



Kaiso is expressed in lung cancer: Its expression and localization is affected by p120ctn

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ABSTRACT

Background: Kaiso is a recently identified transcription factor that binds to p120-catenin (p120ctn), an Armadillo catenin and cell adhesion cofactor. However, clinical studies of human solid tumors have not been reported to investigate relationships between these proteins.

Methods: Expression and localization of Kaiso and p120ctn were examined in 196 lung cancer specimens (including 55 cases of paired lymph node metastases and 80 cases with complete follow-up records) by immunohistochemistry. Three lung cancer cell lines, BE1, SPC, and A549 were used to establish p120ctn stably ablated or overexpressed cell lines. Co-immunoprecipitation was used to confirm p120ctn bind Kaiso in lung cancer tissue and cell lines. Localization and expression levels of Kaiso were detected via immunofluorescence, cytoplasmic vs. nuclear fractionation Western blot analysis and reverse transcription-polymerase chain reaction.

Results: Cytoplasmic Kaiso expression was evident in 115 (58.7%), and abnormal p120ctn expression was noted in 168 (85.7%). Cytoplasmic Kaiso and abnormal p120ctn expressions were associated with higher degree of malignancy, T3–T4 stage and lymph node metastases, all $P < 0.05$. Abnormal p120ctn and cytoplasmic Kaiso expressions were higher in matched autologous nodal metastases than in primary growths. In lung cancer-related 5-year survival rate was significantly lower in patients who were cytoplasmic p120ctn positive (20%; $P = 0.029$) or abnormal p120ctn expression (20.6%; $P = 0.001$). Multivariate analysis showed abnormal p120ctn expression was an independent factor defining the clinicopathological characteristics of patients. Cytoplasmic Kaiso expression was correlated with cytoplasmic p120ctn, they formed Kaiso–p120ctn complex in lung cancer tissues and cell lines. In addition, p120ctn ablation and overexpression altered Kaiso subcellular localization and protein level. Although both isoforms can regulate subcellular localization and protein levels of Kaiso, we found that only p120ctn isoform 3, but not isoform 1 directly interacts with Kaiso.

Conclusion: p120ctn and Kaiso might co-participate in the progression and lymph node metastasis of lung cancer. p120ctn regulates expression and localization of Kaiso in lung cancer cells.

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1. Introduction

It is known that primary lung cancer is one of the most malignant solid tumors, and that its range of abilities in invasion and metastasis is very wide. Dysfunction of adhesion molecules and impairment of cell adhesion may be the initial steps of such tumor progression [1–3]. Of these molecules, p120-catenin (p120ctn) may play an important role in tumor progression and metastasis due to its core function in cadherin turnover and cell migration [4–9].

In most human tumors, p120ctn levels are reduced or even eliminated [5]. We have reported previously that abnormal expression of p120ctn (reduction of p120ctn isoforms 1 and 3) is associated with lymph node metastasis in lung cancer cells [10–12].

p120ctn is an Armadillo protein first identified as a prominent tyrosine kinase substrate implicated in cell transformation by Src [13], and in ligand-induced receptor signaling through various tyrosine kinase receptors [14,15]. p120ctn binds to the juxtamembrane domain (JMD) of E-cadherin [6,16], where it modulates cell–cell adhesion by regulating cadherin turnover and stability at the cell surface [7–9]. Additionally, p120ctn modulates the activities of the RhoA, Rac and Cdc42 GTPases, which are the key mediators of cytoskeletal organization [17–19]. Furthermore, recent evidence has revealed that nuclear p120ctn acts to relieve transcriptional repression mediated by Kaiso, a member of the broad complex,

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tramtrak, bric a' brac/pox virus and zinc finger family (BTB/POZ) [20].

Kaiso contains an amino-terminal, protein–protein interaction BTB/POZ domain and a carboxyl-terminal DNA-binding C₂H₂ zinc finger domain [20]. Interaction between p120ctn and Kaiso occurs via the Arm repeats 1–7 of p120ctn and a noncontiguous Kaiso domain flanking the carboxyl-terminal DNA-binding zinc finger domain. This close physical juxtaposition of the p120ctn-binding site of Kaiso with its DNA-binding motif may explain why DNA binding of Kaiso is inhibited by nuclear p120ctn [21,22]. It is shown that nuclear p120ctn can modulate non-canonical Wnt signaling [23], and, along with T cell factor (TCF)/β-catenin complexes, coordinately regulate canonical Wnt gene targets such as *cyclin D1* and *mmp-7* by Kaiso [24,25]. There is mounting evidence that Kaiso is a nuclear protein that plays a role in transcription repression [23–26] and Kaiso is frequently detected in the nuclei of various mammalian cell lines. However, immunohistochemical study of human normal and tumor tissues show that Kaiso is predominantly localized in the cytoplasm rather than in cell nuclei. Interestingly, Kaiso's cytoplasmic localization almost always coincided with cytoplasmic p120ctn [27]. Thus, it remains a question that whether Kaiso interacts with p120ctn in cytoplasm.

The human p120ctn gene comprises 21 exons, potentially encoding up to 32 protein isoforms as the result of alternative product splicing. All p120ctn isoforms share the central Armadillo repeat domain but have a divergent N- and C-terminal end [6]. Human p120ctn isoforms, designated from 1 to 4, differ from each other by the start codon used. In our previous study, we determined that only two isoforms (isoforms 1 and 3) expressed in normal and cancerous lung tissues [10] and the roles of these two isoforms on invasion are somewhat different [28]. Because p120ctn is expressed as multiple isoforms with different capacities to bind to the cytoskeleton and to enter the nucleus [29–32], these isoforms might have a different potential to modulate the function of Kaiso *in vivo*. We also try to provide some experimental data to clarify this issue in this study.

To date, little clinicopathological report has referred to the relationship between Kaiso and p120ctn expression in human solid tumors. In this study, we examined the expression pattern and prognostic significance of p120ctn and Kaiso expression in patients with lung cancer. Meanwhile, Kaiso and p120ctn expressions in 55 cases of lymph node metastases were tested to investigate differences between primary lung cancers and paired lymph node metastases. Co-immunoprecipitation was performed to confirm the binding between p120ctn and Kaiso in lung cancer tissues and three lung cancer cell lines. To investigate the effect of p120ctn on Kaiso, we depleted total p120ctn expression and overexpressed two p120ctn isoforms in lung cancer cell lines to evaluate the expression and subcellular localization of Kaiso.

