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Original Article

Functional promoter rs189037 variant of *ATM* is associated with decrease in lung diffusing capacity after irradiation for non–small-cell lung cancer

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Abstract

Objective: Single-nucleotide polymorphisms (SNPs) in the ataxia telangiectasia–mutated gene *ATM* have been linked with pneumonitis after radiotherapy for lung cancer but have not been evaluated in terms of pulmonary function impairment. Here we investigated potential associations between SNPs in *ATM* and changes in diffusing capacity of the lung for carbon monoxide (DLCO) in patients with non–small-cell lung cancer (NSCLC) after radiotherapy.

Methods: From November 1998 through June 2009, 448 consecutive patients with inoperable primary NSCLC underwent definitive (≥60 Gy) radiotherapy, with or without chemotherapy. After excluding patients with a history of thoracic surgery, radiation, or lung cancer; without DNA samples available for analysis; or without pulmonary function testing within the 12 months before and the 12 months after radiotherapy, 100 patients were identified who are the subjects of this study. We genotyped two SNPs of *ATM* previously found to be associated with radiation-induced pneumonitis (rs189037 and rs228590) and evaluated potential correlations between these SNPs and impairment (decreases) in DLCO by using logistic regression analysis.

Results: Univariate and multivariate analyses showed that the AA genotype of *ATM* rs189037 was associated with decreased DLCO after definitive radiotherapy than the GG/AG genotypes [univariate coefficient, −0.122; 95% confidence interval (*CI*), −0.236 to −0.008; $P = 0.037$; and multivariate coefficient, −0.102; 95% *CI*, −0.198 to −0.005; $P = 0.038$]. No such correlations were found for rs228590 (univariate coefficient, −0.096; 95% *CI*, −0.208 to 0.017; $P = 0.096$).

Conclusions: The AA genotype of *ATM* rs189037 was associated with higher risk of lung injury than were the GG/AG genotypes in patients with NSCLC treated with radiotherapy. This finding should be validated prospectively with other patient populations.

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Introduction

Radiotherapy for locally advanced non−small-cell lung cancer (NSCLC) inevitably results in partial irradiation of normal lung tissue near the tumor, and this radiation exposure often leads to lung toxicity, the most severe type of which is radiation-induced pneumonitis (RP). Indeed, the probability of RP limits radiation dose escalation, and lung toxicity is one of the most common causes of impairments in quality of life and chronic complications such as lung fibrosis.[1]

Others have found an association between RP risk and several therapy- and patient-related factors, including performance status, dosimetric variables, age, and smoking status.[2−5] However, only a few patients whose lungs are exposed to a certain dose and volume of radiation go on to develop RP, suggesting that genetic makeup may be important in individual responses to radiotherapy. Many studies have been done seeking associations between RP and single-nucleotide polymorphisms (SNPs) in different categories of genes. Substantial improvements have been made in identifying genetic factors contributing to differences in radiation response of the normal lung. Two SNPs in the ataxia telangiectasia−mutated gene *ATM* were found to be associated with higher risk of RP in patients with NSCLC treated with definitive radiotherapy, with or without chemotherapy.[6] In that study, patients with the AA genotype of rs189037 or the CC genotype of rs228590 in *ATM* were at significantly higher risk of severe (grade $\geq$3) RP compared with patients with the AG/GG genotypes of rs189037 or the CT/TT genotypes of rs228590, after adjustment for differences in mean lung dose (MLD). Because SNPs in *ATM* have been linked with RP after radio(chemo)therapy for NSCLC, and because change in the diffusing capacity of the lung for carbon monoxide (DLCO) has been used as an objective measure with which to grade RP after definitive radio(chemo)therapy,[7−11] we hypothesized that SNPs associated with RP would also be associated with changes in DLCO after radiotherapy. As far as we know, no studies have investigated potential associations between genetic factors and impaired (reduced) DLCO. Therefore, the primary aim of the current study was to assess potential associations between two SNPs in *ATM* and the extent of change in DLCO after radiotherapy, and to assess whether *ATM* genotypes could be used to predict the extent of change in DLCO. We took care to control for dosimetric and clinical variables which were known or suspected of being associated with the study endpoint and thus had the potential to confound our study results.

