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Expression of *PANDA*, *LincRNA-p21*, *PUMA* in lung tissues of lung cancer patients in the Xuanwei and non-Xuanwei areas of Yunnan Province

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ABSTRACT

Aim: To study the expression of *PANDA*, *LincRNA-p21*, and *PUMA* in lung tissue of patients with lung cancer from Xuanwei of Yunnan Province. **Methods:** Forty-five cases of lung cancer patients from Xuanwei and 42 lung cancer cases from non-Xuanwei were enrolled. Extraction of RNA was done using the Trizol kit. Real-time fluorescence quantitative PCR assay was done to obtain the relative expression. **Results:** Expressions of *PANDA*, *LincRNA-p21*, and *PUMA* in male and female patients or in squamous cell carcinoma and adenocarcinoma were not significantly different ($P > 0.05$). However, expression of *LincRNA-p21* in Xuanwei patients was higher than non-Xuanwei patients ($P < 0.05$). Expression of *PUMA* in tumor tissue was lower than that in normal lung tissue ($P < 0.05$), and in Xuanwei patients was lower than non-Xuanwei patients ($P < 0.05$). In patients from non-Xuanwei regions, expression of *LincRNA-p21* in patients with smoking index > 400 was higher than in those < 400 and non-smokers. **Conclusion:** Expressions of *PANDA*, *LincRNA-p21*, and *PUMA* in lung tissues have no gender differences or tissue specificity. High expression of *LincRNA-p21* in Xuanwei patients may have relationship with cell damage caused by coal burning pollution in Xuanwei.

INTRODUCTION

Xuanwei is located in the northeast of Yunnan, China. In the east it is bordered by Panxian County of Guizhou, in the south by Zhanyi and Fuyuan Counties. Xuanwei has a population of 1,518,500. It has an estimated 801,100 males and 717,400 females. The primary source of income in Xuanwei is from agriculture, coal

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mining, and coal-related industries.

Xuanwei is an area with high deposits of coal and also has high incidence of lung cancer as well as high mortality rate from lung cancer. From 2004 to 2005, the mortality rate of lung cancer in Xuanwei was 91.30/10^5, 2.26 times higher than that of Yunnan Province and 2.96 times higher than that of all of China.[1] Current research suggests that the high incidence of lung cancer in Xuanwei is related to air pollution caused by bituminouscoal, with environmental factors perhaps accounting for 70% and 30% depending on susceptibility factors.[2]

Development of lung cancer is a complex biological, multistep process, which not only activates oncogenes and inactivates the tumor suppressor genes, but also has a close relation with imbalance of apoptosis.[3] PANDA (DNA damage activates p21 related non-coding RNA), LincRNA-p21 (Cyclin-dependent kinase inhibitor 1a, CDKN1A/p21), and PUMA (p53 upregulated modulator of apoptosis) play an important role in p53-dependent mitochondrial apoptotic pathway when DNA is damaged. When DNA damage occurs, it activates expression of p53 and LincRNA-p21. In one aspect, the combination of p53 and LincRNA-p21 activates expression of PUMA, which then interacts with antiapoptotic members of the family tore lease Bax and Bak. Baxis embedded in the outer membrane of mitochondria, leading to increased mitochondrial outer membrane permeability. The release of cytochrome C apoptotic factors activates the caspase cascade, eventually leading to cell death.[4] On the other hand, the combination of p53 and LincRNA-p21 activates expression of PANDA which in combination with Nuclear transcription factor Y subunit alpha (NF-YA) prevent apoptosis-related gene expression, thus inhibiting apoptosis.[5]

Our initial research found that smoke and ash of bituminous coal in Xuanwei contained large numbers of polyaromatic hydrocarbons and nano-sized quartz particles, which were also found in lung tissues of people with lung cancer in Xuanwei, which can lead to DNA damage as outlined earlier. We also found mutations and polymorphisms of mitochondrial DNA and miRNA in tissues of lung cancer patients from Xuanwei. By employing real-time quantitative fluorescence PCR techniques, expressions of PANDA, LincRNA-p21, and PUMA were examined in lung cancer tissues of patients from Xuanwei and non-Xuanwei areas. We also sought to investigate the relationship between PANDA, LincRNA-p21, PUMA and lung cancer risk in the Xuanwei area.