2. Materials and methods

2.1. Tissue samples

Lung cancer tissues from 196 patients with primary lung cancer and normal from 20 of these patients were obtained following surgical resection at the First Clinical College affiliated with China Medical University between 2001 and 2005. Among the 196 cases, the lymph node metastases of 55 patients were available. All patients had not received radiotherapy, chemotherapy or immunotherapy before the operation. The average age of the patients was 57.24 years (from 20 to 81 years). The ratio of male to female was 1.87:1. Based on the classification of the World Health Organization [33] and the TNM stage revised by International Union Against Cancer (UICC) in 2002 [34], we classified the patients with primary lung cancer into the following groups: (1) 81 squamous

cell carcinomas, with 27 cases at stage I, 23 cases at stage II, 35 cases at stage III, and 2 cases at stage IV; 14 cases were highly differentiated, 33 cases were moderately differentiated, and 40 cases were undifferentiated; and 49 cases with lymph node metastasis. (2) 115 adenocarcinomas, with 33 cases at stage I, 23 cases at stage II, 48 cases at stage III, and 5 cases at stage IV; 31 cases were highly differentiated, 27 cases were moderately differentiated, and 51 cases were undifferentiated; and 75 cases with lymph node metastasis. A total of 55 samples (24 squamous cell carcinomas, 31 adenocarcinomas) with autologous nodal metastasis were used as paired samples to perform immunohistochemical analysis. 20 cases of tumor samples (including primary cancer and lymph node metastases cancer tissues) were quick frozen in deep freeze refrigerator until protein extraction. Lymph node status was determined by routine pathological examination of dissected pulmonary hilar and mediastinal and intraluminal lymph nodes. In addition, immunohistochemistry was completed on 80 cases of primary NSCLC paraffin specimens which have complete follow-up records. This study was conducted under the regulations of the Institutional Review Board of China Medical University. Informed consent was obtained prior to surgery from all enrolled patients.

2.2. Cell culture, plasmid construction and transfection

2.2.1. Cell culture

The human pulmonary giant cell cancer cell line BE1, which expresses high levels of p120ctn, kindly was provided by Dr Jie Zhou of Department of Pathology, Peking University Health Science Center (Beijing, China). Human lung adenocarcinoma cell line A549 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA), containing 10% fetal calf serum (Invitrogen), 100 IU/ml penicillin (Sigma, St Louis, MO, USA), and 100 IU/ml streptomycin (Sigma).

2.2.2. p120ctn siRNA plasmids and transfection

For the production of the p120ctn-siRNA (GeneBank#: 001331) plasmids used in the experiments, sense and anti-sense oligonucleotides were annealed and inserted between BamHI and HindIII sites of the pGCsi vector by Shanghai GeneChem (Shanghai GeneChem Co. Ltd., Shanghai, China). The sequences of the three double-stranded oligonucleotides were as follows:

A: 5'-GGATCACAGTCACCTTCTA-3'; 5'-TAGAAGGTGACTGTGATCC-3'
 B: 5'-GCACTTGTATTACAGACAA-3'; 5'-TTCTCTGTAATACAAGTGC-3'
 C: 5'-GGATAACAAGATTGCCATA-3'; 5'-TATGGCAATCTGTTATCC-3'

The BE1 was stably transfected with the p120ctn-siRNA plasmids using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. A non-specific scrambled p120ctn-siRNA plasmid (5'-GG TAG AGA GAC AGT CTC GC-3'; 5'-GC GAG ACT GTC TCT CTA CC-3') was used as a negative control. Selection was accomplished with G418 (Invitrogen) at a concentration of 0.3 mg/ml. Drug-resistant cells were tested for the absence of p120ctn expression by Western blot and reverse transcription-PCR.

2.2.3. p120ctn cDNA plasmids and transfection

Following Dr. Reynolds' method [7], mouse p120ctn isoform 1A and p120ctn isoform 3A cDNA (a gift from Dr. Reynolds, Vanderbilt University, Nashville, USA) were stably transfected using Lipofectamine 2000 into human lung adenocarcinoma A549 cells. The stably transfected cells were named A549-1A and A549-3A, respectively. The empty plasmid was used as a negative control.

2.3. Cellular fractionation

Cellular fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific Inc. Meridian Rd., Rockford, USA) according to the manufacturer's instructions. Antibodies against α -tubulin (B-7, Santa Cruz Biotechnology, Inc.) and Lamin B1 (8D-1, Santa Cruz Biotechnology, Inc.) were used in control experiments.

2.4. Immunohistochemical staining and evaluation

Immunohistochemistry was performed using the ultrasensitive avidin–biotin–peroxidase complex method (Maixin Biotechnology, Fuzhou, Fujian, China) according to the manufacturer's instructions. The antibodies used were mouse anti-human Kaiso monoclonal antibody (clone 6F, dilution 1:400, Upstate, Lake Placid, NY, USA) and mouse anti-human p120ctn monoclonal antibody (clone 15D2, dilution 1:400, BD Transduction Laboratories, Lexington KY, USA). For the negative control, phosphate-buffered saline (PBS) was used in place of the primary antibodies.

The percentage of immunoreactive tumor cells was independently assessed by two observers (S.D.D and Q.W.) on all sections. Cases with discrepancies were jointly re-evaluated by the investigators, and a consensus was obtained. Positive samples were analyzed semi-quantitative at low magnification ($\times 100$) to identify areas where Kaiso/p120ctn was evenly stained. We counted 400 tumor cells and calculated the percentage of positively staining cells. Staining scores were determined by the percentage of positive cells per slide for membranous and cytoplasmic staining separately. As proposed previously [12,35,36], normal expression was defined when over 90% of the tumor cells showed cell membrane staining of p120ctn. When less than 90% of the tumor cells were stained for p120ctn at the cell membrane, the sample was labeled with "reduced membranous expression". When more than 10% of the tumor cells stained for cytoplasmic p120ctn expression, the sample was labeled with "ectopic cytoplasmic expression". A designation of either "reduced membranous expression" or "ectopic cytoplasmic expression" was defined as abnormal expression pattern. For evaluation of the staining of Kaiso in tissue sections, we use the criteria as follows: 0: less than 1%; 1: 26–50%; 2: 51–75%; and 3: more than 75%. The staining intensity was categorized by relative intensity as follows: 1 (weak); 2 (intermediate) and 3 (strong). The proportion and intensity scores were then multiplied to obtain a total score. Scores less than 1 were considered as negative, while scores of 2 or more were considered as positive. Cases were scored nuclear positive when >5% of the cells reacted with the anti-Kaiso antibody.

2.5. Immunofluorescent staining

Immunofluorescent staining was performed as described previously [10]. Briefly, cells grown on glass coverslips were fixed with ice-cold acetone/methanol for 15 min at -20°C , followed by permeabilization with 0.2% Triton X-100. Kaiso was detected using two mouse monoclonal antibodies (6F and 12H, each at a concentration of 4 $\mu\text{g}/\text{ml}$. Upstate, Lake Placid, NY and Santa Cruz Biotechnology, Inc. respectively), and a polyclonal antibody (C-18, Santa Cruz Biotechnology, Inc.) and p120ctn was detected using mouse monoclonal antibodies (clone 15D2, BD Transduction Laboratories, Lexington KY, USA). Primary antibodies were applied overnight at 4°C followed by incubation with secondary antibody conjugated to rhodamine/fluorescein isothiocyanate (FITC)-labeled at a dilution of 1:100 (Beijing Zhongshan Golden Bridge Biotechnology Co. Beijing, China). The nuclei were counterstained with propidium iodide (PI, 50 $\mu\text{g}/\text{ml}$, Sigma)/4, 6 diamidino-2-phenylindole (DAPI, 1:200, Sigma). The cells were examined with an Olympus IX51 fluorescent

microscope (Olympus, Tokyo, Japan), and images were recorded with a CoolPIX 5400 camera (Nikon, Japan).

