medicine group and combined medication group. In the model group, a sterile gauze was used to administer normal saline and egg white to tumors. In the cisplatin group, cisplatin and albumin were mixed to form a cream, which was compressed onto the skin for ~2-3 mm thickness. Treatment for every group was performed according to a previously published study (5). The above drugs were administered three times a day, eight h at a time, for 21 days.

Observe the general condition and tumor growth. Before inoculation and after vaccination mice were weighed. General observations were noted, including their hair, diet and activities. For tumor growth calculations, before and after inoculation, mice were weighed once every three days and the dose of administration was adjusted according to weight change. After 21 days administration, the mice were sacrificed, the tumor was removed and weighed, and then the (a) longest and (b) shortest diameter of mice tumors was determined with a vernier caliper. The volume of tumor was calculated according to the equation: V=(0.5ab²) (units in cm³), and the curve was drawn. The tumor growth inhibition rate was calculated as: (Average weight of tumors in model group-average weight of tumors in treatment group)/average weight of tumors in model group x100% (6).

Pulmonary surface metastatic nodules. On day 21, the mice were dissected, the pulmonary tissue was removed, and fixed for 24 h with Bouin’s fixative at 4°C (picric acid, formaldehyde, glacial acetic acid in a ratio of 75:25:5, respectively), then put them into ethyl alcohol to remove the yellow color. Metastases were observed under a microscope as white nodules. The rate of inhibition on metastasis (%) was calculated as: (Average metastatic nodules of pulmonary surface of model group-average metastatic nodules of pulmonary surface of treatment group)/average metastatic nodules of pulmonary surface of model group x100%.

Western blotting assay. The tumor tissue was embedded in paraffin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and cut into 4 µm sections, and incubated with RIPA lysis buffer (Sigma-Aldrich; Merck KGaA), and the total proteins were extracted and the concentration was examined using bicinchoninic acid protein assay kit (cat. no. PA115; Tiangen Biotech Co. Ltd., Beijing, China). Equal quantities of protein (2 µg) were separated using a 10% gel and SDS-PAGE and electro transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked with 5% non-fat milk for 2 h at 4°C. The membranes were incubated with a rabbit anti-mouse Nm23 polyclonal antibody (1:2,000; cat. no. ab154547; Abcam, Cambridge, UK), a rabbit anti-mouse K-ras monoclonal antibody (1:3,000; cat. no. ab199557; Abcam) and a rabbit anti-mouse GAPDH polyclonal antibody (1:2,000; cat. no. ab9485; Abcam) for 2 h at room temperature. The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2,000, cat. no. sc-2030; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 37°C for 1 h. Finally, the western blot bands were visualized by using an enhanced chemiluminescent kit (Thermo Fisher Scientific, Inc.). Finally, the bands were scanned and analyzed by using a UVP gel image scanning system GDS8000 software (UVP, Sacramento, CA, USA).
Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from tissues using TRIzol (Tiangen Biotech Co. Ltd.). The concentration and purity of RNA was measured by determining the optical density by using a spectrophotometer (Nanodrop Lite; Thermo Fisher Scientific, Inc.). cDNA was synthesized by using a Rever Tra Ace-a-reverse transcription kit (cat. no. FKS-101, Toyobo Life Science, Osaka, Japan). The primers were: nm-23: Forward, 5'-CGGCGAGTA TTCAGTGAGATG-3' and reverse, 5'-CTGGTCTCTCCCG CGTCTACTTACA-3'; K-ras: Forward, 5'-CTGGGAGGG GCTTCTTGTG-3' and reverse, 5'-TCCGTAGCCTGTTT CGTGCTCA-3'; β-actin: Forward, 5'-ACCCCGTGCTGCTGA CCGAG-3' and reverse, 5'-TCCGGGCCAGCCAGGTCTCA-3'. The RT reaction conditions were: 42°C for 10 min, 30°C for 20 min, 99°C for 5 min and 4°C for 5 min. The PCR reaction conditions were: 94°C for 5 min, 94°C for 30 sec, 57°C for 30 sec and 72°C for 30 sec for a total of 40 cycles and termination at 72°C for 10 min. After the reaction, the ABI StepOne Plus PCR system was used to analyze and calculate the cycle threshold value of the target gene. The relative mRNA expression of targeting genes was normalized to the β-actin gene by using the comparative threshold cycle (2^ΔΔCq) method (7).