Patients and methods

Selection criteria

The retrospective investigation was approved by the institutional review board of the University of Texas MD Anderson Cancer Center and was in compliance with the Health Insurance Portability and Accountability Act regulations. Informed consent was obtained from all individual participants included in the study. We identified and reviewed records from 448 consecutive patients with inoperable primary NSCLC who were treated from November 1998, when conformal radiation techniques became available for routine clinical use, through June 2009 in the University of Texas MD Anderson Cancer Center. Patients must have had definitive radiotherapy to a dose of $\geq$60 Gy, with or without chemotherapy, and have DNA samples available for analysis; exclusion criteria were prior thoracic surgery, radiotherapy, or lung cancer or not having had pulmonary function test results available within at least 1 year before beginning radiotherapy and again within 1 year after completing radiotherapy. Ultimately, 100 patients with NSCLC for whom DNA samples were available and met the selection criteria were included for this study. MLD was available for all patients. The median total radiation dose received for all patients was 63 Gy (range, 60−84 Gy) at 1.2−2 Gy/fraction (21 patients received 69.6 Gy/58 fractions at 1.2 Gy/fraction twice a day). Ninety-one patients also received concurrent platinum- and taxane-based chemotherapy. Nine patients were treated with radiation only.

Patient evaluation and follow-up

During the course of radiotherapy, patients were seen at least weekly and more often if needed for clinical evaluation and disease management. Standard

follow-up after radiotherapy at the authors' institution includes computed tomography (CT) or positron emission tomography (PET)-CT scans every 3–4 months for the first 2 years, then every 6–12 months thereafter at the treating physician's discretion. Pulmonary function testing was typically done at intervals of 3–6 months or more frequently at the treating physician's discretion.

Pulmonary function testing

On the basis of recommendations from the American Thoracic Society and the European Respiratory Society,[12] we chose DLCO as a measure of diffusion capacity. All values were recorded as a percentage of predicted level (determined by sex, height, and weight). DLCO values before and after treatment were compared as DLCO ratio (post-treatment/pre-treatment) to assess the effect of treatment. Patients had different numbers of follow-up pulmonary function tests depending on survival, duration of follow-up, and number of return visits. We chose to evaluate the pulmonary function tests obtained within the 12 months before radiotherapy and within the 12 months after radiotherapy.

SNPs selection and genotyping methods

We used the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP), Hapmap database (http://www.hapmap.org/, Rel 27), and SNP Function Prediction tool (http://snpinfo.niehs.nih.gov/snpfunc.htm) to select two *ATM* SNPs (rs189037G > A and rs228590C > T) on the basis of at least two of the following three criteria: (1) minor allele frequency of >5% among non-Hispanic whites, (2) variant located in the promoter or coding regions of the gene, and (3) previously linked with RP in patients with NSCLC. Genomic DNA was extracted from peripheral blood leukocytes by a Blood Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Genotyping was done by the polymerase chain reaction-restriction fragment length polymorphism method. The detailed information was listed in Table 1.

Statistical analyses

Stata/SE 11.1 (StataCorp, College Station, TX, USA) was used for data analyses. Pulmonary function test values after radiotherapy (percentage of predicted) were evaluated for each patient and compared with that same patient's test values before radiotherapy. For patients who had more than one post-treatment values, the lowest value within the 12 months after radiotherapy was used for analysis and compared with the baseline value. We used mixed effect linear regression to evaluate changes in pulmonary function test values by time and its association with *ATM* SNPs. Potential confounders such as age, radiation fractionation, use of chemotherapy, and dosimetric variables (e.g., MLD, fractionation, etc.) were included into multi-variate model and removed if $P \geq 0.05$. P values of <0.05 were considered statistically significant.