2.6. RT-PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen). cDNA was prepared using the RNA PCR Kit (AMV) Version 3.0 (TaKaRa Bio Inc., Dalian, Liaoning, China), according to the manufacturer's instructions. PCR was carried out with the following primers: Kaiso, 5'-TGCCTATTATAACAGAGTCTTT-3' and 5'-AGTAGG TGTGATATTGTTAAAG-3'. p120ctn, 5'-TGCCCTGCTGGATTGTCCT-3' and 5'-CGAGTGGTCCGAGCTG-3'. β -actin, 5'-AGAGCTACGAGCTGCCTGAC-3' and 5'-AGTACTCTCTCAGGAGGA-3'. After electrophoresis on a 1.2% agarose gel, the PCR product bands were visualized using Bioimaging System with Labworks Image Acquisition and Analysis Software (UVP Inc., Upland, CA, USA). The relative mRNA levels were normalized to the relative amount of β -actin mRNA.

2.7. Immunoprecipitation and immunoblot analysis

Cells were washed twice with 5 ml of PBS followed by incubation on ice with lysis buffer containing 0.5% NP-40, 50 mM Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM sodium orthovanadate, and 1 mM PMSF for 5 min. Cells were harvested from the plates, and transferred to a 1.5 ml tube. The lysate was centrifuged at 16,000 g for 30 min at 4°C ; the supernatant transferred to a new tube. Lysates were measured by Bradford assay and equal amounts of total protein were used for immunoprecipitation with the anti-p120ctn mAb 15D2, BD Transduction Laboratories, Lexington KY, USA; mAb 6F (a gift from Dr. Reynolds, Vanderbilt University, Nashville, USA) or anti-Kaiso pAb (a gift from Dr. Juliet M. Daniel, Department of Biology, McMaster University). The immunocomplexes were then subjected to SDS-PAGE.

After SDS-PAGE, proteins were transferred from the gel to a nitrocellulose membrane using the Hoeffer semi-dry transfer apparatus (Amersham/Pharmacia, San Francisco, CA). The membrane was then briefly blocked at room temperature with 3% milk in TBS pH 7.4 before incubating at 4°C overnight with anti-Kaiso mAb (a gift from Dr. Juliet M. Daniel, Department of Biology, McMaster University) used at 2 $\mu\text{g}/\text{ml}$ in 3% milk-TBS. The primary antibodies were removed by washing with TBS and the membranes incubated for 2 h at RT with peroxidase-conjugated donkey anti-mouse secondary antibody, diluted 1:40,000 in 3% milk-TBS. Membranes were finally rinsed five times with water, once with TBS pH 7.4 for 5 min, and processed using the enhanced chemiluminescence (ECL) system (Amersham/Pharmacia) according to the manufacturer's protocols.

2.8. Statistical analysis

SPSS v13.0 software for Windows (SPSS, Inc. Chicago, IL, USA) was used for statistical analysis. The χ^2 -test was used to analyze the relationship between the expression of Kaiso and p120ctn and clinicopathological factors. McNemar's test was used to assess the difference between expression of Kaiso and p120ctn in primary lung cancer and lymph node metastasis cancer. The independent-samples *T* test was used to analyze the results of RT-PCR and Western blot. The prognostic significance of p120ctn and Kaiso expression concerning other pathological variables was assessed using multivariate Cox proportional hazard's analysis. A *P* value of less than 0.05 was considered statistically significant. All tests and *P* values were bilateral.

3. Results

3.1. Expressions of Kaiso and p120ctn were correlated with each other, and associated with the malignancy of lung cancer

Kaiso was expressed weakly in the epithelial cells of bronchus from all 20 normal pulmonary tissues and primarily localized on the apiculus of these cells (Fig. 1A). According to our evaluation criteria, they were judged as negative expression. The positive rate of cytoplasmic Kaiso expression in lung cancer tissue was 58.7% (115/196), significantly higher than that in normal bronchial epithelial cells ($P < 0.001$). We also examined the relationship between cytoplasmic Kaiso expression and clinicopathological factors (Table 1). We found that cytoplasmic Kaiso expression occurred more frequently in samples with advanced tumor stage and lymph node metastasis ($P = 0.004$, $P < 0.001$, respectively), while no significant associations were found with regard to age, sex, histological type or differentiation (Table 1). Cytoplasmic Kaiso expression was significantly higher in stages II + III + IV lung cancers than in stage I ($P = 0.004$, Table 1). In 124 cases with lymph node metastasis, 85 (68.5%) showed cytoplasmic Kaiso expression, and 39 (31.5%) showed no Kaiso expression. While in 72 cases without lymph node metastasis, 30 (41.7%) showed cytoplasmic Kaiso expression, and 42 (58.4%) showed no Kaiso expression. There was a significant correlation between cytoplasmic Kaiso expression and lymph node metastasis ($P < 0.001$, Table 1). Nuclear staining of Kaiso was seen in occasional tumor cells but only with a 5.61% (11/196) positive expression rate and not associated with any clinicopathological features of lung cancer (data not shown). In the 55 paired comparison samples, the positive rate of cytoplasmic Kaiso expression was 72.7% (40/55) in primary sites and 90.9% (50/55) in lymph node metastasis lesions. Lymph node metastases showed an increased expression rate in cytoplasmic Kaiso, compared to primary tumors ($P = 0.002$; Table 2B).

Expression of p120ctn occurs mainly in the cell membrane of normal bronchial epithelium (Fig. 1C). Of 196 lung cancers, 28 (14.29%) showed normal or preserved membranous expression only, and 168 (85.71%) showed abnormal expression, including reduced membranous expression, preserved membranous expression accompanied with ectopic cytoplasmic expression, and reduced or absent membranous expression together with ectopic cytoplasmic expression (Fig. 2A and E). Next, we examined associations between abnormal expression of p120ctn and clinicopathological factors (Table 1). We found that abnormal p120ctn expression occurred more frequently in samples with advanced tumor stage and lymph node metastasis ($P = 0.016$, $P < 0.001$, respectively), while no significant association was found with regard to age, sex, histological type and differentiation (Table 1).

The expression of p120ctn in the 55 cases for which paired data were available is summarized in Table 2A. Normal p120ctn expression (reduced membranous expression and ectopic cytoplasmic expression) was detected in 81.8% (45/55) in primary sites and 96.36% (53/55) in the lymph node metastases. Lymph node metastases showed significantly more abnormal p120ctn expression, compared to primary tumors ($P = 0.002$; Table 2B).

