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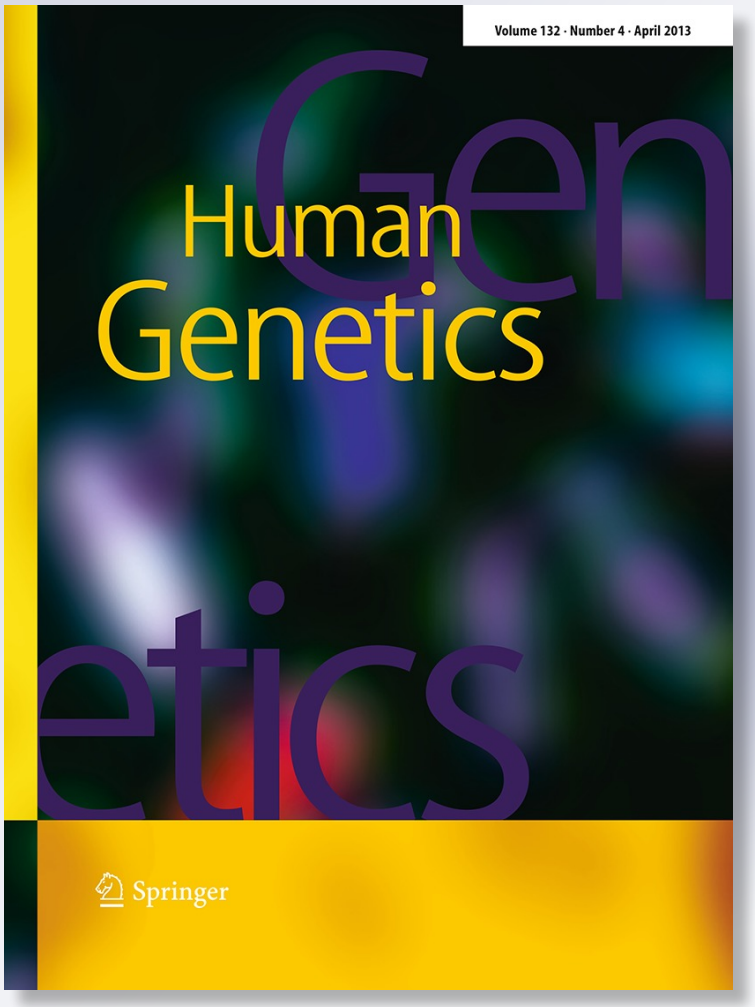

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ORIGINAL INVESTIGATION

Functional polymorphisms in *NFκB1/IκBα* predict risks of chronic obstructive pulmonary disease and lung cancer in Chinese

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Abstract Lung inflammation is the major pathogenetic feature for both chronic obstructive pulmonary disease (COPD) and lung cancer. The nuclear factor-kappa B (NFκB) and its inhibitor (IκB) play crucial roles in inflammatory. Here, we tested the hypothesis that single nucleotide polymorphisms (SNPs) in *NFκB/IκB* confer consistent risks for COPD and lung cancer. Four putative functional SNPs (*NFκB1*: −94del>insATTG; *NFκB2*: −2966G>A; *IκBα*: −826C>T, 2758G>A) were analyzed in southern and validated in eastern Chineses to test their associations with COPD risk in 1,511 COPD patients and 1,677 normal lung function controls, as well as lung cancer risk in 1,559 lung cancer cases and 1,679 cancer-free controls. We found that the −94ins ATTG variants (ins/del + ins/ins) in *NFκB1* conferred an increased risk of COPD (OR 1.27, 95 % CI 1.06–1.52) and promoted COPD progression by accelerating annual FEV1 decline ($P = 0.015$). The 2758AA variant in *IκBα* had an increased risk of lung cancer (OR 1.53, 95 % CI 1.30–1.80) by decreasing *IκBα* expression due to the modulation of microRNA hsa-miR-449a but not hsa-miR-34b. Furthermore, both adverse genotypes exerted effect on increasing lung cancer risk in individuals with pre-existing COPD, while the −94del>insATTG did not in those without pre-existing COPD. However, no significant association with COPD or lung cancer was observed for −2966G>A and −826C>T. Our data suggested a common susceptible mechanism of inflammation in lung induced by genetic variants in *NFκB1* (−94del>ins ATTG) or *IκBα* (2758G>A) to predict risk of COPD or lung cancer.

D. Huang, L. Yang and Y. Liu contributed equally to this work.

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Introduction

Chronic obstructive pulmonary disease (COPD) and lung cancer, two major smoking-related diseases, are the leading causes of morbidity and mortality in China and worldwide (Ferlay et al. 2010). Tobacco smoking is the most important risk factor of COPD and lung cancer (Zhang and Cai 2003; Zhong et al. 2007). Smoking can induce inflammatory response in the airways, which play decisive roles in the developments of both COPD and lung cancer (Grivennikov et al. 2010; Lee et al. 2012). Moreover, patients with COPD develop a greater degree of inflammation and thus provide protumorigenic effects in lung (Hogg et al. 2004), and it has been reported that COPD patients would develop lung cancer with a high incidence rate of 16.7 cases per 1,000 person-years (de Torres et al. 2011). COPD and lung cancer are both inheritable (Chen 1999; Lichtenstein et al. 2000), therefore, to reveal the common genetic susceptible factors of them would be useful for prevention of both diseases, especially for preventing the COPD patients to develop lung cancer.