Statistical analysis. Data in the present study were analyzed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA). Data was presented as the mean ± standard deviation. All data were obtained from at least three independent experiments. Tukey's post hoc test was used following a one-way analysis of variance between the two groups. P<0.05 was considered to indicate a statistically significant difference.

Results

General observations. A total of 40 mice were inoculated with Lewis lung carcinoma cells and all developed tumors. Mice experienced symptoms of lassitude, asthenia, somnolence, lethargy and withered hair. Compared with the model group, these symptoms were reduced in the Traditional Chinese medicine group and combined medication group. However, the cisplatin group not only exacerbated the above symptoms, but also suffered side-effects of chemotherapy, such as decreased appetite, loss of weight and loss of hair (data not shown).

Comparison of tumor growth in each group. The weight of tumors in the cisplatin, Yuxiao San group and the combination group were lower than that of the model group (P<0.01; Table I). The weight of tumor in the Yuxiao San group and combination group were lower than the cisplatin group (P<0.01; Table I). The combination group had a lower tumor weight than the cisplatin group and Yuxiao San group (P<0.01; Table I).

The volume of tumor in the cisplatin group, Yuxiao San group and the combination group were lower than that of the model group (P<0.01; Table I). The volume of tumor in the Yuxiao San group and combination group were lower than that in the cisplatin group (P<0.01; Table I). The combination group had a lower volume of tumor than the cisplatin group and Yuxiao San group (P<0.01; Table I). The inhibition rate of tumor growth in the combination group was 55.88% and was higher than the cisplatin group (33.82%) and Yuxiao San group (43.14%) (P<0.01; Table I).

Comparison of pulmonary surface metastatic nodules in each group. The rate of inhibition on tumor metastasis in the cisplatin group, Yuxiao San group and the combination group (35.90, 57.38 and 68.85%, respectively) was significantly increased compared with that of the model group (24.60%) (P<0.01; Table II). The combination group also inhibited pulmonary surface metastatic nodules to a greater extent than the other groups (Table II).

Observation for K-ras and nm-23 expression in tumor tissue by using immunohistochemistry. K-ras and nm-23 were detected via immunohistochemical staining (Fig. 1). The mean absorbance of K-ras expression in the model group, cisplatin group, Yuxiao San group and combination group were 1.29±0.01, 1.02±0.24, 0.87±0.17 and 0.61±0.02, respectively (Table III). Compared with the model group, expression of K-ras and nm-23 in the other groups were significantly decreased (P<0.01). Compared with the model group, the cisplatin group and the Yuxiao San group, the combination group exhibited the most significantly decreased expression levels of K-ras and nm-23 (P<0.01; Table III). Compared with the cisplatin group, the Yuxiao San group and combination group exhibited significantly decreased expression of the two proteins (P<0.01). Compared with the Yuxiao San group, the combination group exhibited the most significantly decreased expression levels. For the decreased expression of K-ras and nm-23, the combination of cisplatin and Yuxiao San was superior to single drug treatment (P<0.01; Table III).

The average absorbance values of nm-23 in the model group, cisplatin group, Yuxiao San group and combination group were 0.58±0.02, 0.65±0.01, 0.89±0.01 and 1.08±0.02, respectively (Table III). Compared with the model group, the other groups exhibited significantly greater expression levels of nm-23 (P<0.01; Table III). Compared with the model group, cisplatin group and Yuxiao San group, the combination group exhibited the most significantly increased expression of nm-23 (P<0.01; Table III). Compared with the cisplatin group, the Yuxiao San group and combination group exhibited significantly increased nm-23 expression levels (P<0.01; Table III). Compared with Yuxiao San group, the combination group demonstrated the most significantly increased nm-23 expression levels (P<0.01; Table III). For the decreased expression of K-ras and nm-23, the combination of cisplatin and Yuxiao San is superior to single drug treatment (P<0.01; Table III).

