

## The Contribution of Interleukin-12 Genetic Variations to Taiwanese Lung Cancer

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**Abstract.** *Background/Aim:* Lung cancer is the leading cause of cancer-related death and a better marker for advanced personalized therapeutic approaches, such as immunotherapies, is in urgent need. Interleukin-12 (IL-12) is a cytokine that has been reported to exhibit potent tumoricidal effects, however, the contribution of IL-12 genotypes to lung cancer is still largely unrevealed. The aim of this study was to investigate whether single nucleotide polymorphisms (SNPs) in IL-12A and IL-12B are associated with lung cancer in a Taiwanese population. *Materials and Methods:* Genotypes of 358 lung cancer patients and 716 controls were determined by the polymerase chain reaction–restriction fragment length polymorphism method. *Results:* The distributions of genotypic ( $p=0.0036$ ) and allelic ( $p=0.0005$ ) frequencies of IL-12A rs568408 demonstrated significant differences between cases and controls. In detail, the AA genotype of IL-12A rs568408 was associated with a significantly elevated risk of lung cancer

compared with the GG genotype (odds ratio(OR)=2.41, 95% confidence interval(CI)=1.36-4.29,  $p=0.0021$ ). No difference was observed regarding IL-12A rs2243115 and IL-12B rs3212227 genotypes between the case and control groups. In addition, the results of interaction analysis showed that the AA genotype of IL-12A rs568408 was associated with elevated lung cancer risk, especially among those with smoking habits ( $p=0.0043$ ). *Conclusion:* IL-12A rs568408 AA genotype may contribute to the etiology and serve as a genomic determinant of lung cancer in Taiwanese, especially smokers.

Lung cancer remains a serious public health problem since it has been the leading cause of cancer mortality worldwide (1, 2). Although the first-line chemotherapeutic approaches such as paclitaxel (PTX) and cisplatin (CDDP) doublet chemotherapy are effective for non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (3-5), the 5-year survival rate is still very low (6-8). Thus, a better target or marker for advanced personalized therapeutic approaches such as immunotherapies are in urgent need. To fulfill this aim, mounting studies have reported that specific genotypes are associated with increased lung cancer risk for cigarette smokers relative to non-smokers (9-16) and *vice versa* (17-20). These studies elucidating the contribution of both genomic and behavioral factors to lung cancer etiology may provide the basis for better therapeutic decisions.

Interleukin 12 (IL-12) is a cytokine originally identified as a natural killer (NK) cell stimulatory factor (NKSF) and a cytotoxic lymphocyte maturation factor (21, 22). IL-12 has

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been reported to stimulate NK and T cell proliferation, enhance their cytolytic activity and induce cytokine production, particularly IFN- $\gamma$  (23, 24). Also, IL-12 plays an important role in bridging innate and adaptive immunity by promoting the differentiation of T helper 1 (TH1) cells (25, 26). IL-12 has the potential of antitumor capacity since mice lacking IL-12 subunit p35 develop earlier and higher numbers of papilloma compared to the wild-type mice (27). It has also been shown that the growth of B16 melanomas is faster in mice that are deficient in IL-12 receptor chain IL-12R $\beta$ 2 compared to wild-type mice (28). In mice tumor models, exogenous administration of IL-12 exhibited antitumor effects against the growth of various types of cancer including sarcoma, melanoma, lung carcinoma and breast carcinoma (29-31). Clinically, although IL-12 has certain side-effects, its curative effect is impressive and significant. The results from IL-12 phase I/II trials in patients with B-cell lymphoma or Kaposi sarcoma were very successful and promising for future clinical practice (32, 33). Combined with IL-18, IL-12 treatment can restore the intratumoral NK cell functions in MHC (major histocompatibility complex) class I-deficient tumors (34, 35). In 2016, it was reported that IL-12 inhibited tumor angiogenesis with the help of interferon- $\gamma$  produced by natural killer cells (36).