As a crucial inflammatory mediator, nuclear factor kappa-B (NFκB) and its endogenous inhibitors NFκBI (IκB) are recognized as molecular link between COPD and lung cancer (Garcia-Rio et al. 2010; Petrescu et al. 2010; Tanni et al. 2010). Many signal transduction pathways, originating from a wide variety of cellular stimuli, converge on the NFκB/IκB complex in the bronchial epithelium, inflammatory cells, premalignant lesions of the bronchial epithelium and neoplastic cells (Karin 2009); and subsequent activation of NFκB might cause chronic inflammation in the lower airways that further promote the developments of COPD and lung cancer. Furthermore, NFκB mediates tumor-promoting inflammation during every stages of carcinogenesis, including initiation, promotion, malignant conversion, invasion and metastasis (Chaturvedi et al. 2011).

NFκB1 and *NFκB2* are two major forms of NFκB family in human (Chen et al. 1999), they can be inactivated by the most common protein of IκB family-NFkB inhibitor α (IκBα) (Hayden et al. 2006). Previous studies have identified several single nucleotide polymorphisms (SNPs) in *NFκB1*/*NFκB2* and *IκBα* to be associated with various diseases including inflammatory disorders and cancer (He et al. 2009; Karban et al. 2004; Song et al. 2011). However, the pathophysiological effects of these SNPs on chronic pulmonary diseases are unclear. We hypothesized that the SNPs in *NFκB*/*IκBα* genes may influence the development of COPD and lung cancer.

In this study, we tested the associations between four putative functional SNPs (−94del>ins ATTG in *NFκB1*; −2966G>A in *NFκB2*; −826C>T and 2758G>A in *IκBα*) and COPD risk in a total of 1,511 COPD patients and 1,677 normal lung function controls, as well as lung cancer risk with totally 1,559 lung cancer cases and 1,679 cancer-free controls. We further performed a series of biological assays to identify the biological effects of these polymorphisms.

Methods

Study subjects

Retrospective case–control studies were conducted for COPD and lung cancer in two stages. The discovery set included 1,025 COPD patients and 1,061 normal lung function controls (Yang et al. 2012b), as well as 1,056 lung cancer cases and 1,056 cancer-free controls of southern Chinese (Liu et al. 2012; Lu et al. 2011; Yang et al. 2012a). The validation set comprised 486 COPD patients and 616 normal controls, as well as 503 lung cancer cases and 623 healthy controls (Liu et al. 2012; Lu et al. 2011; Yang et al. 2012a). The detailed information of subjects' recruitment was presented in Electronic Supplementary Material. Definition of COPD and its severity stage were according to the global initiative for chronic obstructive lung disease (Rabe et al. 2007). Individual's demographic characters and surrounding variables were obtained during an interview after a written informed consent was signed. The definitions of variables such as pre-existing COPD were described in Electronic Supplementary Material and elsewheres (Liu et al. 2012; Lu et al. 2011; Yang et al. 2012a). Demographic and selected variables of study subjects were presented in Supplementary Table S1, S2. Furthermore, all subjects that we recruited in previous studies (Buist et al. 2007; Zhong et al. 2007; Zhou et al. 2010) with at least 4 years spirometric follow-up data between 2002 and 2010 were selected for further phenotype analysis (i.e., 116 COPD patients and 357 controls). The study was approved by the institutional review boards of Guangzhou Medical University and Soochow University.

SNP selection

Several SNPs located in *NFκB* or *IκBα* gene have been identified in previous studies. Among them, four polymorphisms [i.e., −94del>ins ATTG (rs28362491) of *NFκB1*; −2966G>A (rs12769316) of *NFκB2*; −826C>T (rs2233406) and 2758G>A (rs696) of *IκBα*] were putatively functional and reported to be associated with various human diseases (He et al. 2009; Hung et al. 2010; Marcos et al. 2009; Sampath et al. 2011; Song et al. 2011; Zhang et al. 2009). Therefore, we selected these four SNPs in our study.

Genotype and phenotype detection

The Taqman allelic discrimination assay was used to detect the genotypes of −94del>ins ATTG (rs28362491) of

NFκB1; −2966G>A (rs12769316) of *NFκB2*; −826C>T (rs2233406) and 2758G>A (rs696) of *IκBα*. Primers and probes were designed by using Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) and synthesized by Shanghai GeneCore Biotechnologies (Shanghai, China) as shown in Supplementary Table S3. The PCR was performed in the ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems) and the genotypes were automatically determined by Sequence Detection Systems software 2.0.1 (Applied Biosystems; Supplementary Figure S1). To confirm the genotyping results, we randomly selected 10 % samples to repeat by Taqman assay and 60 samples to re-sequence, as expected, the results were all 100 % concordant (Supplementary Figure S1).

Because the biological effect of the −94del>ins ATTG variants has been identified (Karban et al. 2004), we focused on assaying the effect of 2758G>A polymorphism. The *IκBα* expression level was detected in mRNA level by real-time PCR, in protein level by western blotting like described previously (Lu et al. 2011). We also performed immunohistochemistry to detect the *IκBα* expression in situ. The detail protocols were described in Electronic Supplementary Material.