Results

Baseline characteristics of the 100 patients are listed in Table 2. The dataset consisted of 58 men and 42 women, with a median age of 64 years (range, 38–83 years). Eighty-six patients were non-Hispanic whites and 82% (82/100) had stage III or IV disease according to the 6th edition of the American Joint Committee on Cancer (AJCC) staging manual.[13] The median MLD was 17.5 Gy (range, 4.7–29.5 Gy). Age, receiving of chemotherapy, radiation modality, daily fraction and MLD were factors for the DLCO decline after radiotherapy in univariate analysis. These factors were later applied into multivariate analysis.

The median value of DLCO was 67% (range, 18%–124%) before radiation; 50% (range, 12%–119%) during 3–6 months after radiation and 53% (range, 12%–119%) during 6–12 months after radiation. Fig. 1 showed the DLCO distributions before radiation treatment, 3–6 months and 6–12 months after radiation treatment among each genotype groups of *ATM*

Table 1
Information about the *ATM* SNPs and PCR-RFLP genotyping methods.

Polymorphisms	rs number	Tm	Restriction enzyme	PCR primer pair
−111G > A	rs189037	64°C	*Msc*I	5′-GCTGCTTGGCGTTGCTTC-3′ 5′-CATGAGATTGGCGGTCTGG-3′
D1853N	rs228590	63°C	*DRA*I	5′- CAGAGCGAGACTGTCTCAAAACA-3′ 5′- AAGTCAGAAGAACCACCAGTGAATTT-3’.

SNPs: single-nucleotide polymorphisms; PCR: polymerase chain reaction; RFLP: restriction fragment length polymorphism; Tm: temperature.

Table 2
Patient characteristics and association with decrease in diffusing capacity within 12 months after radiotherapy ($n = 100$).

Characteristics	Value or No. of patients	Coefficient (95% *CI*)	*P* value
Age, years, mean (range)			
	64 (38–83)	−0.007 (−0.013 to −0.003)	0.003
Sex, *n*			
Male	58	Ref	
Female	42	0.069 (−0.040 to 0.177)	0.214
Race, *n*			
White	86	−0.069 (−0.225 to 0.086)	0.383
Black	14	Ref	
Disease stage, *n*			
I–II	18	Ref	
III–IV	82	−0.034 (−0.176 to 0.108)	0.642
Tumor histology, *n*			
Adenocarcinoma	40	Ref	
Squamous	39	−0.032 (−0.152 to 0.088)	0.604
Other	21	−0.074 (−0.219 to 0.071)	0.316
Gross tumor volume, cm^3, mean (range)			
	154 (5–860)	−0.0003 (−0.0006 to 0.00004)	0.09
Karnofsky performance status score			
<80	19	Ref	
≥80	81	−0.009 (−0.149 to 0.131)	0.899
Smoking status, *n*			
Ever	85	Ref	
Never	15	0.061 (−0.089 to 0.212)	0.424
Chemotherapy, *n*			
No	9	Ref	
Yes	91	−0.233 (−0.421 to −0.044)	0.016
Radiation treatment, *n*			
3D CRT	45	Ref	
IMRT	37	0.077 (−0.038 to 0.192)	0.191
PT	18	0.197 (0.052–0.342)	0.008
Radiation fractionation, *n*			
Once daily	79	Ref	
Twice daily	21	−0.158 (−0.286 to −0.031)	0.015
History of COPD, *n*			
No	84	Ref	
Yes	16	0.076 (−0.072 to 0.223)	0.314
Mean lung dose, Gy, mean (range)			
	17.5 (4.7–29.5)	−0.021 (−0.031 to −0.011)	0.001

CI: confidence interval; Ref: reference; 3D CRT: three-dimensional conformal radiotherapy; IMRT: intensity-modulated radiotherapy; PT: proton therapy; COPD: chronic obstructive pulmonary disease.