Human *IL-12A* and *IL12B* genes are located at chromosome 3 and 5, respectively. Among the SNPs of *IL-12*, *IL-12A* rs568408, *IL-12B* rs2243115 and *IL-12B* rs3212227 are the three polymorphic sites most examined. *IL-12A* rs568408 and *IL-12B* rs3212227, located in the 3'-untranslated region (3'UTR), may disrupt exonic splicing and influence the production level of IL-12 (37, 38). *IL-12A* rs2243115 is situated in the 5'UTR, and its functional significance has not been well examined or conclusively reported. As for the contribution of IL-12A and IL-12B to lung cancer, TT genotype at *IL-12A* rs662959 was found to be associated with higher risk of cancer progression in NSCLC patients (39). However, the effects of *IL-12A* rs662959 TT genotype on the expression level of IL-12 has not yet revealed. In the current study, the contribution of *IL-12A* and *IL-12B* genotypes to the risk of lung cancer, as well as the interaction of *IL-12* genotypes and smoking were investigated Taiwanese patients.

## Materials and Methods

**Investigated population.** Three hundred and fifty-eight patients diagnosed with lung cancer were recruited by the surgery team at the Outpatient Clinics of General Surgery at the China Medical University Hospital during 2005-2008. The clinical characteristics of patients, including histological details, were all graded and defined by expert surgeons. The patients with history of any other cancer and pulmonary diseases, such as chronic obstructive pulmonary disease (COPD), pneumothorax and asthma, were excluded from the databank. All participants were Taiwanese and voluntarily completed a self-administered questionnaire and provided their blood sample for

genotyping studies. At the same time, twice as many non-lung cancer healthy volunteers as controls matched for age, gender and smoking behavior were selected after initial random sampling from the Health Examination Cohort of China Medical University Hospital. The exclusion criteria of the controls included previous malignancy, metastasized cancer from other or unknown origin and any genetic or familial diseases. The study was approved by the Institutional Review Board of the China Medical University Hospital with the document coded DMR100-IRB-284 and written informed consent was obtained from all participants. Selected characteristics of all the investigated subjects are summarized in Table I.

***IL-12* polymerase chain reaction-restriction fragment length polymorphism genotyping conditions.** Genomic DNA from peripheral blood leucocytes was prepared using the QIAamp Blood Mini Kit (Blossom, Taipei, Taiwan) (40, 41) and further processed in typical polymerase chain reactions (PCR) as we have previously described (42-44). The primer sequences for *IL-12* genotyping were those designed by Chen and his colleagues (37). For *IL-12A* rs2243115 and rs568408, the forward primers were introduced a mismatched A to replace C and a mismatched T to replace C, respectively, at -3 bp from the polymorphic sites to create *Bse* I and *Nde* I (New England BioLabs, Ipswich, MA, USA) digestible restriction sites. For *IL-12A* rs568408, the primers were 5'-AGAAAAGACCTGTGAACAAAACGACT-3' (forward) and 5'-AGATGGCTCACTAGATGCCAGG-3' (reverse). For *IL-12A* rs2243115, the primers were 5'-GAAGGATGGGACYAT TACATCCATAT-3' (forward) and 5'-CAGGATGGATATTTTCC CTTCT-3' (reverse). The wild-type allele of *IL-12A* rs2243115T generated a fragment of 122 bp while the variant allele *IL-12A* rs2243115G produced two fragments of 93 and 29 bp after PCR and digestion. As for *IL-12A* rs568408, the wild-type allele *IL-12A* rs568408G produced 2 fragments of 98 and 23 bp and the variant allele *IL-12A* rs568408A resulted in a fragment of 121 bp. The primers for *IL-12B* rs3212227 were 5'-GATATCTTTGCTGTATT TGTATAGTT-3' (forward) and 5'-AATATTTAAATAGCATG AAGGC-3' (reverse). The PCR that included these primers generated a 118-bp fragment which was then digested by *Taq* I (New England BioLabs, Ipswich, MA, USA). The variant allele *IL-12B* rs3212227C produced 2 fragments of 92 and 26 bp, and the wild-type allele *IL-12B* rs3212227A a single 118-bp fragment.

The PCR cycling were set as: one cycle at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec; and a final extension at 72°C for 10 min. The PCR and digestion fragments were analyzed in a 3% agarose gel. The genotype analysis was performed by three researchers independently and blindly. For each *IL-12* SNP, five percent of the PCR products were randomly selected for direct sequencing and the results from PCR-RFLP and direct sequencing were 100% concordant.

