The close association between IL-12Rβ2 and p38MAPK, and higher expression in the early stages of NSCLC, indicates a good prognosis for survival

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Abstract. Interleukin-12 receptor (IL-12R) and p38 mitogen-activated protein kinase (p38MAPK) serve an important role in non-small cell lung cancer (NSCLC). It has previously been suggested that IL-12Rβ2 may be involved in key regulatory pathways and interacts with the p38MAPK signaling pathway. The present study aimed to elucidate the possible association and roles of IL-12Rβ2 and p38MAPK in NSCLC. The protein expression levels of IL-12Rβ2 and p38MAPK were measured in 230 NSCLC tissue samples by immunohistochemistry (IHC) and western blot analyses. In addition, an immunofluorescence assay was used to observe the expression levels of these proteins in A549 and H358 cells. The associations between IL-12Rβ2, p38MAPK and clinical characteristics, were evaluated by Pearson χ^2 and Spearman correlation tests. Kaplan-Meier plots (log-rank test) and Cox proportional hazard models were used to analyze overall survival (OS). Compared with in benign pulmonary tissues, the expression levels of IL-12Rβ2 and p38MAPK were not demonstrated to be significantly different in I+II pathological tumor-node-metastasis (pTNM) stage NSCLC tissues; however, reduced expression was detected in III+IV pTNM stage NSCLC tissues. Analysis of the association between advanced stage pTNM and the expression of both proteins demonstrated a significantly decreased Allred score (both P<0.0001), which was confirmed by IHC and western blot analyses. The IHC results demonstrated a significant correlation between IL-12Rβ2 and p38MAPK expression (r=0.415, P=0.0143). By analyzing IL-12Rβ2, p38MAPK expression and clinical characteristics, it was identified that IL-12Rβ2 was significantly associated with gender (P=0.0168), age (P=0.0341), histological type (P<0.0001) and pTNM stage (P<0.0001). p38MAPK demonstrated a strong association with gender (P=0.0082) and pTNM stage (P<0.0001). The results of a Kaplan-Meier analysis indicated that positive IL-12Rβ2 and p38MAPK expression was associated with increased OS compared with negative protein expression. The Cox proportional hazard models revealed that IL-12Rβ2 and p38MAPK predicted a long OS. To the best of our knowledge, the present study is the first to reveal a close association between IL-12Rβ2 and p38MAPK, and their possible function in NSCLC progression. It further demonstrated that expression of both proteins was lower with advanced pTNM staging, whereas a high expression of both proteins was associated with improved prognosis in NSCLC.

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Introduction

Lung cancer is a very common and highly lethal malignant tumor worldwide (1,2). Non-small cell lung cancer (NSCLC) is a major type of lung cancer. As a result of its complex tumorigenesis and other mechanisms, it is exceedingly difficult to treat and predict the prognosis of NSCLC. In order to identify therapeutic targets and prognostic biomarkers, numerous studies regarding NSCLC have focused on specific key molecules, particularly growth factor receptors, including epidermal growth factor receptor and insulin-like growth factor 1 receptor (3,4). Previous studies have indicated that interleukin (IL)-12 may be involved in specific regulatory pathways and may serve vital biological roles via the cross-interaction between interstitial inflammatory factors and IL-12 receptors (IL-12Rs); therefore, it may be considered a target for lung cancer treatment (5,6).

It is well known that the biological functions of human IL-12 are mediated by IL-12Rs, which are composed of two subunits, the β1 and β2 chains, which confer high-affinity binding and responsiveness to IL-12. The β2 chains may be considered the main molecules encoding the IL-12R chain, and IL-12Rβ2 is essential for IL-12 signaling transduction and functions as a tumor suppressor (7,8). In previous studies (9,10), the roles of IL-12Rβ2 were investigated and IL-12Rβ2-deficient mice

were demonstrated to develop lung adenocarcinoma. However, the mechanism by which IL-12Rβ2 acts is unclear; it has been reported to be potentially associated with certain immunological factors or cells, including interferon-γ or natural killer cells (9-11). In addition, other studies have identified that by binding to IL-12Rs, including IL-12Rβ2, IL-12 activates downstream molecules or interstitial inflammatory factors and regulates NSCLC progression (11-14).