SNPs. The DLCOs during 3–6 months and 6–12 months after radiation treatment were significantly lower than pre-treatment values in all genotype groups ($P < 0.05$); but not much differences existed between 3–6 months and 6–12 months after radiation therapy ($P > 0.05$). The median percentage change in DLCO from before to ≤1 year after radiotherapy was −19% (range, −78% to 79%). The early changes of DLCO after radiation therapy (change in DLCO at 3–6 months after radiation therapy) had the same range but the median value was −23%.

The AA, AG and GG genotype frequencies of *ATM* rs189037 were 29%, 49% and 22%, respectively; the CC, CT and TT genotype frequencies of *ATM* rs228590 were 32%, 49% and 19%, respectively. In univariate Cox proportional hazard analyses, only the AA genotype of *ATM* rs189037 was associated with change in DLCO within 1 year after definitive radiotherapy [coefficient, −0.122; 95% confidence interval (*CI*), −0.236 to −0.008; $P = 0.037$] (Table 3). This effect was virtually unchanged after adjustment for potential confounding factors from previous investigations (MLD, age, receipt of chemotherapy, and twice-daily radiotherapy) (coefficient, −0.102; 95% *CI*, −0.198 to −0.005; $P = 0.038$) (Table 4). However, similar results were not observed for rs228590 SNP (coefficient, −0.096; 95% *CI*, −0.208 to 0.017; $P = 0.096$) (Table 3). Multivariate analysis also indicated that MLD, age, chemotherapy, and twice-daily fractionation were all associated with lower DLCO after definitive radiotherapy (Table 4).

When DLCO values were compared between baseline and 3–6 months after radiotherapy (data available for 64 patients, Table 5), again, the AA genotype of *ATM* rs189037 was associated with increased risk of impaired DLCO compared with the GG/AG genotypes (coefficient, −0.208; 95% *CI*: −0.373 to −0.044; $P = 0.013$) (Table 3). The CC genotype of *ATM* rs228590 was also associated with impaired DLCO compared with the CT/TT genotypes (coefficient, −0.178; 95% *CI*: −0.339 to −0.018; $P = 0.029$). Both associations retained significance after adjustment for MLD (Table 4).

The dynamic trend of DLCO changes within 1 year after start of radiation was showed in scatter plots (Fig. 2). DLCO kept decreasing during the first year after radiation in all patients. The decreased slopes of DLCO were not significantly different in all genotype groups ($P > 0.05$). But patients with AA genotype of rs189037 ($P = 0.005$) and patients with CC genotype of rs228590 ($P = 0.014$) had larger decrease than other genotype groups after receiving radiation treatment.