**Statistical analyses.** Seven hundred and sixteen of the controls and 358 lung cancer patients were included in the analysis. The Student's *t*-test was used to compare the difference of age between case and control groups. Pearson's Chi-square test was used to compare the distribution of *IL-12* genotypes between the lung cancer and control groups. The associations between the *IL-12* genotypes and lung cancer risk were estimated by computing odds ratios (ORs) and their 95% confidence intervals (CIs) from logistic regression analysis. Any comparison with *p*<0.05 was considered as statistically significant.

Table I. Distribution of selected demographic data of the 358 patients with lung cancer and the 716 matched controls.

Characteristics	Controls (n=716)			Patients (n=358)			<i>p</i> -Value <sup>a</sup>
	n	%	Mean (SD)	n	%	Mean (SD)	
Age (years)			64.8 (6.8)			64.0 (6.9)	0.5871
Gender							
Male	488	68.1%		254	70.9%		
Female	228	31.9%		104	29.1%		0.3642
Smoking status							
Ever smokers	563	78.6%		293	81.8%		
Non-smokers	153	21.4%		65	18.2%		0.2282
Histology							
Adenocarcinoma				218	60.9%		
SCC				106	29.6%		
Other				34	9.5%		

<sup>a</sup>Based on Chi-square test; SCC: squamous cell carcinoma; SD: standard deviation.Table II. Distribution of *IL-12A* rs568408, *IL-12A* rs2243115 and *IL-12B* rs3212227 genotypes among the 358 lung cancer patients and the 716 controls.

Genotype	Cases		Controls		OR (95%CI)	<i>p</i> -Value <sup>a</sup>
	n	%	n	%		
<i>IL-12A</i>						
rs568408						
GG	242	67.6%	539	75.3%	1.00 (reference)	
AG	90	25.1%	153	21.4%	1.31 (0.97-1.77)	0.0784
AA	<b>26</b>	<b>7.3%</b>	<b>24</b>	<b>3.3%</b>	<b>2.41 (1.36-4.29)</b>	<b>0.0021*</b>
AG+AA	<b>116</b>	<b>32.4%</b>	<b>177</b>	<b>24.7%</b>	<b>1.46 (1.10-1.93)</b>	<b>0.0077*</b>
<i>P</i> <sub>trend</sub>						<b>0.0036*</b>
<i>IL-12A</i>						
rs2243115						
TT	310	86.6%	609	85.1%	1.00 (reference)	
TG	33	9.2%	78	10.9%	0.83 (0.54-1.28)	0.3980
GG	15	4.2%	29	4.0%	1.01 (0.54-1.92)	0.9608
TG+GG	48	13.6%	107	14.9%	0.88 (0.61-1.27)	0.4994
<i>P</i> <sub>trend</sub>						0.6956
<i>IL-12B</i>						
rs3212227						
AA	136	38.0%	258	36.0%	1.00 (reference)	
AC	140	39.1%	297	41.5%	0.89 (0.67-1.19)	0.4483
CC	82	22.9%	161	22.5%	0.97 (0.69-1.35)	0.8417
AC+CC	222	62.0%	458	64.0%	0.92 (0.71-1.20)	0.5308
<i>P</i> <sub>trend</sub>						0.7416

<sup>a</sup>Based on Chi-square without Yate's correction test; the significant *p*-value and odds ratio are bolded and marked with a star.

## Results

The distributions of age and gender for the investigated 358 lung cancer patients and the 716 matched controls are summarized in Table I. As all the controls and cases were matched by age, gender and smoking behavior, there were

no differences between the two groups in these aspects (*p*=0.5871 and 0.3642, respectively) (Table I). The non-smoker percentages in the lung cancer patients and matched controls were 18.2% and 21.4%, respectively (*p*=0.2282).

The distribution of genotypic frequencies of the 3 SNPs (rs2243115 and rs568408 in *IL-12A* and rs3212227 in *IL-*

Table III. Distributions of *IL-12A* rs568408, *IL-12A* rs2243115 and *IL-12B* rs3212227 allelic frequencies among the 358 lung cancer patients and the 716 controls.