p38 mitogen-activated protein kinase (p38MAPK) can be activated by various environmental insults and inflammatory cytokines, and controls specific cell functions, including the cell cycle, apoptosis and proliferation (15-17). It has been demonstrated that activation of p38MAPK in multiple myeloma promotes destructive osteoclast differentiation and recruitment (18). Furthermore, p38MAPK has been identified as a regulator of dickkopf-related protein 1 expression (18,19). In various tumor types, including prostate and breast cancer, p38MAPK activity is deregulated and it has been reported to present both tumorigenic and tumor-suppressor roles (20). With respect to lung cancer, certain studies (8,13,14) have demonstrated that IL-12 can activate downstream signaling pathways via specific molecular interactions, including with p38MAPK, and it is possible that further interactions exist between p38MAPK and IL-12.

In the present study, the expression and distribution of IL-12Rβ2 and p38MAPK were analyzed, and their association was observed via western blotting, immunohistochemistry (IHC) and immunofluorescence (IF). Furthermore, through Kaplan-Meier and Cox proportional hazard models, the association between these proteins and overall survival (OS) was observed. The data from the present study provided novel insights into interpreting the mechanisms underlying NSCLC.

Materials and methods

Human tissue samples and antibodies. The present study was approved by the medical ethics committee of First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). A total of 230 patients with NSCLC that underwent thoracic surgical procedures at the General Thoracic Surgery Department of First Affiliated Hospital, Sun Yat-sen University were recruited to the present study between April 2007 and October 2016. A total of 72 benign pulmonary (BPL) tissue samples (Of the 72 specimens from the paraplastic lung tissue distant from the tumor tissue >5 cm, 42 were male, 30 were female) and 102 NSCLC frozen tissue samples (Of the 102 subsequent frozen specimens from April 2016 and October 2016, there were 60 males and 42 females (74 cases of lung adenocarcinoma, 28 squamous cell carcinoma) were also collected between April 2016 and October 2016. Written informed consent was obtained from all patients. None of the patients included in the present study received radiotherapy or chemotherapy. The relevant clinicopathological data were collected including age, gender, tumor node metastasis (TNM) staging, smoking index (Smoking index=number of cigarettes per day and number of years of smoking), histological type and OS, as illustrated in Table I. Anti-human IL-12Rb2 (cat. no. ABIN1828221; Shanghai univ-bio Co., Ltd., Shanghai, China) and anti-p38MAPK (cat. no. AN1020; Abgent, Inc., San Diego, CA, USA) antibodies were demonstrated to be highly specific in IHC, WB and IF assays.

IHC. Following dewaxing and hydration and antigen retrieval: All tissues placed 4% polyformaldehyde (pH 7.4), fixed for 12 h in the refrigerator at 4 C, embedded in paraffin and made of continuous slice (4 um), and then randomly sampled from the front and back with equidistance. Each specimen was extracted with 10 slices. The slices were dewaxing for 10 min x 2 times in xylene and hydrated in the gradient concentration alcohol (100% alcohol I→100% alcohol II→95% alcohol I→95% alcohol II, each 5 min, 90% alcohol →80% alcohol, each 3 min, 70% alcohol, 2 min). The antigen was repaired, and the pre-prepared antigen retrieval solution was heated to 95 C in the water bath, and the slice was immersed into the 0.01M citrate buffer solution (pH 6.0) repair solution, and 5 min was carried out. After the repair, the slice was removed, the temperature was cooled at room temperature for 40 min, and then 0.01M PBS wash, 5 min 3 times. The rest is described as IHC steps in the method. Subsequently, sections were treated with 3% hydrogen peroxide for 15 min to inactivate endogenous enzymes and blocked in 5% bovine serum albumin (BSA, 36101ES25, YE SEN Bio Technology Co., Ltd., ShangHai, China) for 20 min at room temperature. Sections were then incubated with the following primary antibodies: Anti-IL-12Rβ2 (1:100) and anti-p38MAPK (1:250) overnight at 4˚C. Negative control staining consisted of sections incubated with PBS instead of primary antibodies. Sections were then washed in PBS and incubated with secondary antibodies (A0277, Biotinlabeled Goat AntiRabbit IgG (H+L), 1:100; Beyotime, Shanghai, China) at room temperature for 20 min. After further washing in PBS, sections were treated with a streptavidin-biotin complex (xy-PRO-283; X-Y Biotechnology, Shanghai, China; shxysw.biomart.cn) at room temperature for 20 min, washed in PBS and treated with 3,3'-diaminobenzidine (DAB). Following dehydration, clearing and mounting, observation was performed under a light microscope.