As shown in Table 2, Kaiso and p120ctn often exhibited cytoplasmic co-localization not only in primary lesions but also in lymph node metastases. The immunohistochemical staining results of consecutive sections showed that p120ctn ectopic cytoplasmic expression frequently accompanied with cytoplasmic Kaiso expression. We confirmed this observation widely existed in lung cancer tissues regardless of clinical and histological features (such as histological type and lymph node metastasis status). Next, we carried out statistical analysis to confirm the significantly relationship between cytoplasmic Kaiso expression and ectopic cytoplasmic p120ctn expression. Of 196 primary lung cancers, cytoplasmic Kaiso expression correlates with ectopic cytoplasmic p120ctn expression ($\kappa = 0.269$, $P < 0.001$; Table 2A). The same was true for pri-

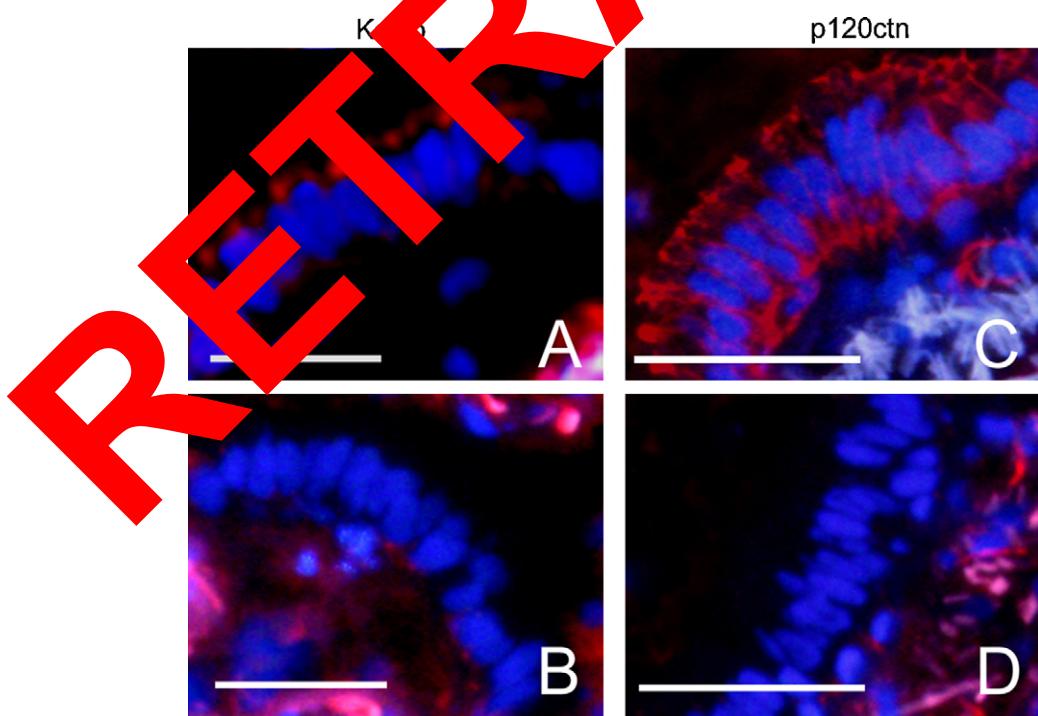


Fig. 1. Immunofluorescent staining of Kaiso and p120ctn in normal bronchial epithelial cells. (A) Weak red fluorescence of Kaiso is detected at the cytoplasm of normal bronchial epithelial cells (they were judged as negative expression in this study). (B) In negative control, no specific fluorescence was observed at the normal bronchial epithelial cells. (C) Clear and continuous red fluorescence of p120ctn is detected at the cell membrane of normal bronchial epithelial cells. (D) In negative control, no specific fluorescence was observed at the normal bronchial epithelial cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1

Clinical and histologic features in 196 patients with lung cancer.

Variables	All patients	Cytoplasmic Kaiso expression		P*	p120ctn expression		P
		Negative	Positive		Normal	Abnormal	
Total	196	81	115		28	168	
Age (year)				0.661			0.195
≤55	85	37	48		9	76	
>55	111	44	67		19	92	
Gender				0.446			0.581
Male	128	50	78		17	111	
Female	68	31	37		11	57	
Stage				0.004			0.016
I	60	34	26		14	46	
II/III/IV	136	47	89		14	122	
Histology				0.568			0.318
Squamous cell carcinoma	81	34	47		3	77	
Adenocarcinoma	115	53	62		3	91	
Grade				0.062			0.179
Well, moderate	103	49	54		18	85	
Poor	93	32	61		1	83	
Lymph node metastasis				<0.001			<0.001
Yes	124	39	85		9	115	
No	72	42	30		19	53	

* P values were obtained from the χ^2 test (two-sided).

mary and lymph node metastases samples ($\text{Kappa} = 0.267, P = 0.037$) ($\text{Kappa} = 0.393, P = 0.001$; Table 2C).

To test for the existence of p120ctn: Kaiso interactions *in vivo*, we performed co-immunoprecipitation from lung cancer tissue extracts by using anti-p120ctn antibody. The reverse endogenous precipitation of Kaiso, followed by Western blot detection of p120ctn, was more difficult to interpret due to the appearance of background signals (data not shown). Kaiso co-precipitated efficiently with p120ctn-specific mAb from all 13 pairs of lung cancer tissues. Immunohistochemical study clearly demonstrated that Kaiso and p120ctn were synchronously expressed in 13 of 20 cases of lung cancer tissues, both primary and lymph node metastases samples. These findings may clearly support the coassociation of Kaiso and p120ctn in lung cancer tissues. The co-immunoprecipitation results are shown in Fig. 3B.

Expression of Kaiso and p120ctn and survival time

In order to obtain prognostic data more quickly, immunohistochemistry was performed on partial lung cancer paraffin embedded tissues 5 years ago to determine the expression of Kaiso and p120ctn. The effects of Kaiso and p120ctn on prognosis of the patients with lung cancer were analyzed by inspecting follow-up data. With regard to the expression of p120ctn, the lung cancer-related 5-year survival rates were 51.8% in the normal expression and 20.6% for the abnormal expression. A significant difference was observed between the two groups ($P = 0.001$; Fig. 4). With regard to the expression of cytoplasmic Kaiso, the lung cancer-related 5-year survival rate was 51.4% for in case of the negative expression and 22.9% for the positive expression, and patients with the positive cytoplasmic of Kaiso had a significantly poor prognosis than did those with the negative cytoplasmic of Kaiso ($P = 0.029$; Fig. 4).

Table 2

Correlation between cytoplasmic Kaiso and p120ctn in lung cancer.

Antigen	Cytoplasmic p120ctn-positive	Cytoplasmic p120ctn-negative	Total	Kappa	P
A. Correlation between cytoplasmic Kaiso and p120ctn in 196 cases of primarily lung cancer					
Cytoplasmic Kaiso-positive	81	18	115	0.269	<0.001
Cytoplasmic Kaiso-negative	115	32	81		
Total	196	50	196		
B. Correlation between cytoplasmic Kaiso and abnormal p120ctn expression in 55 cases of primarily and lymphatic metastasis lung cancer					
Primarily Lung cancer	Cytoplasmic Kaiso-positive	35	5	40	
	Cytoplasmic Kaiso-negative	10	5	15	
	Total	45	10	55	
Lymphatic Metastasis Lung cancer	Cytoplasmic Kaiso-positive	48	2	50	
	Cytoplasmic Kaiso-negative	5	0	5	
	Total	53	2	55	
C. Correlation between cytoplasmic Kaiso and cytoplasmic p120ctn expression in 55 cases of primarily and lymphatic metastasis lung cancer					
Primarily Lung cancer	Cytoplasmic Kaiso-positive	36	4	40	0.267
	Cytoplasmic Kaiso-negative	10	5	15	
	Total	46	9	55	
Lymphatic Metastasis Lung cancer	Cytoplasmic Kaiso-positive	42	8	50	0.393
	Cytoplasmic Kaiso-negative	1	4	5	
	Total	43	12	55	