RNA interference and luciferase assays

The protocol for construction of two luciferase reporter genes comprising the 3′-UTR of *IκBα* with different 2758G or A allele was presented in Electronic Supplementary Material. The *IκBα* in vitro luciferase assays were performed first without any microRNA treatment (Lu et al. 2011). Because the bioinformatics analysis showed that the 2758G>A would change the binding of the microRNA miR-449a and miR-34b, we added the RNA interference assay to show their effect interacted with the SNP. The mimics and inhibitors of miR-449a and miR-34b synthesized by GenePharma Co. (Shanghai, China) were co-transferred with the luciferase reporters to show the effect of microRNA on *IκBα* reporter genes in vitro. A549 (2758GG genotype) and NCI-520 (2758AG genotype) were seeded into 24-well plates at 1×10^5 cells/well and cultured at 37 °C in 5 % CO_2 for 24 h. The cells were then transiently transfected with 1.5 μg of reporter plasmids (G or A allele) alone or co-transfected with or without microRNA mimics or inhibitors using Lipofectamine 2000 according to the protocol (Invitrogen, Carlsbad, CA, USA). The activities of reporter genes with renilla luciferase and the internal standard firefly luciferase were quantified by a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). Independent triplicate experiments were done for each plasmid construct. We further detect the effect of the operant microRNA on *IκBα* expression with real-time PCR analysis in A549 (2758GG genotype) and NCI-520 (2758AG genotype).

Statistical analysis

Differences in the distributions of demographic characteristics and genotypes between cases and controls were evaluated using the Chi-square test or the Student's *t* test. The association between each SNP and diseases risk was estimated using an unconditional logistic regression model with adjustments for surrounding factors. A multiplicative interaction was suggested to detect the possible gene–environment interaction (Lu et al. 2011). Homogeneity test was performed with Breslow–Day test. The statistical power was calculated by the PS Software (Dupont and Plummer 1990). The One-way ANOVA test, Student's *t* test and linear regression analysis were used to assay the deviation of annual decline of pre-bronchodilator FEV1, which refers to the volume exhaled during the first second of a forced expiratory maneuver started from the level of total lung capacity and *IκBα* expression in groups with different genotypes. The Student's *t* test was also used to examine the difference in levels of luciferase reporter genes expression between different constructs. All tests were two-sided by using the SAS software (version 9.3; SAS Institute, Cary, NC, USA). $P < 0.05$ was considered statistically significant.

Results

NFκB/IκBα genotypes and COPD or lung cancer risk

As summarized in Table 1, the observed genotype frequencies of all SNPs were in agreement with the Hardy–Weinberg equilibrium in controls ($P > 0.05$ for all). In discovery set, according to criteria of the smallest AIC value, the −94ins variant genotypes (ins/ins + ins/del) of *NFκB1* conferred a 1.23-fold increased risk of COPD compared with del/del genotype (OR 1.23; 95 % CI 1.03–1.54; $P = 0.037$) in a dominant genetic model. And the 2758AA genotype of *IκBα* conferred a 1.49-fold increased risk of lung cancer compared with 2758G (GG + GA) genotypes (OR 1.49; 95 % CI 1.22–1.82; $P = 9.5 \times 10^{-5}$) under a recessive genetic model. However, no other significant association between these polymorphisms and the risk of either COPD or lung cancer was observed. The results in validation set confirmed the above findings, the −94ins variant genotypes conferred a 1.33-fold increased risk of COPD compared with del/del genotype (OR 1.33; 95 % CI 1.04–1.81; $P = 0.047$). Meanwhile, the 2758AA adverse genotype had a 1.61-fold risk of lung cancer compared to 2758G genotypes (OR 1.61; 95 %

Table 1 Associations between genotypes in *NFκB1/NFκB2 and IκBα* genes and COPD as well as lung cancer risk