IHC evaluation. The results of the IHC were evaluated in a double-blind manner. Cells that were positive for IL-12Rβ2 and p38MAPK exhibited red-brown or brown cellular granules. To count the positive cells, 10 fields of view were randomly selected at a magnification of x200, and 100 cancer cells were counted in each field (a total of 1,000 cells). Under a light microscope, the product of the staining intensity score and the proportion of positive cells was used as the end standard score. The percentage of positive cells was recorded as follows: ≤20%, 1; 20-50%, 2; 50-75%, 3 and >75%, 4. The staining intensity scores were recorded as follows: Negative staining, 1; weak staining, 2; moderate staining, 3; and strong staining, 4. These two results were then multiplied to obtain the Allred score (1 to 16), and negative (-), 1-4; (+), 5 to 8; (++), 9 to 12; (+++), 13 to 16.

Western blot analyses. Proteins were extracted from frozen lung cancer tissues in 100 mmol/l Tris (pH 7.5), 300 mmol/l NaCl, 4 mmol/l EDTA, 2% NP40, 0.5% Na deoxycholate and 1 mmol/l sodium orthovanadate. The protein samples were quantified by BCA, Laemmli buffer (3 ul) was added to 10 μg protein/lane and the samples were boiled for 5 min. Proteins were resolved by SDS-PAGE (4-15% gradient polyacrylamide gels) and transferred onto a Hybond-ECL membrane. Membranes were blocked at room temperature for 2 h and antibodies were diluted in PBS containing 5% milk and 0.1% Tween 20 and were washed in PBS

Table I. Association between IL-12Rβ2 and p38MAPK, and clinicopathological parameters.

	IL-12Rβ2			p38MAPK		
Variable	-	+	P-value	-	+	P-value
Sex						
Male	59	53	0.0168	65	47	0.0082
Female	43	75		47	71	
Age (years)						
<55	56	52	0.0341	56	52	0.4280
≥55	46	76		56	66	
Smoking index						
<400	65	66	0.0813	70	61	0.1108
≥400	37	62		42	57	
Histological type						
SCC	70	53	<0.0001	62	61	0.5989
AC	32	75		50	57	
TNM staging						
IA-IIB	22	73	<0.0001	28	67	<0.0001
IIIA-IV	80	55		84	51	

AC, adenocarcinoma; IL-12R, interleukin-12 receptor; p38MAPK, p38 mitogen associated protein kinase; SCC, squamous cell carcinoma; TNM, tumor node metastasis.

containing 0.1% Tween 20. The membranes were incubated at 4˚C for 20 h with anti-IL-12Rβ2 (1:300), anti-p38MAPK (1:1,000) and mouse anti-β-actin (clone AC-15; 1:10,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Following further washing, blots were incubated with horseradish peroxidase-conjugated goat anti mouse immunoglobulin G (cat. no. 20060105; Beijing Bayer Corporation, BeiJing, China; 1:1,000). Gelpro 32 analysis was performed for gel image analysis and download from internet (www.bioon.com/Soft/Class1/Class16/200408/155.html).

Cell culture. Human NSCLC cell lines, H358 and a549 were obtained from shanghai institute of cell biology. They were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, 10099141, Thermo Fisher scientific, Inc.) at 37˚C in humidified atmosphere of 5% CO_2.

IF. A total of 5 x10^4/ml cells (H358 and A549 cells) were fixed in 4% paraformaldehyde for 20 min and permeabilized in 0.5% Triton X-100 at room temperature for 20 min. Cells were then incubated with anti-IL-12Rβ2 (1:50) and anti-p38MAPK (1:250) in 10% sheep serum No: 005-000-121, Amyjet scientific, WuHan, China; amyjet.bioon.com.cn) overnight at 4˚C, washed with PBS and incubated with GY3-conjugated anti-mouse immunoglobulin G (1:100; Abcam, Cambridge, UK) for 1 h at 20˚C. Subsequently, cells were washed with PBS and counterstained with DAPI for 4 min at 4˚C. A fluorescence microscope (Leica DM1000; Leica Microsystems GmbH, Wetzlar, Germany) was used for examination.