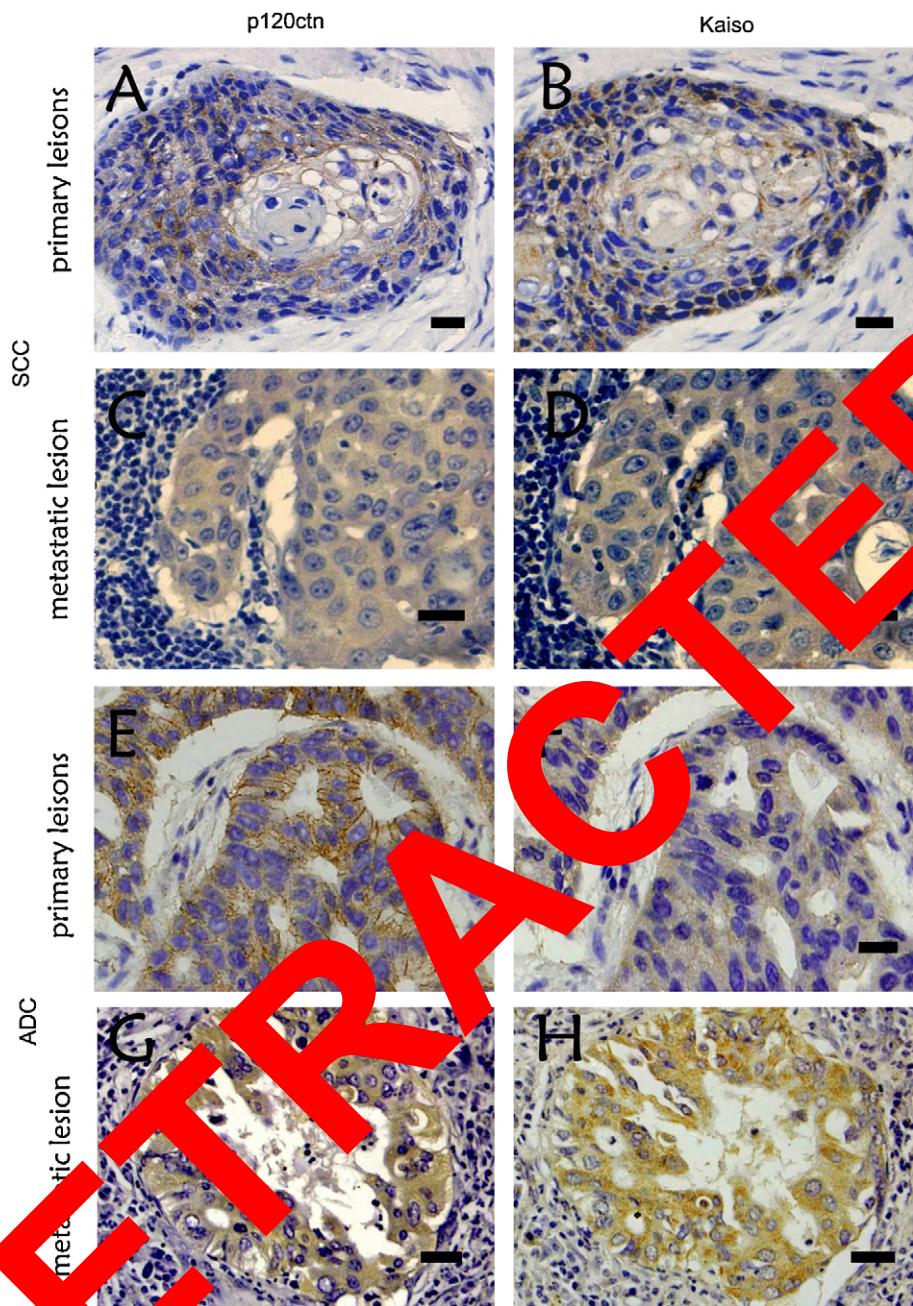


Fig. 2. Immunohistochemical staining of p120ctn and Kaiso in primary lung cancer and lymph node metastases. Consecutive sections showed that p120ctn (A and E) and Kaiso (B and F) expression patterns in similar region of the same sample of primary growth, as well as the expression patterns of p120ctn (C and G) and Kaiso (D and H) in similar region of the same sample of metastasis were similar, mainly as a cytoplasmic staining. Original magnification, 400 \times ; scale bar, 20 μ m.

Table 3
Cox regression model for prediction of survival of 80 patients with lung cancer.

Factor	Risk	95% CI	P-value
Age	0.671	0.336–1.436	0.161
Gender	0.751	0.424–1.653	0.402
Histology	1.315	0.312–1.421	0.362
Differentiation	2.847	1.361–3.344	0.034
TNM stage	3.500	1.264–6.735	0.002
Lymphatic metastasis	4.004	1.351–7.811	0.001
Abnormal p120ctn expression	0.335	0.412–0.723	0.006
Positive cytoplasmic Kaiso expression	0.301	0.459–1.556	0.062

To further evaluate the expression of p120ctn and Kaiso as prognostic factors, a multivariate Cox regression analysis was carried out. In an analysis of 80 patients, which included age, gender, histology, differentiation, TNM stage, lymph node metastasis, abnormal expression of p120ctn and positive cytoplasmic Kaiso, abnormal p120ctn expression showed an independent prognostic factor ($P=0.006$) as did lymph node metastasis ($P=0.001$), tumor stage ($P=0.002$), and differentiation ($P=0.034$; Table 3).

3.3. Expression and localization of Kaiso protein is affected by its binding partner, p120ctn

Since Kaiso was associated with p120ctn expression in lung cancer tissues, and p120ctn was Kaiso's specific binding partner, this

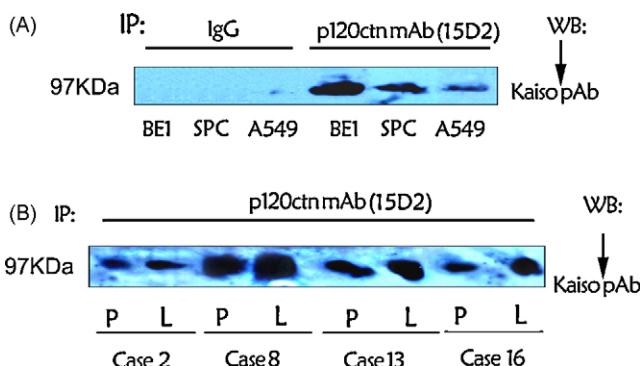


Fig. 3. p120ctn coprecipitates Kaiso from lung cancer cell lines and lung cancer tissues. Whole cell lysates were immunoprecipitated with the indicated antibodies, separated by SDS-PAGE, transferred to nitrocellulose, and then Western blotted with Kaiso-specific polyclonal antibody (pAb). (A) Kaiso was coprecipitated by p120ctn mAb (15D2) from BE1, SPC and A549 cells (lanes 4, 5 and 6). (B) Kaiso was coprecipitated by p120ctn from both primary lung cancer and lymph node metastases cancer tissues. The band positions are indicated on the left with molecular weight markers. (P: primary lung cancer tissue; L: lymph node metastases tissue).