METHODS

Objectives

Forty-five patients from Xuanwei and 42 from non-Xuanwei regions were recruited for surgery in the Department of Thoracic Surgery, the Third Affiliated Hospital of Kunming Medical University (Cancer Hospital of Yunnan) from March 2014 to December 2014. Details of the patients were obtained, including age, sex, personal habits, living condition, use of coal for household purposes, dietary habits, occupation, and economic conditions of the family. Chest X-ray, ultrasonography, CT scan, fiber optic bronchoscopy, and sputum cytology were done before surgery. All patients were chemotherapy-and radiotherapy-naive [Table 1].

Signed informed consents were obtained from all patients. Lung cancer tissues and normal lung tissues (5 cm away from tumor margins) was resected and preserved in RNA, later (Ambion, United States) in in vitro solution at a temperature of 4 ºC for 8 h and then at -80 ºC for medium or long term preservation. Clinical and pathological data were collected post operatively and the patients had regular follow-ups.

Extraction of RNA

Total extraction of RNA was done using the Triazol reagent kit (Invitrogen Company, USA) and reverse transcription was performed using the QuantScript RT kit (Tiangen Company, China). Using ABI7900HT (ABI Company, USA) according to established methods, three RNA (PANDA, LincRNA-p21, PUMA) and $2^{-\triangle CT}$ value indicated the quantity of relative expression.

Statistical analysis

Statistical analysis was done using SPSS 17.0 software. After log transformation, normal distribution was analyzed. Comparison between the two groups was done using *t*-test and Spearman analysis of correlation was performed between the groups.

RESULTS

The basic situation of cases

A total of 45 patients with lung cancer were selected

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
Community: Han	Silicosis, severe chronic obstructive pulmonary disease, tuberculosis
Primary carcinoma of the lungs, non-small cell lung cancer	Surgical procedure: lobectomy, segmentectomy, pneumonectomy
Absence of concurrent tumors	No history of chemotherapy, radiotherapy, targeted drug therapy

from Xuanwei and 42 patients with lung cancer were from non-Xuanwei regions. All patients were ethnic Han Chinese. Non-Xuanwei regions included Kunming, Yuxi, Baoshan, Dali, Chuxiong, Honghe, and other areas. Eighteen cases of squamous cell carcinoma and 27 cases of adenocarcinoma were from Xuanwei. Seventeen cases of squamous cell carcinoma and 25 cases of adenocarcinoma were from non-Xuanwei. There were 24 males and 21 females from Xuanwei and 24 males and 18 females from non-Xuanwei regions. Meanage of patients from Xuanwei was 54.91 ± 9.62 years while in the non-Xuanwei group, the mean age was 58.33 ± 10.13 years. There were 21 patients from Xuanwei and 22 from non-Xuanwei who were smokers. Eighteen patients from Xuanwei consumed alcohol whereas 16 from non-Xuanwei did [Table 2].

Expressions of *LincRNA-p21*, *PANDA*, and *PUMA* in Xuanwei and non-Xuanwei patients

Expression of LincRNA-p21 in lung tissues of Xuanwei patients was increased ($P < 0.05$). There was no significant difference in expression of it between 2 groups of patients in lung cancer tissues and normal lung tissues ($P > 0.05$). There was no significant difference in the expression of PANDA in lung cancer tissue and normal lung tissue between the 2 groups ($P > 0.05$). Expression of PUMA in lung cancer tissue was lower than normal tissue ($P < 0.05$). Expression of PUMA in patients from Xuanwei was lower than that of non-Xuanwei patients, not only in cancer tissue, but also in normal lung ($P < 0.05$) [Tables 3 and 4].

Table 2: Comparison between the two groups

	Xuanwei	Non-Xuanwei
n	45	42
Squamous cell carcinoma	18	17
Adenocarcinoma	27	25
Male	24	24
Non-smoking	3	2
Smoking* < 400	7	9
Smoking > 400	14	13
Female	21	18
Non-smoking	21	18
Smoking < 400	0	0
Smoking > 400	0	0
Age	54.91 ± 9.62	58.33 ± 10.13
Alcohol	18	16

*Smoking index: the number of cigarettes smoked per day x number of years of smoking, high-risk groups > 400

Expression of *LincRNA-p21*, *PANDA*, and *PUMA* in squamous cell carcinoma and adenocarcinoma

There was no significant difference in the expression of three genes in squamous cell carcinoma and adenocarcinoma ($P > 0.05$) [Table 5].