Statistical analysis. Graphpad Prism6.0 was used for analysis. Continuous variables (WB results) were compared among the groups using the Kruskal-Wallis test, with a post hoc Mann-Whitney U test and Bonferroni correction, and the Pearson χ^2 test was used to analyze categorical variables. The correlation of protein expression was determined using Spearman correlation tests. OS curves were calculated using Kaplan-Meier analyses. Univariate survival analysis was conducted with the log-rank test and Cox proportional hazards regression which were used to assess the prognostic power of these parameters. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of IL-12Rb2 and p38MAPK in NSCLC. In the IHC analysis, overexpression of IL-12Rβ2 and p38MAPK proteins was detected in BPL tissues and early pTNM stage NSCLC. In H358 and A549 cells the expression of two proteins was also observed (Figs. 1A and 2).

The expression levels of IL-12Rβ2 and p38MAPK were markedly decreased in pTNM stage III+IV NSCLC tissues compared with in the BPL and pTNM I+II stage NSCLC tissues, as determined by IIHC and western blotting (P<0.05) (Fig. 1A and B). In addition, with increasing pTNM stage the Allred scores of IL-12Rβ2 and p38MAPK expression were significantly decreased (Table II). The number of cases (Patients with IL-12Rβ2 or p38MAPK positive expression) with positive expression (Allred score-vs. +, ++ and +++ as cut-off for negative/positive expression). Of IL-12Rβ2 and p38MAPK was decreased (both P<0.0001; Table II). Further analysis via Spearman's correlation analyses demonstrated a significant correlation between IL-12Rβ2 and p38MAPK (r=0.415, P=0.0143; Table III).