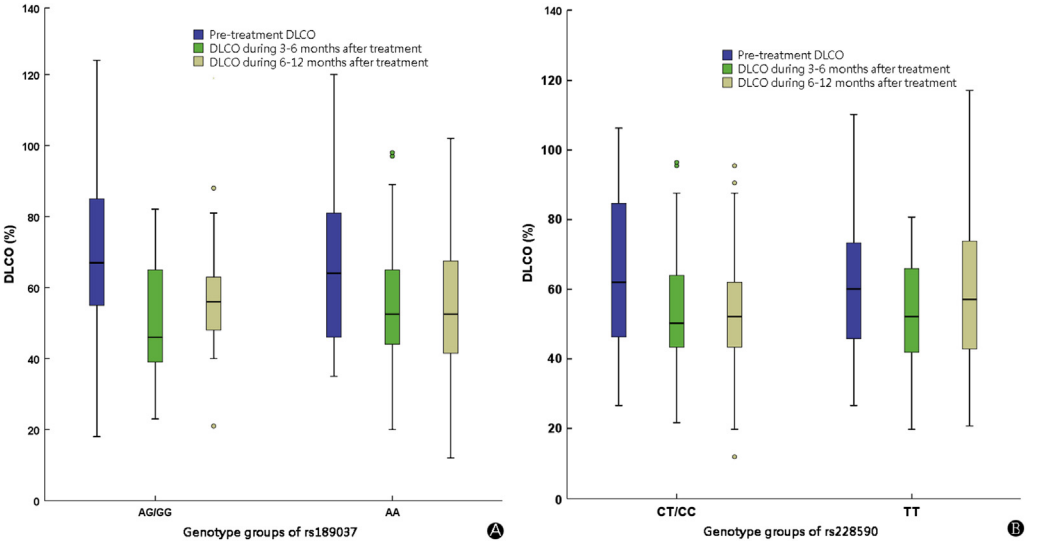

Fig. 1. Box plots of DLCO distributions before and after radiation therapy in each genotype groups of *ATM* SNPs (A) rs189037 and (B) rs228590. DLCO: diffusing capacity of the lung for carbon monoxide; SNPs: single-nucleotide polymorphisms.

Table 3
Univariate analysis of associations between SNPs in *ATM* and reduced diffusing capacity after radiotherapy.

Variates	3–6 Months after radiotherapy ($n = 64$)			≤12 Months after radiotherapy ($n = 100$)		
	No. of patients	Coefficient (95% *CI*)	*P* value	No. of patients	Coefficient (95% *CI*)	*P* value
rs189037						
AG/GG	47	Ref	–	71	Ref	–
AA	17	−0.208 (−0.373 to −0.044)	0.013	29	−0.122 (−0.236 to −0.008)	0.037
rs228590						
CT/TT	44	Ref	–	68	Ref	–
CC	20	−0.178 (−0.339 to −0.018)	0.029	32	−0.096 (−0.208 to 0.017)	0.096

SNPs: single-nucleotide polymorphisms; *CI*: confidence interval; Ref: reference; -: not applicable.

Table 4
Multivariate analysis of associations between SNPs in *ATM* and reduced diffusing capacity after radiotherapy.

Variates	3–6 Months after radiotherapy ($n = 64$)			≤12 Months after radiotherapy ($n = 100$)		
	No. of patients	Coefficient (95% *CI*)	*P* value	No. of patients	Coefficient (95% *CI*)	*P* value
rs189037						
AG/GG	47	Ref	–	71	Ref	–
AA	17	−0.157 (−0.301 to −0.014)	0.031	29	−0.102 (−0.198 to −0.005)	0.038
Mean lung dose	–	−0.024 (−0.036 to −0.01)	0.0001	–	−0.016 (−0.025 to −0.007)	0.001
Age	–	–	–	–	−0.009 (−0.013 to −0.004)	0.0001
Chemotherapy	–	–	–	–	−0.195 (−0.368 to −0.022)	0.027
Twice-daily radiation	–	–	–	–	−0.124 (−0.234 to −0.013)	0.028
rs228590						
CT/TT	44	Ref	–	68	Ref	–
CC	20	−0.150 (−0.295 to −0.005)	0.042	32	−0.071 (−0.166 to 0.024)	0.144
Mean lung dose	–	−0.025 (−0.038 to −0.013)	0.0001	–	−0.016 (−0.025 to −0.006)	0.001
Age	–	–	–	–	−0.009 (−0.013 to −0.004)	0.0001
Chemotherapy	–	–	–	–	−0.183 (−0.358 to −0.009)	0.040
Twice-daily radiation	–	–	–	–	−0.128 (−0.240 to −0.017)	0.023

SNPs: single-nucleotide polymorphisms; *CI*: confidence interval; Ref: reference; -: not applicable.

Table 5
Patient characteristics and association with decrease in diffusing capacity 3–6 months after radiotherapy ($n = 64$).