Allele	Cases	%	Controls	%	OR (95%CI)	p-Value <sup>a</sup>
<i>IL-12A</i>						
rs568408						
Allele G	574	80.2%	1231	86.0%	1.00 (reference)	
Allele A	<b>142</b>	<b>19.8%</b>	<b>201</b>	<b>14.0%</b>	<b>1.52 (1.20-1.92)*</b>	<b>0.0005*</b>
<i>IL-12A</i>						
rs2243115						
Allele T	653	91.2%	1296	90.5%	1.00 (reference)	
Allele G	63	8.8%	136	9.5%	0.92 (0.67-1.26)	0.5987
<i>IL-12B</i>						
rs3212227						
Allele A	412	57.5%	813	56.8%	1.00 (reference)	
Allele C	304	42.5%	619	43.2%	0.97 (0.81-1.16)	0.7346

<sup>a</sup>Based on Chi-square without Yate's correction test; the significant *p*-value and odds ratio are bolded and marked with a star.

*12B*) for all the investigated subjects are summarized in Table II. The genotypic frequencies for the three SNPs agreed with the Hardy–Weinberg equilibrium in each group. Interestingly, there was a significant difference in the distribution of *IL-12A* rs568408 genotypes between lung cancer and control groups (*p* for trend=0.0036), but not for those of *IL-12A* rs2243115 or *IL-12B* rs3212227 (*p* for trend >0.05). In detail, the frequencies of the heterozygous variant AG and homozygous variant GG of *IL-12A* rs568408 were 25.1 and 7.3% in the lung cancer group and a little lower (21.4 and 3.3%) in the control group. The AA (OR=2.41, 95% CI=1.36-4.29, *p*=0.0021) but not the AG (OR=1.31, 95%CI=0.97-1.77, *p*=0.0784) genotype at *IL-12A* rs568408 seemed to be a potential biomarker for lung cancer among the Taiwanese. The fact that the combined variant AG+AA at *IL-12A* rs568408 also elevated the risk of lung cancer among Taiwanese compared to wild-type GG genotype validated the importance of AA genotype (OR=1.46, 95%CI=1.10-1.93, *p*=0.0077) (Table II, top part).

The distribution of allelic frequencies of rs568408, rs2243115 in *IL-12A* and rs3212227 in *IL-12B* are summarized in Table III. Consistent with the findings in Table II, the allele A at *IL-12A* rs568408 was associated with an increased risk of lung cancer, compared with allele G (OR=1.52, 95%CI=1.20-1.92, *p*=0.0005). In detail, the frequencies of the A and G alleles of *IL-12A* rs568408 were 19.8 and 80.2% among lung cancer patients and 14.0 and 86.0% among controls (Table III). On the contrary, the variant G allele of *IL-12A* rs2243115 and the variant C allele of *IL-12B* rs3212227 were not associated with significantly altered risk for lung cancer (Table III, middle and bottom parts).

Since smoking behavior may contribute to lung cancer risk, the interaction of the genotype of *IL-12A* rs568408 with the age, gender and smoking behavior of the investigated subjects

Table IV. Distribution of *IL-12A* rs568408 genotypes among the 358 lung cancer patients and the 716 controls after stratification by smoking status.

Behavior group	<i>IL-12A</i> rs568408 genotype			<i>p</i> -Value <sup>a</sup>
	GG (%)	AG (%)	AA (%)	
Non-smokers				
Controls	112 (73.2%)	35 (22.9%)	6 (3.9%)	0.5069
Cases	46 (70.8%)	14 (21.5%)	5 (7.7%)	
Smokers				
Controls	427 (75.8%)	118 (21.0%)	18 (3.2%)	<b>0.0043*</b>
Cases	196 (66.9%)	76 (25.9%)	21 (7.2%)	

<sup>a</sup>Based on Chi-square without Yate's correction test; the significant *p*-value and odds ratio are bolded and marked with a star.

was examined. The joint effects of *IL-12A* rs568408 with smoking status are summarized in Table IV. First, lung cancer patients and matched controls were stratified according to their smoking status and ORs were computed. The results showed that ever smokers carrying the homologous AA genotype at *IL-12A* rs568408 were of increased risk of lung cancer after adjusted for smoking habits (*p*=0.0043) (Table IV, bottom part). On the contrary, there was no significantly elevated lung cancer risk for non-smokers with variant AG or AA genotypes at *IL-12A* rs568408 (Table IV, top part).