Expression of *LincRNA-p21*, *PANDA*, *PUMA* in smokers and non-smokers

The expression of LincRNA-p21, PANDA, PUMA in smokers and non-smokers was showed in Table 6.

Analysis of correlation in expression of *LincRNA-p21*, *PANDA* and *PUMA* in tissues of lung cancer patients from Xuanwei and non-Xuanwei areas

LincRNA-p21 and PANDA in lung cancer tissues and normal lung tissues were characterized by positive correlation. PANDA with PUMA in lung cancer and normal lung tissue showed no corresponding correlation [Table 7].

Expression of LincRNA-p21, PANDA and PUMA in lung cancer patients from non-Xuanwei areas: LincRNA-p21 was positively correlated with PUMA in normal tissue, but not in lung cancer tissue. LincRNA-p21 and PANDA in lung cancer and normal lung tissue all showed positive correlation. PANDA was positively correlated with PUMA in normal lung tissue but not in lung cancers [Table 8].

Table 3: Expression of *LincRNA-p21*, *PANDA*, *PUMA* in males and females

Gene		Male	Female	*P*
LincRNA-p21	Xuanwei	15.18 ± 1.44	15.19 ± 1.65	0.63
	Non-Xuanwei	13.33 ± 1.85	13.01 ± 1.05	0.51
PANDA	Xuanwei	14.36 ± 1.65	14.38 ± 2.79	0.88
	Non-Xuanwei	14.14 ± 1.23	14.03 ± 1.67	0.85
PUMA	Xuanwei	5.32 ± 1.85	5.23 ± 1.43	0.74
	Non-Xuanwei	7.45 ± 2.18	7.34 ± 2.42	0.77

Expression of the 3 genes in lung tissues of both males and females showed no statistical difference ($P > 0.05$)

Table 4: Expression of *LincRNA-p21*, *PANDA*, *PUMA* in Xuanwei and non-Xuanwei patients

Gene		Xuanwei	Non-Xuanwei	*P*
LincRNA-p21	Cancer tissue	15.12 ± 1.27	12.21 ± 1.51	0.03
	Normal tissue	15.07 ± 1.42	12.19 ± 1.62	0.04
PANDA	Cancer tissue	14.36 ± 1.46	14.08 ± 1.27	0.52
	Normal tissue	14.44 ± 1.55	14.11 ± 2.32	0.47
PUMA	Cancer tissue	5.14 ± 1.37	7.38 ± 2.74	0.01
	Normal tissue	6.19 ± 1.43*	8.82 ± 2.89**	0.01

*$P = 0.04$ (cancer tissue *vs.* normal tissue); **$P = 0.03$ (cancer tissue *vs.* normal tissue)

Table 5: Expression of *LincRNA-p21*, *PANDA* and *PUMA* in squamous cell carcinoma and adenocarcinoma

Gene		Adenocarcinoma	Squamous cell carcinoma	*P*
LincRNA-p21	Xuanwei	15.23 ± 1.31	15.09 ± 1.53	0.56
	Non-Xuanwei	12.31 ± 1.73	12.18 ± 2.05	0.58
PANDA	Xuanwei	14.38 ± 1.64	14.22 ± 1.88	0.74
	Non-Xuanwei	14.11 ± 1.31	14.01 ± 2.15	0.82
PUMA	Xuanwei	5.09 ± 1.15	5.33 ± 1.84	0.64
	Non-Xuanwei	7.39 ± 1.98	7.36 ± 2.76	0.86

Table 6: Expression of *LincRNA-p21*, *PANDA*, *PUMA* in smokers and non-smokers

Gene		Non-Smoker	Smoking < 400	Smoking > 400
LincRNA-p21	Xuanwei	15.19 ± 1.01	15.14 ± 1.21	15.16 ± 2.02
	Non Xuanwei	12.95 ± 2.13	13.16 ± 1.73	14.74 ± 2.11*
PANDA	Xuanwei	14.37 ± 1.21	14.37 ± 1.53	14.38 ± 1.74
	Non Xuanwei	14.08 ± 0.88	14.15 ± 1.29	14.14 ± 1.29
PUMA	Xuanwei	5.31 ± 1.47	5.33 ± 1.91	5.32 ± 1.12
	Non Xuanwei	7.31 ± 1.82	7.33 ± 1.46	7.34 ± 2.05

*$P < 0.05$ (comparing non-smokers with smokers with smoking index < 400 and > 400)