Genotypes	COPD case–control study						Lung cancer case–control study					
	Discovery set (Southern Chinese)			Validation set (Eastern Chinese)			Discovery set (Southern Chinese)			Validation set (Eastern Chinese)		
	Cases *n* (%)	Controls *n* (%)	Adjusted OR (95 % CI)[a]	Cases *n* (%)	Controls *n* (%)	OR (95 % CI)[a]	Cases *n* (%)	Controls *n* (%)	Adjusted OR (95 % CI)[a]	Cases *n* (%)	Controls *n* (%)	OR (95 % CI)[a]
Total no. of subjects	1,025	1,061		486	616		1,056	1,056		503	623	
NFκB1 −94del > insATTG												
del/del	170 (16.6)	214 (20.2)	1.00 (ref)	82 (16.9)	133 (21.6)	1.00 (ref)	225 (21.3)	210 (19.9)	1.00 (ref)	104 (20.7)	145 (23.3)	1.00 (ref)
ins/del	454 (44.3)	496 (46.7)	1.13 (0.89–1.44)	214 (44.0)	261 (42.4)	1.33 (0.96–1.86)	459 (43.4)	491 (46.5)	0.87 (0.69–1.10)	230 (45.7)	289 (46.4)	1.08 (0.79–1.47)
ins/ins	401 (39.1)	351 (33.1)	**1.38 (1.07–1.77)**	190 (39.1)	222 (36.0)	**1.39 (1.00–1.95)**	372 (35.5)	355 (33.6)	0.97 (0.77–1.24)	169 (33.6)	189 (30.3)	1.25 (0.90–1.73)
Trend test *P* value			**0.006**			0.095			0.982			0.173
ins/del + ins/ins	855 (83.4)	847 (79.8)	**1.23 (1.03–1.54)**	404 (83.1)	483 (78.4)	**1.33 (1.04–1.81)**	831 (78.7)	846 (80.1)	0.92 (0.74–1.13)	404 (83.1)	483 (78.4)	1.14 (0.86–1.52)
NFκB2-2966G>A												
GG	412 (40.2)	451 (42.5)	1.00 (ref)				440 (41.7)	459 (43.5)	1.00 (ref)			
AG	480 (46.8)	475 (44.8)	1.11 (0.91–1.34)				494 (46.8)	481 (45.5)	1.11 (0.91–1.34)			
AA	133 (13.0)	135 (12.7)	1.08 (0.79–1.48)				122 (11.5)	116 (11.0)	1.08 (0.79–1.48)			
Trend test *P* value			0.687						0.798			
IκBα-826T>C												
TT	788 (76.9)	822 (77.5)	1.00 (ref)				815 (77.2)	808 (76.5)	1.00 (ref)			
TC	210 (20.5)	225 (21.2)	0.99 (0.80–1.23)				217 (20.5)	229 (21.7)	0.95 (0.77–1.17)			
CC	27 (2.6)	14 (1.32)	1.76 (0.90–3.47)				24 (2.3)	19 (1.8)	1.26 (0.69–2.33)			
Trend test *P* value			0.227						0.976			
IκBα 2758G>A												
GG	311 (30.3)	305 (28.8)	1.00 (ref)	150 (30.9)	171 (27.8)	1.00 (ref.) (ref.)	294 (27.9)	293 (27.8)	1.00 (ref)	132 (26.2)	206 (33.1)	1.00 (ref.) (ref.)
AG	503 (49.1)	532 (50.1)	0.93 (0.76–1.14)	244 (50.2)	324 (52.6)	0.88 (0.66–1.16)	466 (44.1)	544 (51.5)	0.86 (0.70–1.06)	226 (44.9)	290 (46.5)	1.23 (0.93–1.63)
AA	211 (20.6)	224 (21.1)	0.91 (0.71–1.17)	92 (18.9)	121 (19.6)	0.87 (0.61–1.24)	296 (28.0)	219 (20.7)	**1.36 (1.07–1.72)**	145 (28.8)	127 (20.4)	**1.82 (1.31-2.53)**
Trend test *P* value			0.448			0.350			**0.018**			**4.0×10^{-5}**
GG + AG	814 (79.4)	837 (78.9)	1.00 (ref)	394 (81.1)	495 (80.4)	1.00 (ref)	760 (72.0)	837 (79.3)	1.00 (ref)	358 (71.2)	496 (79.6)	1.00 (ref)
AA	211 (20.6)	224 (21.1)	0.96 (0.77–1.19)	92 (18.9)	121 (19.6)	0.95 (0.70–1.29)	296 (28.0)	219 (20.7)	**1.49 (1.22–1.82)**	145 (28.8)	127 (20.4)	**1.61 (1.22–2.12)**

Significant statistics with tested *P* values less than 0.05 were presented in bold

[a] Adjusted in a logistic regression model that included age, sex, smoking status and drinking status

CI 1.22–2.12; $P = 0.001$). Because the associations of above adverse genotypes in the two datasets were homogeneous ($P = 0.734$ for −94del>ins ATTG, $P = 0.726$ for 2758G>A), we then merged the two sets, the −94ins variant genotypes of *NFκB1* conferred a 1.27-fold increased risk of COPD (OR 1.27; 95 % CI 1.06–1.52; $P = 0.009$; Supplementary Figure S2), while the 2758AA genotype had a 1.53-fold increased risk of lung cancer (OR 1.53, 95 % CI 1.30–1.80; $P = 3.0 \times 10^{-4}$; Supplementary Figure S3).

We further analyze the effect of pre-existing COPD condition on the risk genotypes for lung cancer because COPD promotes inflammation and thus provide protumorigenic effects (Hogg et al. 2004). Interestingly, both the adverse genotypes were significant in those subjects with pre-existing COPD ($P < 0.05$ for all), while the −94ins variant genotypes was not in those without pre-existing COPD (Table 2). When combined the number of risk genotypes of these two SNPs, we found that the subjects carrying two risk genotypes with pre-existing COPD had a higher risk of lung cancer (OR 3.15, 95 % CI 1.48–6.70) than those without pre-existing COPD (OR 1.43, 95 % CI 1.12–1.48) on a borderline statistically significance (Breslow–Day test: $P = 0.087$, Table 2). In the stratification analysis, we did not observe any significant differences for associations between the −94del>ins ATTG or 2758G>A polymorphism and risk of COPD or lung cancer in each stratum (Supplementary Figure S2, S3; homogeneity test $P > 0.05$ for all), and no significant interaction between these risk genotypes and surrounding factors on both diseases risk. In addition, there was no significant association between the risk genotypes and smoking status as well as pack years smoked ($P > 0.05$, data not shown).