Detection of K-ras and nm-23 in tumor tissue by western blotting. K-ras expression levels were examined by using western blotting (Fig. 2A). The results suggested that K-ras protein expression levels in the cisplatin group, Yuxiao San group and cisplatin and Yuxiao San combination group were significantly decreased compared with the model group (P<0.01; Fig. 2B). In addition, K-ras expression levels in the Yuxiao San group and the cisplatin and Yuxiao San combination group were also decreased compared with the cisplatin group (P<0.01; Fig. 2B).

Protein expression levels of nm-23 in the cisplatin group, Yuxiao San group, and cisplatin and Yuxiao San combination group were significantly increased compared with the model group (P<0.01; Fig. 2B). Protein expression levels of nm-23 in the Yuxiao San group and cisplatin and Yuxiao San combination group were lower than that of the model group (P<0.01; Fig. 2B).
Discussion

The traditional Chinese Medicine holds the view that tumors are associated with the depression and stress indicated in patients. Lung cancer also belongs to a kind of pulmonary disease, according to a Chinese medical theory, indicating an abscess in the lung (8). Yuxiao San powder is a common application for tumors and is composed of *Curcuma aromatica*, mint, rhubarb, pubescent holly root, dandelion and Glauber’s salt (9,10). Modern medical research has established that the effective constituent of *Curcuma aromatica* (8), mint (9) and dandelion (10) work against tumor growth and induce cell apoptosis.

The K-ras gene causes cancer when mutated. K-ras genes make proteins, which are involved in cell signaling, growth and death (apoptosis). The Ras oncogene is well known to be involved in cancer development, including H-RAS, K-RAS and N-RAS, which encode a family of 21 kDa guanosine triphosphate-binding proteins called p21. In physiologic conditions, these proteins may be activated by binding with guanosine triphosphate (GTP) and initiate cell proliferation via the Ras-dependent kinase cascade. Subsequently, GTP is hydrolyzed to guanosine diphosphate by its intrinsic GTPase activity and Ras proteins return to an inactive state. However, in tumors, when a point mutation occurs in these genes, which often results in loss of its intrinsic GTPase activity, Ras proteins may acquire transforming potential, leading to continuous activation of Ras signaling (11).

K-ras gene mutations occur frequently in non-small cell lung cancer (NSCLC), mainly in adenocarcinoma, and rarely in squamous cell carcinoma (12). Approximately 80% of K-ras mutations in NSCLC involve codon 12, others are located in codons 13 or 61 (13). Evidence from animal model studies of NSCLC demonstrated that K-ras mutations enhance cellular proliferation and induce malignant transformation, and their continuous activation serves a key role in tumor development and maintenance. The carcinogenesis of K-ras mutations was also demonstrated in human NSCLC (14).

Targeted knockdown of K-ras in NSCLC cell lines has provided critical preclinical confirmation of the role of this driver in tumorigenesis, resulting in the suppression of tumor growth and sensitization to inhibitors of other signaling pathways (15). However, in practical terms, K-ras itself is an intractable target for the development of therapeutics (16), and considerable effort has thus focused on inhibition of its down-stream effectors to perturb persistent activation of oncogenic signaling pathways. The canonical RAF/MEK/ERK kinase cascade is the primary mitogenic pathway stimulated by K-ras under both physiologic and pathologic conditions (17,18).

The nm-23 gene was first discovered as a cancer metastasis suppressor in murine melanoma cell lines. As many as five nm-23 genes in human have been identified, including nm-23-h1 to nm-23-h5. Protein nm-23 gene products are known as abnormal wing disc, which is found in *Drosophila* (19). Factor 1 (inhibitor of differentiation) and c-myc purine-binding transcription factor (PUF) are
the transcription factors that regulate c-myc. Factor 1 and PUF include a large family of proteins that have activity for nucleoside diphosphate kinase (NDPK). NDPK is found in almost all cells and serves to catalyze the phosphorylation of 5’triphosphate nucleoside to 5’diphosphate nucleoside, through an intermediary mechanism involving high-energy oxidative phosphorylation enzymes (19,20).