DISCUSSION

The Xuanwei region of Yunnan, China, has a high incidence of lung cancer.[2] The characteristics of lung cancer in Xuanwei are: (1) the mortality rate of lung cancer in Xuanwei was 28.20/100,000 in 1973-1975, in 1990-1992 it was 40.29/100,000, and in 2004-2005 it was 83.28/100,000, which were higher than the average of the entire Yunnan and the national average; (2) lung cancer caused death 10-15 years earlier than the national average age; (3) lung cancer incidence is high among female population whereas the sex ratio (male:female) is low in other places like Netherland (16.32), Sweden (4.15), China (2.01), whereas in it is 1.09. In the small town of Laibin in Xuanwei the sex ratio for the incidence of lung cancer is 0.87, with females being affected more than males; (4) the incidence of lung cancer in Xuanwei area may be familial.

Indoor burning of bituminouscoal, leading to indoor air pollution, is considered to be the main reason for the high incidence of lung cancer in Xuanwei. Polyaromatic hydrocarbons (PAHs), nano silica particles and other substances in bituminouscoal dust and smoke are thought to be the causes of lung cancer.[6,7] In 1970, the Chinese government implemented a policy at all levels to improve coal burning stoves which could lead to improvement of indoor air quality. Despite these efforts, lung cancer incidence is still high and with an increasing trend.

The development of lung cancer is a complex biological process which is multistep and multifactorial. It not only includes the activation and inactivation of oncogenes and tumor suppressor gene respectively, but also has close relationship with apoptosis. There are two pathways of apoptosis of which one is the activation of the apoptotic caspase through extracellular signaling while the other is release of cytochrome C and activation of apoptotic enzyme

Table 7: Expression of *LincRNA-p21*, *PANDA* and *PUMA* in lung cancer patients from Xuanwei

Gene	Tissue type	Spearman correlation	
		R	*P*
LincRNA-p21	Cancer tissue	0.08	0.62
and *PUMA*	Normal tissue	0.12	0.33
PANDA and	Cancer tissue	0.07	0.58
PUMA	Normal tissue	0.14	0.28
LincRNA-p21	Cancer tissue	0.81	0.00
and *PANDA*	Normal tissue	0.77	0.00

Table 8: Expression of *LincRNA-p21, PANDA* and *PUMA* in lung cancer patients from non-Xuanwei areas

Gene	Tissue type	Spearman correlation	
		R	*P*
LincRNA-p21	Cancer tissue	0.06	0.67
and *PUMA*	Normal tissue	0.35	0.04
PANDA and	Cancer tissue	0.08	0.62
PUMA	Normal tissue	0.42	0.00
LincRNA-p21	Cancer tissue	0.84	0.00
and *PANDA*	Normal tissue	0.71	0.00

through mitochondrial caspase.[8,9] It is well known that repeated DNA damage can be carcinogenic, and tumor suppressor p53 increases as a response to DNA damage. Burning of coal produces numerous harmful pollutants and carcinogens which causes damage to DNA. At the molecular level, DNA damage is sensed by human protein kinase ATM (ataxia-telangiectasia, mutated), leading to phosphorylation and activation of ATM. Downstream phosphorylation and activation of TP53, together with RUNX3, activate transcription of downstream mediators as a response to injury to DNA.[10]

In our previous study with 25 patients of lung cancer (adenocarcinoma and squamous cell cancer) from Xuanwei, we found differences in 33 genes, of which *LincRNA-p21* was one of them. As a result, we chose to study LincRNA-p21. In this study, we focused on three selected mediators of TP53 response: PUMA, LincRNA-p21 and PANDA. PUMA is a member of the BCL2 family and is an important mediator of apoptosis.

Research has found that PUMA is more important than NOXA in DNA damage that induces activation of the p53-mediated mitochondrial apoptotic pathway. PUMA is located downstream of the *p53* gene and has a powerful effect in promoting apoptosis and inhibition of cell growth. Low expression of PUMA exists in some tumor tissues and it is associated with the occurrence and development of tumor. Thus, increasing of PUMA can inhibit tumor growth.[11] Long non-coding RNA p21 interacts with hnRNP-K to activate p21 to enforce cell cycle arrest at the G1/S phase. LincRNA-p21 is an important member of the cell cycle and its expression is directly induced by p53. The result of flow cytometry and apoptosis-related enzyme activity assay confirmed LincRNA-p21 induced apoptosis in the p53 dependent pathway, and related to the formation of tumor. PANDA is a noncoding RNA that inhibits expression of apoptotic genes by sequestering NF-YA.