NFκB1 genotypes and pulmonary functions

Only the effect of −94del>ins ATTG on pulmonary function was presented because no significant association was observed in other SNPs for pulmonary function. In subjects with pulmonary function follow-up data, there was a significant decreasing trend of annual average decline of pre-bronchodilator FEV1 in COPD patients according to −94ins ATTG allele-dependent manner (−94ins/ins ATTG: $n = 62$, -0.126 ± 0.113 L, −94ins/del ATTG: $n = 42$, -0.091 ± 0.083 L, −94del/del ATTG: $n = 12$, -0.040 ± 0.036 L, ANOVA test $P = 0.015$, linear regression test $P = 0.017$), but not in controls (Fig. 1). And the −94del>insATTG genotypes were significantly correlated with COPD Gold stages ($P = 0.048$). Furthermore, as shown in Supplementary Table S4, sex, smoking status and pack-year smoked were correlated with the annual decline of pre-bronchodilator FEV1 in both COPD cases and controls.

IκBα genotypes and its expression

As shown in Fig. 2, the mRNA levels of IκBα were much lower in lung cancer tissues compared to their adjacent

Table 2 Effect of *NFκB1*/*IκBα* polymorphisms on risk of lung cancer with or without pre-existing COPD

Genotypes	Non pre-existing COPD individuals			Pre-existing COPD individuals			Homogeneity test *P* value
	Lung cancers *n* (%)	Controls *n* (%)	OR (95 % CI)[a]	Lung cancers *n* (%)	Controls *n* (%)	OR (95 % CI)[a]	
Total no. of subjects	1,342	1,510		217	169		
NFκB1 −94del>insATTG							
del/del	289 (21.6)	309 (20.5)	1.00 (ref.)	40 (18.4)	46 (27.2)	1.00 (ref.)	
ins/del	599 (44.6)	653 (43.2)	1.05 (0.91–1.22)	90 (41.5)	77 (45.6)	0.86 (0.57–1.30)	0.267
ins/ins	454 (33.8)	548 (36.3)	0.90 (0.77–1.05)	87 (40.1)	46 (27.2)	**1.80 (1.15–2.80)**	**0.003**
del/del	289 (21.6)	309 (20.5)	1.00 (ref.)	40 (18.4)	46 (27.2)	1.00 (ref.)	
ins/del + ins/ins	1,053 (78.5)	1,201 (79.5)	0.93 (0.78–1.12)	177 (81.6)	123 (72.8)	**1.69 (1.03–2.78)**	**0.030**
IκBα 2758G>A							
GG+AG	968 (72.1)	1,202 (79.6)	1.00 (ref.)	150 (69.1)	131 (77.5)	1.00 (ref.)	
AA	374 (27.9)	308 (20.4)	**1.52 (1.28–1.80)**	67 (30.9)	38 (22.5)	**1.60 (1.00–2.58)**	0.934
No. of the combined risk genotypes[b]							
0	210 (15.6)	251 (16.6)	1.00 (ref.)	26 (12.0)	33 (19.5)	1.00 (ref.)	
1	837 (62.4)	1,009 (66.8)	0.98 (0.80–1.21)	138 (63.6)	111 (65.7)	1.60 (0.89–2.89)	0.132
2	295 (22.0)	250 (16.6)	**1.43 (1.12–1.84)**	53 (24.4)	25 (14.8)	**3.15 (1.48−6.70)**	0.087

Significant statistics with tested *P* values less than 0.05 were presented in bold

[a] Adjusted in a logistic regression model that included age, sex, smoking status and drinking status

[b] Genotype combinations of the two polymorphisms in the *NFκB1 and IκBα*: ins variant genotypes (ins/del + ins/ins) and 2758AA genotype are defined as risk genotypes: i.e., the carriers of del/del and 2758GG/AG have zero risk genotype, the carriers of ins/del (ins/ins) and 2758GG/AG, or del/del and 2758AA have one risk genotype; and the ins/del (ins/ins) and 2758AA carriers have two risk genotypes