raised the possibility that p120ctn may affect Kaiso's subcellular localization and/or expression in lung cancer cells. Thus we chose three lung cancer cell lines, BE1, SPC and A549 cells, to validate our supposition. BE1 cells derived from the PG cell line (express high levels of p120ctn) and human lung adenocarcinoma cell line SPC (express relative middle levels of p120ctn) were suitable to carry out the siRNA experiment (Fig. 5). Human lung adenocarcinoma cell line A549 normally express only a minor amount of p120ctn, was used to transfect with p120ctn 1A and 3A plasmids (Fig. 5). As shown in Fig. 3A, the co-immunoprecipitation result demonstrated that p120ctn coprecipitated with Kaiso in these three cell lines. We also detected the levels of Kaiso mRNA and protein in these cell lines, the results demonstrate that these par-

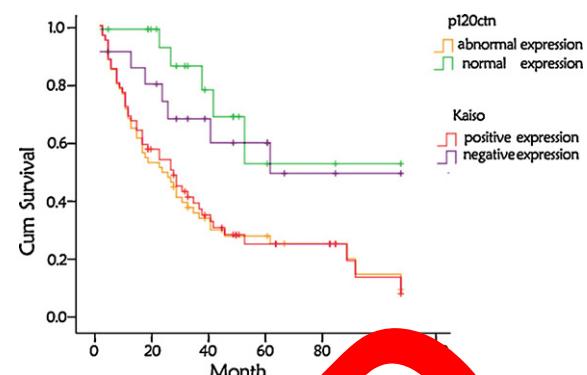


Fig. 4. Survival curves of abnormal expression of p120ctn and cytoplasmic expression of Kaiso. Kaplan-Meier curves for analysis of 83 patients with non-small-cell-lung-cancer (NSCLC).

cell lines expressed different levels of Kaiso mRNA and protein (Fig. 5).

RT-PCR results revealed that whether p120ctn was depleted or overexpressed, the levels of p120ctn mRNA varied little, as compared with control cells (all $P > 0.05$, Fig. 5A). However, after p120ctn depletion in BE1 and SPC cells, Kaiso protein expression was significantly lower than control cells ($P < 0.001$, Fig. 5B). We also found that Kaiso protein expression significantly upregulated in A549-1A and A549-3A cells, which overexpressed p120ctn 1A and 3A respectively ($P < 0.003$, $P < 0.001$, respectively, Fig. 5B).

Localization of Kaiso protein is affected by its binding partner. p120ctn immunofluorescence showed that nuclear Kaiso significantly reduced in siRNA-BE1 cells (Fig. 6A) and increased in siRNA-SPC cells (Fig. 7A). These observations were confirmed by cytoplasmic vs. nuclear fractionation Western blot analysis (Fig. 6B and Fig. 7B). Effective separation of the cytoplasmic and nuclear fractions was verified by immunoblotting

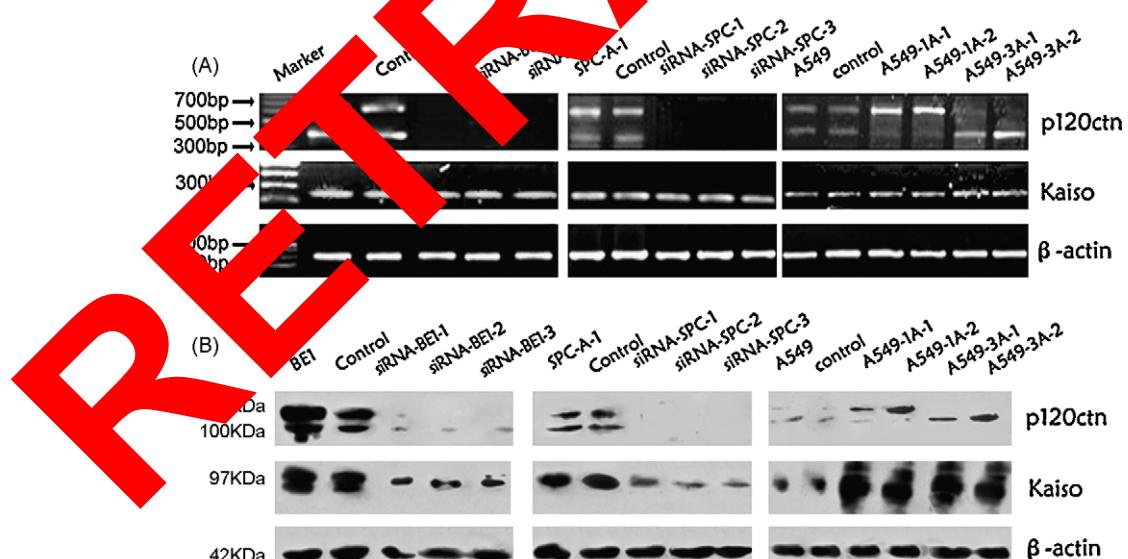


Fig. 5. Kaiso protein expression was regulated by p120ctn in three lung cell lines. (A) RT-PCR analysis indicates that mRNA expression of p120ctn produced several specific bands of 586 bp and 370 bp, which correspond to isoforms 1.3 and 3.1, respectively, in BE1 cells, SPC cells, A549 cells and control cells, while no corresponding bands were detected in siRNA-BE1-1, siRNA-BE1-2 and siRNA-BE1-3 cells, as well as siRNA-SPC-1, siRNA-SPC-2 and siRNA-SPC-3 but enhanced specific bands of 586 bp and 370 bp were detected in A549-1A-1, A549-1A-2 and A549-3A-1, A549-3A-2, respectively. Nevertheless, the mRNA expression of Kaiso (248 bp) showed no obvious alterations in any of these cells. The band positions are indicated on the left with molecular weight markers. (B) Expression of p120ctn and Kaiso protein in siRNA-p120ctn and overexpression-p120ctn cells. Following stably transfection with p120ctn-siRNA-1, p120ctn-siRNA-2 and p120ctn-siRNA-3, the BE1 cells and SPC cells showed a reduction or the complete absence of all p120ctn isoforms. Accordingly, Kaiso protein also was reduced significantly in these cells (lanes 3, 4 and 5, as well as lanes 8, 9 and 10). And the control cells which were transfected with scrambled p120ctn-siRNA plasmids, showed no obviously p120ctn and Kaiso protein change (lanes 2 and 7). Protein bands representing p120ctn isoforms were low in A549 and control cells (lanes 11 and 12). After stably transfected with either p120ctn isoform 1A or 3A, A549 cells showed enhanced expression, respectively, at approximately 120 kDa (isoform 1, lanes 13 and 14) and 100 kDa (isoform 3, lanes 15 and 16). Kaiso (approximately 97 kDa) protein levels remarkably increased in p120ctn-1A-transfected and p120ctn-3A-transfected cells compared to A549 cells, control cells. The band positions are indicated on the left with molecular weight markers.