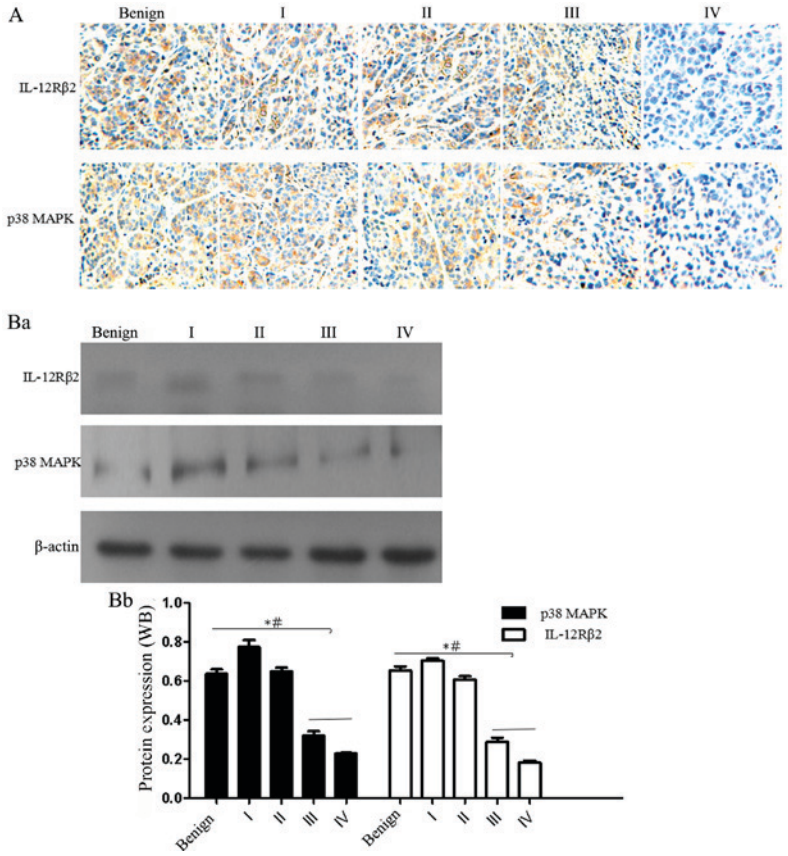

Figure 1. Expression of IL-12Rb2 and p38MAPK in NSCLC and BPL tissues, as determined by IHC and western blotting. The immunopositive signal of each protein was graded according to the Allred scoring system. Only an Allred score greater than the cut-off point was considered positive. (A) BPL and NSCLC tissues (TNM stage I-IV) were analyzed by IHC (streptavidin-biotin complex; magnification, x200). (Ba) IL-12Rb2 and p38MAPK expression was detected in BPL and NSCLC frozen tissues using western blotting. As TNM stage advanced, protein expression levels were gradually reduced. (Bb) Semi-quantification of western blotting. Compared with in III+IV stage tissues, protein expression levels were significantly increased in BPL and I+II stage tissues. *P<0.05 vs. benign, #P indicates P<0.05 vs. I+II BPL, benign pulmonary; IHC, immunohistochemistry; IL-12R, interleukin-12 receptor; NSCLC, non-small cell lung cancer; p38MAPK, p38 mitogen-activated protein kinases; TMN, tumor-node-metastasis.

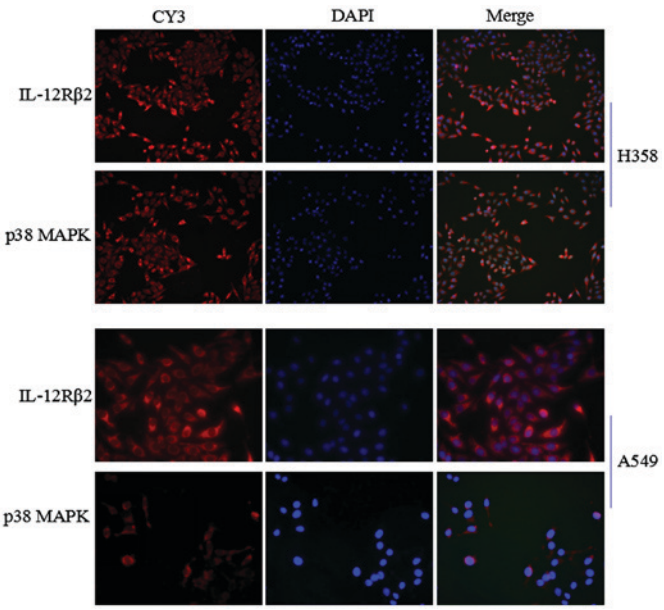

Figure 2. Expression of IL-12Rb2 and p38MAPK in NSCLC cells, as determined by IF. IF was performed in the H358 cell line and was confirmed in the A549 cell line. GY3-conjugated anti-mouse immunoglobulin G was used as a secondary antibody. Both proteins were expressed and distributed in the cytoplasm (magnification, x200). IF, immunofluorescence; IL-12R, interleukin-12 receptor; NSCLC, non-small cell lung cancer; p38MAPK, p38 mitogen-activated protein kinases.

Association between IL-12Rb2 and p38MAPK expression, and patient clinical characteristics. The association between IL-12Rβ2 and p38MAPK expression, and a range of standard clinicopathological parameters were measured. Using the χ^2 test, significant associations between gender (P=0.0168), age (P=0.0341), histological type (P<0.0001), TNM staging (P<0.0001) and IL-12Rβ2 expression were demonstrated (Table I). Similar results were observed when the associations between p38MAPK expression and clinicopathological factors were analyzed. For example, gender (P=0.0082) and TNM staging (P<0.0001) were significantly associated with p38MAPK expression (Table I). In addition, with advanced TNM staging, the expression of both proteins was significantly decreased, particularly in the III+IV stage tissues compared with in the I+II and BPL tissues (P<0.0001; Fig. 1B; Tables I and II).

Correlation analysis between IL-12Rb2, p38MAPK and OS. The Kaplan-Meier method was used to investigate the association between patient survival and IL-12Rβ2- and p38MAPK-positive expression compared with negative expression in a univariate model. The results indicated that IL-12Rβ2- and p38MAPK-positive expression were significantly associated with longer OS (IL-12Rβ2: Log-rank, 5.203 and P=0.0016; p38MAPK: Log-rank, 4.503 and P=0.0040; Fig. 3 and Table IV). In addition, a similar result was observed

Table II. Association between IL-12Rβ2 and p38MAPK expression, and TNM stage.

Variable	IL-12Rβ2				p38MAPK			
Allred score	I	II	III	IV	I	II	III	IV
-	10	12	30	50	13	15	30	54
+	6	12	24	9	6	11	22	7
++	12	16	9	3	11	18	14	3
+++	14	13	5	3	12	9	4	1
Positive (%)	76.19	77.35	55.88	23.07	69.04	71.69	57.14	16.92
χ^2	67.49				65.22			
P-value	<0.0001				<0.0001			

IL-12R, interleukin-12 receptor; p38MAPK, p38 mitogen-activated protein kinases; TMN, tumour-node-metastasis.