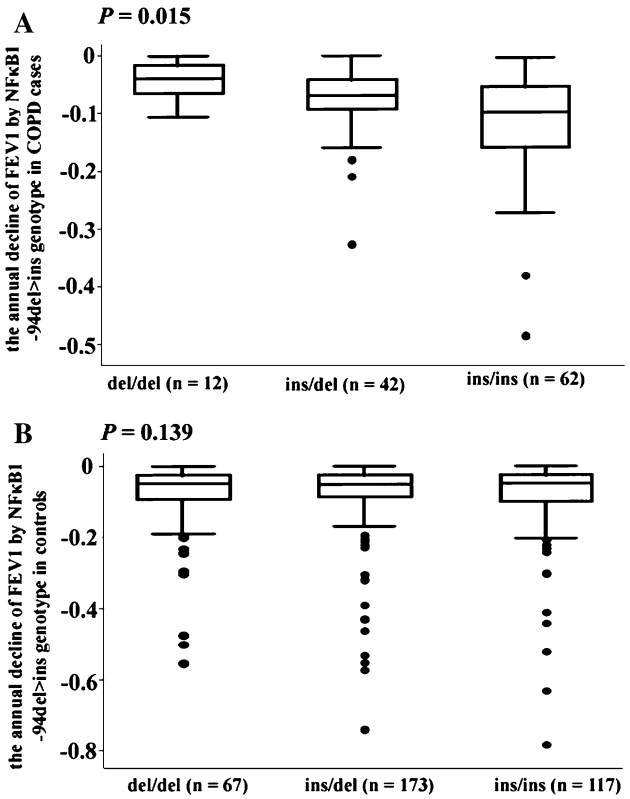

Fig. 1 Association between the *NFκB1* −94del>ins ATTG polymorphism and the annual average decline of pre-bronchodilator FEV1. **a** The annual average decline of pre-bronchodilator FEV1 by −94del>ins ATTG genotypes in COPD patients. **b** The annual average decline of pre-bronchodilator FEV1 by −94del>ins ATTG genotypes in healthy controls. A significant decreased trend of annual average decline of pre-bronchodilator FEV1 in COPD patients according to −94ins ATTG allele-dependent manner (ANOVA test $P = 0.015$) but not in controls (ANOVA test $P = 0.139$) was observed

normal tissues ($P = 0.032$), and they were significantly lower in cases carrying 2758AA genotype than in cases with 2758G genotypes of both normal and cancer tissues ($P < 0.05$ for all). The IκBα protein expression confirmed the above findings as they were significantly decreased in carriers of 2758AA genotype compared with those of 2758G genotypes ($P < 0.05$ for all). Furthermore, the immunohistochemical stain also shown that 2758AA genotype exerted a significantly lower IκBα expression in situ than 2758G genotypes (Supplementary Figure S4; $P = 0.045$).

Luciferase activity

As shown in Fig. 3b, the transcription activity of the reporter gene integrated the *IκBα* 3′-UTR with 2758A allele was significantly lower than that with G allele both in A549 cell and NCI-520 cell (P value is 0.022, 0.036, respectively). The miR-449a mimics could further reduce the reporter genes' activity with 2758A allele ($P < 0.05$), and the miR-449a inhibitor reversed and up-regulated reporter genes' activity ($P < 0.05$). However, the miR-34b failed to exert any effect on the reporter genes either with 2758A allele or G allele ($P > 0.05$ for all). Furthermore, the expressions of miR-449a were observed in A549 and NCI-520 (Fig. 3c). As shown in Fig. 3d, the mimics of miR-449a suppressed the mRNA expression of *IκBα* and its inhibitors could reverse and up-regulate *IκBα*'s expression only in NCI-520 with 2758AG genotype ($P < 0.01$). However, in A549 with 2758GG genotype, the miR-449a mimics could significantly suppress *IκBα*, but the miR-449a inhibitors did not significantly reverse the *IκBα* expression.

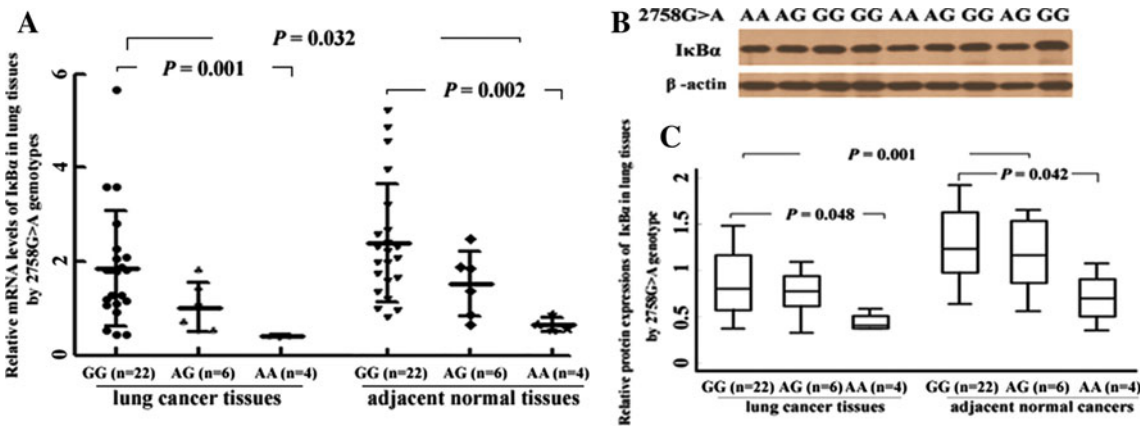

Fig. 2 Association between the 2758G>A genotypes and *IκBα* expressions. **a** Relative mRNA levels of the *IκBα* expression in lung tissues by 2758G>A genotypes. **b** The western blotting assay on detecting *IκBα* protein with *β*-actin as an internal reference. **c** The *IκBα* protein expression by 2758G>A genotypes in lung tissues. Both the mRNA and protein expressions of IκBα were significantly decreased in carriers of 2758AA genotype than those of 2758G genotypes in lung cancer tissues (ANOVA test $P < 0.05$ for all) as well as in adjacent normal tissues (ANOVA test $P < 0.05$ for all)