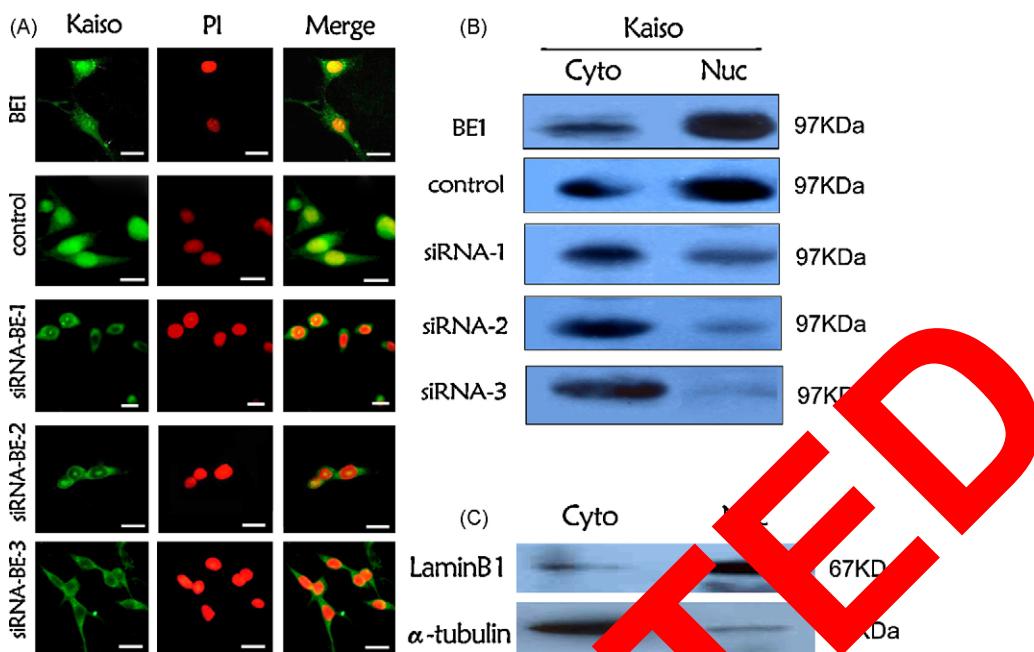


Fig. 6. p120ctn depletion alters Kaiso subcellular localization in BE1 cell lines. (A) In BE1 and control cells, Kaiso was distributed diffusely in the nucleus, with less visible staining in the cytoplasm. In siRNA-BE1-1, siRNA-BE1-2 and siRNA-BE1-3 cells, Kaiso staining in the nucleus significantly increased and no longer diffuse but aggregated or even invisible. Bar, 18.75 μm. (B) Cytoplasmic vs. nuclear fractionation Western blot analysis confirmed the results of immunofluorescence staining. After p120ctn depletion, protein bands representing Kaiso in nuclear extract significantly decreased in siRNA-BE1-1, siRNA-BE1-2 and siRNA-BE1-3 cells. (C) To verify effective separation of the cytoplasmic and nuclear fractions, cytoplasmic and nuclear extracts were immunoblotted for α-tubulin and Lamin B1.

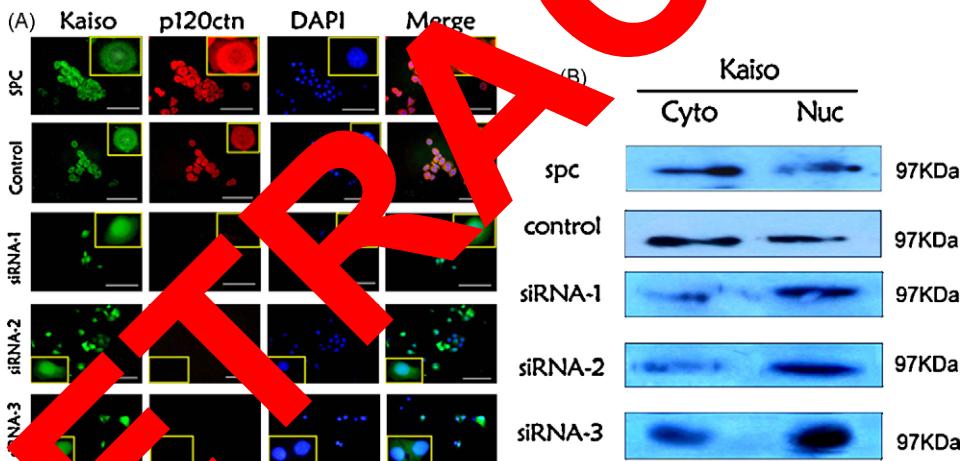


Fig. 7. p120ctn depletion alters Kaiso subcellular localization in SPC cell lines. (A) In SPC and control cells, Kaiso was distributed in the cytoplasm, with less visible staining in the nucleus. In siRNA-1, siRNA-2 and siRNA-SPC-3 cells, Kaiso staining in the nucleus significantly increased. Bar, 18.75 μm. (B) Cytoplasmic vs. nuclear fractionation Western blot analysis confirmed the results of immunofluorescence staining. After p120ctn depletion, protein bands representing Kaiso in nuclear extract significantly increased in siRNA-SPC-1, siRNA-SPC-2 and siRNA-SPC-3 cells.

each fraction was α-tubulin, a cytoplasmic protein, and Lamin B1, a nuclear protein. Fig. 6C shows that each fraction was only a little of contamination, as α-tubulin and Lamin B1 were detected only in the cytoplasmic and nuclear fractions, respectively.

After p120ctn 1A or 3A stable overexpression, more Kaiso can be observed in both cytoplasm and nucleus by immunofluorescence (Fig. 8A). In p120ctn-3A cells, Kaiso and p120ctn even co-localized at the site of cell-cell contacts (Fig. 8A). As shown in Fig. 8B, more Kaiso can be coprecipitated by p120ctn mAb 15D2, which recognize all isoforms of p120ctn, from cytoplasmic and nuclear extract of A549, A549-1A and A549-3A cells. But difference existed between p120ctn-1A and p120ctn-3A cells. In p120ctn-1A cells, although more Kaiso can be coprecipitated from both cytoplasmic and nuclear extract, significant difference

only found in cytoplasmic extract. As for p120ctn 3A overexpression A549 cells, significant difference existed in both cytoplasmic and nuclear extract. Besides, p120ctn mAb 6H11, which recognize only the p120ctn isoform 1, did not coprecipitate Kaiso. These results demonstrated that Kaiso interacts only with p120ctn isoform 3.

4. Discussion

Kaiso is a nuclear protein that plays a role in transcription repression [23–25,37,38]. It belongs to the BTB/POZ (Broad Complex, Tramtrak, Bric a' brac/Pox virus and Zinc finger) family of proteins [39]. BTB/POZ proteins contain an amino-terminal BTB/POZ domain that engages in protein–protein interactions (dimerization and corepressor interactions) and a carboxyl-