## Discussion

In the current study, the contribution of *IL-12A* rs568408, *IL-12A* rs2243115 and *IL-12B* rs3212227 genotypes to lung cancer risk among Taiwanese was investigated. The examined samples included 358 lung cancer patients and 716 age-,

gender- and smoking behavior-matched healthy controls (Table I). From the genotyping results it was found that the *IL-12A* rs568408 AA genotype, but not the AG or the genotypes of *IL-12A* rs2243115 or *IL-12B* rs3212227 (Tables II and III), was a novel genomic biomarker for detection and prediction of lung cancer risk among Taiwanese. It was further found that the AA genotype of *IL-12A* rs568408 was associated with elevated lung cancer risk, especially among those with smoking habits (Tables IV). These findings support the idea that potentially functional polymorphisms in *IL-12* may be involved in the carcinogenesis of lung cancer risk.

Among the three SNPs examined in this study, the genotype-phenotype correlation of *IL-12B* rs3212227 was mostly studied but controversial results were reported. First, the *IL-12B* rs3212227 AA genotype was reported to associate with higher expression of IL-12 in the serum of type 1 diabetes patients, compared with the AC or the CC genotypes (45, 46). On the contrary, peripheral blood mononuclear cells from individuals carrying the CC genotype at *IL-12B* rs3212227 secreted significantly higher levels of IL-12 upon stimulation with lipopolysaccharide and purified protein derivatives than those with the AC or the AA genotypes (47). Interpretation of the results is complicated by evidence suggesting that IL-12A and IL-12B may control the secretion of each other. It has been shown that the presence of the variant genotype *IL-12B* rs3212227 correlated with increased IL-12A secretion, but not IL-12B itself (48). Cancer genomic studies have shown that the CC/AC genotypes of *IL-12B* rs3212227 are associated with increased risk of many types of cancer, including esophageal (49), gastric (50), breast (51), bladder (52), cervical (37, 53) and osteosarcoma (54), but some controversial findings have also been reported (38, 55-57). A meta-analysis has shown that *IL-12B* rs3212227 serves as a potential biomarker for cancer risk among Asians, especially for cervical and nasopharyngeal cancer (58). In 2017, a more updated analysis reported that genotypes at *IL-12B* rs3212227 were significantly correlated with overall cancer risk, especially among Asian ethnicities (59). Our results did not show any association between the genotypes of *IL-12B* rs3212227 and lung cancer risk (Tables II and III). The inconsistent conclusions may come from different ethics and/or populations investigated, and the genetic background, life-style, environmental exposure and immunostatus were different among the investigated subjects. Regarding *IL-12A* rs2243115, Shi and his colleagues failed to detect any significant association between this polymorphism and cancer risk in either the overall or any subgroup analyses in a meta-analysis in 2018 (60), which is consistent with our findings (Tables II and III).

The highlight of the current study is that a practical biomarker, the AA genotype at *IL-12A* rs568408, for early detection and prediction lung cancer among Taiwanese is proposed (Tables II and III). The elevated risk was also found in several other types of cancer including colorectal (57),

cervical (37), esophageal (49) cancer, hepatocellular carcinoma (38) and osteosarcoma (54). In the most updated meta-analysis of *IL-12A* rs568408 and cancer, the genotypes at *IL-12A* rs568408 were found to significantly correlate with overall cancer risk, especially among Asian ethnicities (59).

Smoking is a well-known risk factor for lung cancer. Therefore, the interaction of the genotype of *IL-12A* rs568408 and cigarette smoking status of the participants was further analyzed. The results showed that the genotypic distribution of the variant genotype of *IL-12A* rs568408 was significantly different between lung cancer and control sub-groups who have smoking habits (Table IV). On the contrary, no differential distribution was observed among non-smokers (Table IV). The interaction of the genotype of *IL-12A* rs568408 with age, gender and alcohol drinking status was not found to be significant (data not shown). In the current study, the genotypic and phenotypic data are not yet sufficient enough to establish genotype-phenotype correlations among Taiwanese. Further studies are recommended, using cells from patients in addition to commercial lung cancer cell lines, to explore the differential effects of tobacco compounds on cells with different *IL-12A* rs568408 genotypes.

In conclusion, the study provides evidence that the AA genotype of *IL-12A* rs568408 is associated with an increased lung cancer risk among the Taiwanese, especially those with smoking habits.

## Conflicts of Interest

The Authors declare no conflicts of interest regarding this study.

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