When DNA damage occurs, it activates expression of p53 and LincRNA-p21. In one aspect, the combination of p53 and LincRNA-p21 activates expression of PUMA. PUMA interact with members of the antiapoptotic family, and release Bax and Bak. Baxis embedded in the outer membrane of mitochondria, leading to increased permeability of the outer mitochondrial membrane. Release of cytochrome C apoptotic factors activates the caspase cascade, eventually leading to cell death.

This study found that expression of PANDA, LincRNA-p21 and PUMA showed no significant difference in lung tissue of male and female ($P > 0.05$). Expression in squamous cell carcinoma and adenocarcinoma also showed no significant difference ($P > 0.05$), indicating that there is no gender difference or tissue specificity. Expression of LincRNA-p21 and PUMA in cancer tissues is not related but in the normal tissue it is positively correlated, perhaps due to existence of regulatory changes and disorder in cancer tissues.

In this study, we found that expression of LincRNA-p21 was higher in patients from non-Xuanwei regions with smoking index > 400 than in patients with smoking index < 400 and non-smokers, while in patients from Xuanwei, there was no significant difference among the three groups ($P > 0.05$). The probable reason might be that smoking is not the main reason for lung cancer in Xuanwei. The incidence of lung cancer is higher in women from Xuanwei, even though the majority of women were non-smokers. In an experiment, we exposed cells to the byproduct obtained from combustion of bituminous coal, the result of which was secretion of mediators of inflammation, damage to the cell membrane, damage to mitochondria, and mutation in nuclear DNA and mt-DNA.

Expression of LincRNA-p21 in lung cancer patients from Xuanwei was higher than non-Xuanwei patients ($P < 0.05$). We observed that alveolar epithelial cells and bronchial epithelial cells were severely damaged in patients from Xuanwei due to serious air pollution caused by burning of coal. There was also damage to DNA from pollutants released as a result of combustion of coal. Our initial research in Xuanwei found that ash and smoke of bituminous coal contained large numbers of polyaromatic hydrocarbons and nano-sized quartz particles,[12-14] which were also found in lung tissues of lung cancer patients from Xuanwei. Polyaromatic hydrocarbons and nano-sized quartz particles can lead to damage of nuclear DNA and mitochondrial DNA.[15,16]

Expression of PUMA in lung tissues of Xuanwei patients was lower than non-Xuanwei patients ($P < 0.05$). The correlation analysis revealed that the expression of LincRNA-p21 had no correlation with expression of PUMA in normal lung tissues of Xuanwei patients, while normal lung tissues of non-Xuanwei patients were positively correlated. We considered that PUMA is a highly conserved gene in eukaryotes, possibly due to air pollution in Xuanwei causing damage to PUMA or effect the gene transcription process, but also polymorphism of LincRNA-p21 and PUMA in local population could not be ruled out, for which further research with larger sample is required.

We have successfully cultured a type of Xuanwei lung adenocarcinoma cell line (XW-05). We are now carrying out gene transfection and silencing experiments on XW-05 cells, human adenocarcinoma cells (A549), and human bronchial epithelial cells (BS2B) to observe whether XW-05 cells have specificity.

Part of this project was under the U.S. National Cancer Institute to study environmental exposure, dose-effect relationship, and epidemiology of lung cancer in Xuanwei. We are responsible for the questionnaire, collection of clinical data, specimen collection, treatment, follow-up, and prognosis. Genomic sequencing is carried out by Beijing Gene Square for National Cancer Institute. Currently, Beijing Gene Square is sequencing specimens of 421 cases, the findings of which should be reported soon.

Authors' contributions

The study's conception and design: K.Y. Yang, Z.Q. Shen

Extraction of RNA and PCR: Y.F. He, K.Y. Yang, K. Rizal

Patients' enrollment and lung tissues and data's collection: K.Y. Yang, A.N. Chen, Y.C. Huang, G.Q.

Zhao, Y.J. Lei
Paper's written: K.Y. Yang, K. Rizal
Manuscript's review and edition: K.Y. Yang, Z.Q. Shen, K. Rizal

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Conflicts of interest
There are no conflicts of interest.

Patient consent
Patient consent was obtained.

Ethics approval
Ethics approval was obtained.

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