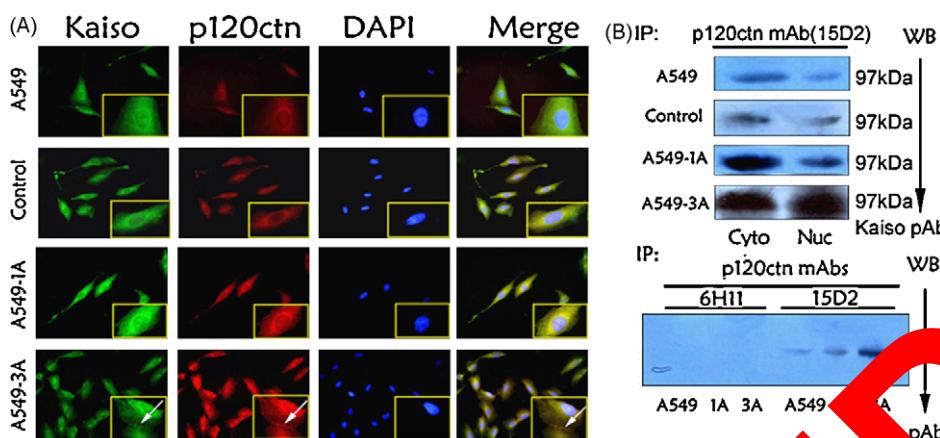


Fig. 8. p120ctn 1A and 3A overexpression alter Kaiso subcellular localization in A549 cell lines. (A) Kaiso and p120ctn were predominantly cytoplasmic co-localization in A549, A549-1A and A549-3A cells. In p120ctn 1A and 3A stable overexpression A549 cells, p120ctn (red) and Kaiso (green) staining were more diffuse, not only in cytoplasm but also in nucleus. As for p120ctn isoform 3A-transfected A549 cells, Kaiso and p120ctn even co-localized at the site of cell-cell contacts (white arrow). (B) Upper, More Kaiso was coprecipitated by p120ctn mAb (15D2) from cytoplasmic and nuclear extract of A549-1A and A549-3A cells than from A549 cells. In p120ctn 1A overexpression A549 cells, although more Kaiso can be coprecipitated from both cytoplasmic and nuclear extract, significant difference was found in cytoplasmic extract. As for p120ctn 3A overexpression A549 cells, significant difference existed in both cytoplasmic and nuclear extract, compared to A549 cells. Lower, p120ctn mAb 6H11, which recognize only the p120ctn isoform 1, did not coprecipitate Kaiso. p120ctn mAb 15D2, which recognize all isoforms of p120ctn, coprecipitated Kaiso from whole cell lysates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

terminal (C_2H_2) zinc finger domain responsible for DNA association [39,40]. Among the BTB/POZ proteins, Kaiso appears to be unique in possessing dual specificity, recognizing both sequence-specific consensus sites and methylated CpG dinucleotides [21,41–43].

In this study, we found that cytoplasmic Kaiso expression correlate with ectopic cytoplasmic p120ctn expression in lung cancer by immunohistochemistry. Next, co-immunoprecipitation assay clearly confirmed that Kaiso and p120ctn coassociated with each other in cytoplasm of lung cancer cells. Immunohistochemical study demonstrated that all 13 lung cancer tissues utilized in the coimmunoprecipitation assay only with synchronistically cytoplasmic Kaiso and p120ctn expression. No nuclear staining was observed in these tissues. This is apparently the only report directly reveals Kaiso–p120ctn complex exists in cytoplasm.

The relationships between the expression of p120ctn and Kaiso, as well as their correlation with clinical and pathological factors of cancer, are poorly defined [20,45]. Herein, we found that Kaiso cytoplasmic expression and p120ctn abnormal expression were more apparent in tumors with high degree of malignancy (high-stage, nodal metastasis) and significantly correlated with poor prognosis of the patients. Moreover, immunohistochemical results of 55 paired cases of lung cancer demonstrated that abnormal p120ctn expression and cytoplasmic Kaiso expression were significantly higher in lymph node metastasis than that in primary cancer. In addition, multivariate analysis reveals that abnormal p120ctn expression is the major and independent factors defining the clinicopathological characters of patients while cytoplasmic Kaiso may be an independent factor affecting prognosis, with a P -value of 0.062. It seems important to collect more patient follow-up records to clarify this correlation between Kaiso expression and a patient's clinical response. Kaiso may exert an oncogenic function in the cytoplasm of lung cancer cells. It may therefore be worthwhile in the future to analyze the effect of Kaiso on cell proliferation and transformation by Kaiso overexpression or knockdown. Our p120ctn knockdown results obtained from SPC cells (siRNA-SPC) showed that p120ctn ablation increased Kaiso localization in nucleus, suggesting p120ctn takes part in Kaiso export from nucleus. It has been reported that no nuclear export signals (NES) existed in Kaiso while two NES presented in p120ctn [46]. Considering the close relationship between Kaiso and p120ctn,

it is plausible that p120ctn participates in Kaiso exit from the nucleus. A functional nuclear localization signal (NLS) is present in the C-terminal of the zinc finger domain in Kaiso and Kaiso enters the nucleus by the classical importin- α/β pathway [26]. However, it is still unknown whether p120ctn plays a role in the entry of Kaiso into the nucleus. Our data obtained from p120ctn knockdown BE1 cells (siRNA-BE1) suggested that p120ctn could assist the entry of Kaiso into the nucleus. We also noticed that results got from these two cell lines were somewhat contradictory. This discrepancy may be due to different cell lines employed in this study, most likely the initial p120ctn levels. As shown in Fig. 5B, BE1 cell lines express high levels of p120ctn (more isoform 1 than isoform 3) and SPC cell lines express middle levels of p120ctn (more isoform 3 than isoform 1). The details still need us to address in our future research.

The results got from stably overexpressed p120ctn isoforms 1A and 3A cells (designated A549-1A and A549-3A cells) demonstrate that both of these two isoforms overexpression could increase Kaiso expression in cytoplasm and nucleus. But difference also can be found, p120ctn isoform 3 seems exert more effort on Kaiso's subcellular localization. Our binding studies further suggest that only p120ctn isoform 3 directly binds to Kaiso. This result consistent with report by Daniel and Reynolds [20]. In that study, they found that Kaiso might only directly bind with p120ctn isoform 3 but did not absolute exclude the possible binding between Kaiso and p120ctn isoform 1, for cell lines employed in their study express very low levels of p120ctn isoform 1. This possible interaction can be eliminated by our result of co-immunoprecipitation assay, for p120ctn isoform 1 had been significantly upregulated in A549-1A cells. How p120ctn isoform 1 affects the localization of Kaiso, although they did not directly interact, is an interesting problem to clarify.

Our study showed that p120ctn depletion or overexpression did not influence Kaiso transcription, but did influence Kaiso protein expression. These results demonstrate that p120ctn only regulates Kaiso at the protein level, rather than by affecting Kaiso gene transcription. Park et al. [47] have demonstrated that Frodo depletion (Frodo-morpholino [Frodo-MO] injection) resulted in significantly decreased p120ctn levels, while, in contrast, the amount of Kaiso protein remained unchanged. This apparently contradictory findings of two different studies could be explained by the fact that,

in the Jae-il Park study, *Xenopus* embryos were used rather than cancer cell lines and the regulation mechanism of p120ctn on Kaiso might not be the same under physiological/pathological conditions [44].

5. Conclusion

Our data showed that abnormal p120ctn expression and cytoplasmic Kaiso expression significantly correlated to higher degree of malignancy (high-stage, nodal metastasis) and poor prognosis of the NSCLC patients. Abnormal p120ctn expression is the major and independent factor defining the clinicopathological characters of patients. Kaiso–p120ctn complex exists in cytoplasm of lung cancer cells. Although both p120ctn isoforms can regulate subcellular localization and protein levels of Kaiso, only p120ctn isoform 3, but not isoform 1, directly interacts with Kaiso.

Conflict of interest statement

None of the authors of this manuscript have a financial interest related to this work.

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