Table III. Spearman correlation analysis between IL-12Rb2 and p38MAPK expression.

Comparison	Patients (n)	r	P-value
IL-12Rβ2 vs. p38MAPK	230	0.415	0.0143

IL-12Rβ2, interleukin-12 receptor; p38MAPK, p38 mitogen activated protein kinase.

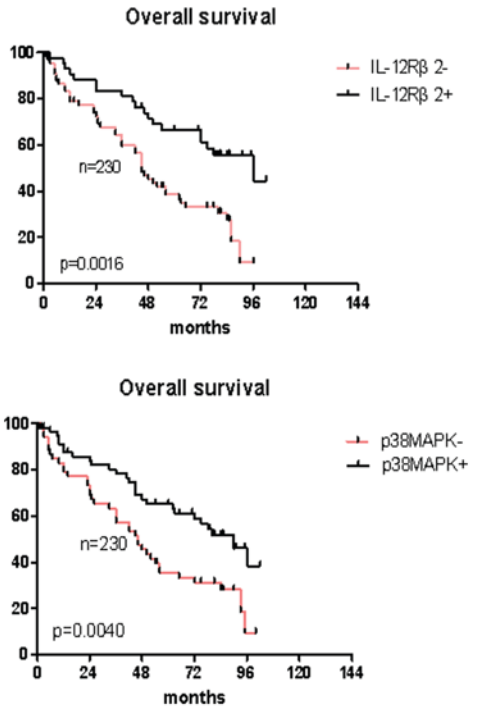

Figure 3. Evaluation of IL-12Rb2 and p38MAPK expression as predictors for OS, as determined by Kaplan-Meier analysis. These proteins were associated with a good prognosis; positive expression of IL-12Rβ2 and p38MAPK was associated with the longest OS. IL-12R, interleukin-12 receptor; OS, overall survival; p38MAPK, p38 mitogen-activated protein kinases.

from the TNM stage analysis (log-rank, 5.565 and P=0.0021; Table IV).

A Cox regression model was performed to determine whether IL-12Rβ2 and p38MAPK are significant, independent prognostic factors. The analysis demonstrated that high expression of IL-12Rβ2 (P=0.0221) and p38MAPK (P=0.0457), as well as TNM stage (P=0.0203), were significant, independent prognostic factors (Table IV).

Discussion

Lung carcinoma is a frequent, lethal malignancy in humans worldwide (21,22). NSCLC is an important type of lung cancer, which comprises two major categories: Lung squamous cell carcinoma and adenocarcinoma. Previous studies have focused on IL-12 in NSCLC; however, the current understanding of the mechanism underlying IL-12R binding to IL-12, and the activated downstream signaling pathways, is incomplete and a number of uncertainties remain (23,24). The present study aimed to elucidate the expression and roles of IL-12Rβ2, and its possible association with p38MAPK, in NSCLC via IHC, WB and IF analyses.

In previous studies (8,9), investigations have been conducted regarding the role of IL-12Rβ2, and notable results have been obtained regarding the development of lung adenocarcinoma in IL-12Rβ2-deficient mice. However, the mechanism by which IL-12Rβ2 performs its' roles remains unclear. In the present study, similar analyses demonstrated that IL-12Rβ2 was overexpressed in BPL and early TNM stage tissues, according to IHC and western blot analyses; the results confirmed that IL-12Rβ2 may be negatively correlated with NSCLC progression, which is consistent with the results of previous studies (7-10) and indicated that IL-12Rβ2 may be involved in complex cellular biological functions, including proliferation, apoptosis and metastasis. Through further analyses, a close correlation was demonstrated between IL-12Rβ2 and p38MAPK expression, thus suggesting that the mechanism by which IL-12Rβ2 acts in NSCLC is closely associated with the p38MAPK signaling pathway.

The results of the IHC analysis revealed that a close correlation may exist between IL-12Rβ2 and p38MAPK expression, according to the Spearman's correlation test, which is consistent with the previous literature (25). In addition, IL-12Rβ2 and

Table IV. Kaplan-Meier and Cox multivariate proportional hazard analysis.