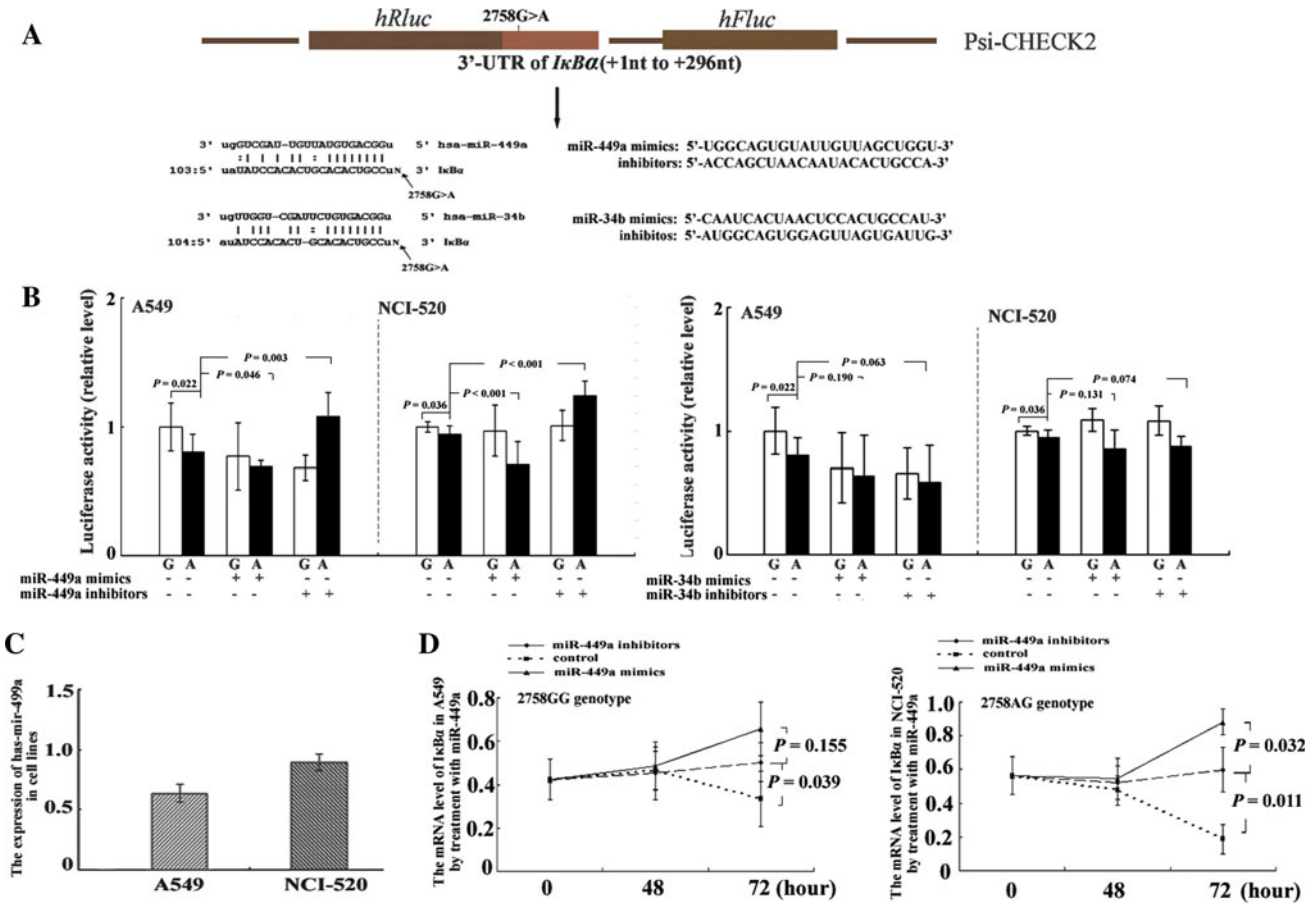

Fig. 3 Effects of the 2758G>A polymorphism and treatment with microRNAs on reporter gene's activity and *IκBα* in different cell lines. **a** Schematic of the reporter gene construct with a 296 bp 3′-UTR of *IκBα* (+1 nt to +296 nt downstream to the translation stop site TGA) including 2758G>A polymorphism and a putative target site of miR-449a and miR-34b highly conserved in the *IκBα* mRNA 3′-UTR. **b** Luciferase expression of the two constructs in lung cancer cells (A549 and NCI-520). The renilla luciferase activity of each construct was normalized against the internal control of firefly luciferase. **c** The gene expressions of has-miR-449a in lung cell lines. **d** The differences of *IκBα* mRNA level in A549 and NCI-520 with different 2758G>A genotypes after the treatment of mimics or inhibitors of miR-449a. Columns, mean from three independent experiments; *bars*, SD; and Student's *t* test was used to test the differences in the expression levels of different constructs as well as *IκBα* mRNA levels. The transcription activity of the reporter gene integrated the *IκBα* 3′-UTR with 2758A allele was significantly lower than that with G allele with a modulation by miR-449a

Discussion

In this study, we found that the −94ins ATTG variants of *NFκB1* conferred an increased risk of COPD and accelerated COPD progression by making carriers more rapid reduction in pulmonary function; and the 2758AA variant of *IκBα* contributed an increased risk of lung cancer by decreasing *IκBα* expression in lung tissues under the regulation of hsa-miR-449a but not hsa-miR-34b. Especially in individuals with pre-existing COPD, both adverse genotypes exerted effect on increasing lung cancer risk while the −94del>insATTG did not in those without pre-existing COPD. All these supported a common susceptible mechanism of inflammation in lung induced by genetic variants in *NFκB1* or *IκBα* for COPD and lung cancer.