NDPK serves a function in signal transduction from the cell membrane into the cell nucleus via a G protein activation pathway and p21, and serves a role in cell division and maintenance of cell shape (21). Protein nm-23 gene products have implications in the proliferation and differentiation of cells, and also serves a role in cancer (22). The nm-23 protein is distributed to the cytosol, mitochondria, plasma membrane and nucleus (23). The nm-23 gene family is highly conserved among a wide variety of eukaryotic species (24,25). The identification of nm23 as a tumor suppressor gene indicated the existence of genes that specifically regulate metastasis (26,27).

The function of nm23 in human cancer is currently unconfirmed (22,28‑31), and certain reports have indicated there is a link between high expression of the nm-23 gene and its...
protein product with low metastatic potential of cancer (28). Nm-23 is known to be associated with early onset of familial breast and ovarian cancer (28). Furthermore, an inverse association between nm-23 expression and metastasis was also observed in various types of cancer (22). Although the mechanism by which nm-23 regulates metastasis is not fully understood, experimental data have shown that nm23 serves an important role in the regulation of metastasis in a number of human cancer types (29). It was also reported that nm-23 expression was a significant factor for predicting a favorable prognosis, suggesting the anti-metastatic potential of nm-23 in NSCLC (30,31). The expression of nm23 in normal lung tissue was much higher than in cancer tissues, and was associated with staging and lymphatic metastasis, and this may suggest that the absence of nm23 expression may serve an important role in the incidence of NSCLC.

The present study determined the effects of Yuxiao San on Lewis lung cancer in mice. This study was conducted in vivo and in vitro, and there were four groups, including the model group, cisplatin group, Yuxiao San group and the combination group (Yuxiao San and cisplatin). The Lewis lung cancer model in C57BL/6 mice was established and mice were treated with Yuxiao San and cisplatin, before determining any side effects of treatment. Comparisons between tumor weight, tumor volume and tumor inhibition rate were made, and the methods of immunohistochemistry, western blotting and RT-qPCR were used to detect K-ras and nm23 gene expression levels in tumor tissue.

Results of the present study revealed that Yuxiao San may reduce tumor weight and tumor volume. Yuxiao San may also decrease K-ras gene expression levels and increase nm-23 gene expression levels. It was found that Yuxiao San downregulated the expression of K-ras, and upregulating expression of nm-23 in Lewis lung cancer mice tissues. The expression levels of nm-23 in normal lung tissue was much higher compared with that in cancer tissues. Combining cisplatin and Yuxiao San was better than the use of single drug treatment, as compared with the model group, the tumor volume, weight and lung metastases of tissues treated with Yuxiao San and cisplatin group in combination were decreased. In addition, compared with the model group, the expression levels of K-ras via immunohistochemical staining and RT-qPCR were decreased. Expression levels of nm-23 in tumors were increased via immunohistochemistry staining, western blotting and RT-qPCR.

Although this study revealed some notable results, there were a number of limitations. Firstly, the sample sizes were relatively small. In a subsequent study, a large sample size should be utilized. Furthermore, the down-stream factors of nm-23 and K-ras have not been clarified. The mechanism for the effect of Yuxiao San following the changes of nm-23 and K-ras should be investigated. Additionally, the effects of Yuxiao San have not been examined in combination with the other chemotherapy drugs, except for cisplatin. In a follow-up study, the combined effects of Yuxiao San with other chemotherapy drugs should be assessed.

To conclude, Yuxiao San and cisplatin injection by external application may effectively inhibit growth of transplanted tumors in mice. The underlying mechanism may be associated with nm-23 upregulation and K-ras downregulation.

Acknowledgements
Not applicable.

Funding
The present study was supported by National Clinical Research Base of traditional Chinese medicine Construction in 2015 (second special research projects; grant no. JDZX2015073) and Project of Chongqing Education Bureau (grant no. yjg143075).

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
MT, SW, YW performed the tests and experiments. YW analyzed the data. MT and JP wrote the manuscript and were the major contributors to the design of the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The present study was performed following the guidelines of the Care and Use of Laboratory Animals of National Institute of Health (NIH) and was approved by the Ethics Committee of Chongqing Medical University (Chongqing, China).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