	Univarivate analysis		Multivariate analysis	
Factor	Log-rank	P-value	Hazard ratio (95%)	P-value
Age (years)				
<55	2.014	0.1302		
≥55				
Sex				
Male	1.245	0.4521		
Female				
IL-12Rβ2				
Negative	5.203	0.0016	4.32 (3.02-6.33)	0.0221
Positive				
p38MAPK				
Negative	4.503	0.0040	3.59 (1.03-5.28)	0.0457
Positive				
pTNM stage				
I-II	5.565	0.0021	4.03 (2.26-6.74)	0.0203
III-IV				

IL-12R, interleukin-12 receptor; p38MAPK, p38 mitogen associated protein kinase; pTNM, pathological tumor-node-metastasis.

p38MAPK were identified as not only being overexpressed in early NSCLC but were also associated with TNM stage and gender; furthermore, it was demonstrated that compared with in stage III+IV NSCLC tissues, the number of cases in which both proteins were positively expressed was increased in stage I+II and BPL tissues. Similar results were also observed via western blotting; protein expression was decreased with increasing TNM stage, particularly at the III+IV stage compared with the I+II stage, thus indicating that the expression of these proteins is negatively associated with the TNM stage, which is in agreement with previous studies on IL-12Rβ2 (7-9,26-28). Notably, differential expression of IL-12Rβ2 and p38MAPK was detected between the genders. The number of cases of positive expression was increased in female patients for both IL-12Rβ2 (female; 61.86%, 75 out of 118) and p38MAPK (female; 60.16%, 71 out of 118) compared with in male patients. In addition, differences between IL-12Rβ2, and tumor types and age were observed; inconsistencies in IL-12Rβ2 expression between patients of different ages, tumor types and gender may imply certain hypotheses e.g., whether IL-12Rβ2 affects tumor progression through the endocrine system, immune environment or intracellular environmental factors. In addition, the present study revealed that IL-12Rβ2- and p38MAPK-positive expression may be strong predictors of OS in NSCLC. The results of a Kaplan-Meier analysis demonstrated that the positive expression of IL-12Rβ2 and p38MAPK was associated with a good OS. In addition, a Cox regression model was performed; both proteins were confirmed as being independent prognostic factors of survival. Based on these results, the correlation between the expression of both proteins, their associations with tumor characteristics (i.e., TNM staging and OS) and the results of previous studies, a close association may exist between IL-12Rβ2 and p38MAPK, which may indicate the presence of cross-talk between IL-12Rβ2 and p38MAPK signaling mechanisms and raise the question as to whether IL-12Rβ2 influences NSCLC via the p38MAPK signaling pathway.

The results of the present study confirmed that IL-12Rβ2 and p38MAPK may be key signaling molecules with important biological functions. However, the biological mechanisms of IL-12Rβ2 are complex and there is a lack of understanding about how these two proteins interact in NSCLC. Although the mechanisms underlying the interaction between these proteins remain unclear, the effects of IL-12Rβ2 on p38MAPK expression is interesting in NSCLC and may imply possible novel methods of diagnosis and treatment in NSCLC.

In conclusion, the correlation between IL-12Rβ2 and p38MAPK expression may help to further explain the mechanisms underlying the effects of IL-12Rβ2 on NSCLC, which may possibly be regulated by p38MAPK. The results of the present study demonstrated that IL-12Rβ2 and p38MAPK may be prognostic factors for survival in NSCLC. Although the detection of IL-12Rβ2 and p38MAPK expression has improved the understanding of the roles of IL-12Rβ2 to a certain extent, several uncertainties remain; in particular, how IL-12Rβ2 exerts its' biological effects via p38MAPK. Therefore, *in vitro* and *in vivo* experiments are required, which will be the focus of future experiments.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZL designed the study, selected surgical specimens and wrote the manuscript. WY performed the statistical analysis and the IF assay. SY performed the western blotting and IHC assay. KC wrote the manuscript, participated in the control of clinical cases and experimental process quality, and coordinated all experimental progress.

Ethics approval and consent to participate

The present study was approved by the medical ethics committee of First Affiliated Hospital, Sun Yat-sen University (Guangzhou, China). Written informed consent was obtained from all patients.

Patient consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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