The NFκB1 and IκBα play critical roles in multiple human diseases by responding to the environmental stimulus and regulating inflammatory responses, cell growth and apoptosis (Escarcega 2010). Up expression or over activation of *NFκB1* that promotes exacerbation of inflammation has been found in both COPD and lung cancer (Brown et al. 2009; Tang et al. 2006), and *NFκB1* also possibly predisposes those COPD patients to further develop lung cancer. Meanwhile, IκBα, which functions to suppress the effect of *NFκB1*, has been reported be inactive or down-regulated during various stimuli induced *NFκB* activation progresses (Abe et al. 2011; Kim et al. 2000), and in consequence loss its protective role in the development of human disease. Previous study has demonstrated that the −94ins ATTG allele has an effect on increasing expression of *NFκB1* and in turn promoting inflammation

(Karban et al. 2004), so, it is conceivable that the −94ins ATTG variant genotypes conferred an increased risk of COPD and decreased pulmonary functions. Correspondingly, the 2758G>A polymorphism exerted an effect on decreasing IκBα expression in lung tissues and might loss its suppression on NFκB dependent inflammation, therefore, the 2758AA variants shared a consistent pro-inflammatory role with the −94ins ATTG variants, thus contributed high risk to lung cancer. The in vitro assays further revealed an adverse effect of 2758A allele on IκBα's expression, and the miR-449a could specially regulate the activities of reporter genes with 2758A but not G allele. The stronger effect of miR-449a on modulating *IκBα* in NCI-520 with 2758AG genotype than that of A549 with 2758AA genotype confirmed this finding. Interestingly, high miR-449a levels have been found in lung cancer tissues and cancer cells (Lize et al. 2010), further suggesting a physical and endogenous function of the miR-449a on regulating the effect of *IκBα*. Two reports of colorectal cancer have showed significant associations of the 2758G>A polymorphism in southern Chinese and Swedish population (Song et al. 2011; Zou et al. 2011). They were consistent with our finding that the 2758G>A polymorphism increased the risk of lung cancer. Taken these together, the genetic variations −94del>ins ATTG of *NFκB1* or 2758G>A of *IκBα* that involved in a common mechanism as pro-inflammation may predispose a susceptibility to COPD or lung cancer. Additionally, the genetic effect of above two risk genotypes was more evident in subjects with pre-existing COPD, indicating a possibly high risk of lung cancer in COPD cases owing to over-activated inflammation.

Many studies have reported the −94ins ATTG variant genotypes conferred an increased risk of inflammatory diseases (He et al. 2009; Karban et al. 2004). Here, we consistently found −94ins ATTG variants were associated with COPD risk and correlated with COPD stages by causing more rapid reduction in pre-bronchodilator FEV1 in COPD cases. The reduction in FEV1 is a marker of airflow obstruction and the FEV1 is used to assess the severity of the airflow obstruction (Ferrer et al. 1997), so the patients with −94ins ATTG genotypes got more serious COPD stages. Our findings of the −94ins ATTG variants significantly associated with FEV1 decline in COPD cases but not in controls implies that the ATTG polymorphism increases the inflammation may involve in the development of COPD.

As a hospital-based case–control study, there were some limitations in current study such as information bias. However, with the fairly large sample size and two study populations, we have achieved high statistical powers (88.0 % for COPD, 99.7 % for lung cancer) and the functional assays also confirmed the associations. In addition, recent genome-wide association studies (GWAS) identified several SNPs in Chromosome 4q24 to be susceptible loci for COPD or lung function (Castaldi et al. 2011; Repapi et al. 2010). The *NFκB1* gene is also located on 4q24. However, the GWAS did not report any loci in *NFκB1*, this may be due to that the SNP (rs28362491) was not included in the Affymetrix 6.0 genechip assay, and there is no LD between the SNP and other SNPs of *NFκB1* comprised in the gene chip. For 2758G>A (rs696), the results from Chinese GWAS that we previously participated in (Hu et al. 2011) showed that the SNP is significant associated with lung cancer risk ($P = 0.016$) although it does not meet the GWAS significant criteria ($P < 10^{-7}$). Therefore, it appears that our finding is unlikely by chance.

In conclusion, in current study, we found significant associations between *NFκB1* and *IκBα* polymorphisms and risk of COPD or lung cancer in Chinese. The findings were consistent in COPD and lung cancer, because the −94ins variants carriers with reduced expression of NF-κB had an increased risk of COPD, and the carriers of 2758AA genotype with decreased expression of IκB and consequently less suppression of NF-κB were associated with an increased risk of lung cancer. Our results suggested that the genetic variation in genes encoding NF-κB/IκB may contribute to the developments of both COPD and lung cancer in Chinese people.

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Conflict of interest The authors have declared no conflicts of interest.

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