Characteristics	Value or No. of patients	Coefficient (95% *CI*)	*P* value
Age, years, mean (range)			
	63 (38–83)	−0.008 (−0.016 to −0.002)	0.012
Sex, *n*			
Male	34	Ref	
Female	30	0.075 (−0.082 to 0.232)	0.351
Race, *n*			
White	54	−0.007 (−0.213 to 0.227)	0.949
Black	10	Ref	
Disease stage, *n*			
I–II	34	Ref	
III–IV	30	−0.025 (−0.255 to 0.205)	0.831
Tumor histology, *n*			
Adenocarcinoma	25	Ref	
Squamous	23	0.029 (−0.152 to 0.211)	0.753
Other	16	−0.077 (−0.277 to 0.121)	0.446
Gross tumor volume, cm^3, mean (range)			
	162 (5–860)	−0.0003 (−0.0007 to 0.0001)	0.207
Karnofsky performance status score			
<80	9	Ref	
≥80	55	0.038 (−0.191 to 0.268)	0.743
Smoking status, *n*			
Ever	57	Ref	
Never	7	−0.030 (−0.286 to 0.226)	0.818
Chemotherapy, *n*			
No	4	Ref	
Yes	60	−0.010 (−0.429 to 0.230)	0.553
Radiation treatment, *n*			
3D CRT	34	Ref	
IMRT	19	0.147 (−0.025 to 0.319)	0.094
PT	11	0.269 (0.059 to 0.478)	0.012
Radiation fractionation, *n*			
Once daily	48	Ref	
Twice daily	16	−0.231 (−0.396 to −0.065)	0.006
History of COPD, *n*			
No	55	Ref	
Yes	9	0.064 (−0.159 to 0.287)	0.574
Mean lung dose, Gy, mean (range)			
	17.5 (4.7–29.5)	−0.027 (−0.040 to −0.014)	0.0001

CI: confidence interval; Ref: reference; 3D CRT: three-dimensional conformal radiotherapy; IMRT: intensity-modulated radiotherapy; PT: proton therapy; COPD: chronic obstructive pulmonary disease.

Discussion

The pertinent findings of this study can be summarized as follows. First, patients carrying the AA genotype at rs189037 of *ATM* had a higher probability of DLCO impairment within 12 months after radiotherapy than did patients with the GG/AG genotypes. Second, this effect was independent of other clinical and treatment factors, such as age, receipt of chemotherapy, MLD, and fractionation schedule (once *vs.* twice a day). Third, both the AA genotype at rs189037 of *ATM* and the CC genotype at rs228590 were associated with higher probability of DLCO impairment when the second DLCO measurement was made at 3–6 months after completion of radiotherapy. Collectively, these findings provide a possible explanation for previous findings[6] that SNPs in *ATM* were associated with a higher risk of RP in patients with NSCLC treated with definitive radio(chemo)therapy.

The criteria for grading RP in the Common Terminology Criteria for Adverse Events are based on clinical symptoms and radiographic data from chest x-rays or CT scans, which can be subjective and hence inconsistent among physicians and institutions. The resulting difficulties in comparing findings between studies have led to extensive efforts to identify objective variables with which to categorize and analyze lung toxicity after radiotherapy. Previous studies of changes in pulmonary function after radiotherapy for NSCLC have shown that the largest and most consistent changes in pulmonary function test values after definitive radiotherapy occurred in DLCO.[7–11] In addition, patients with symptomatic RP tend to experience significant decreases in pulmonary function, which could be quantified in terms of DLCO and used to objectively score RP grade.[8]

Functional experiments have shown that *ATM* acts as a central mediator of the radioprotective machinery of cells in response to radiation, participating in cellular stress responses, control of cell-cycle checkpoints, repair of double-strand breaks, and initiation of apoptosis.[14] *In vitro*, cells acquired from individuals with ataxia-telangiectasia and heterozygous *ATM* genotypes were more radiosensitive than cells from normal subjects.[15] *In vivo*, compared with wild-type mice, those with heterozygous *ATM* genotypes were more susceptible to radiation-induced cataracts.[16] Xiong et al,[6] in studying 362 patients (82% of whom were non-Hispanic whites) found that patients carrying *ATM* rs189037 AG/GG or rs228590 TT/CT genotypes, or rs189037G/rs228590T/rs1801516G (G-T-G) haplotypes, had a lower risk of severe RP. They

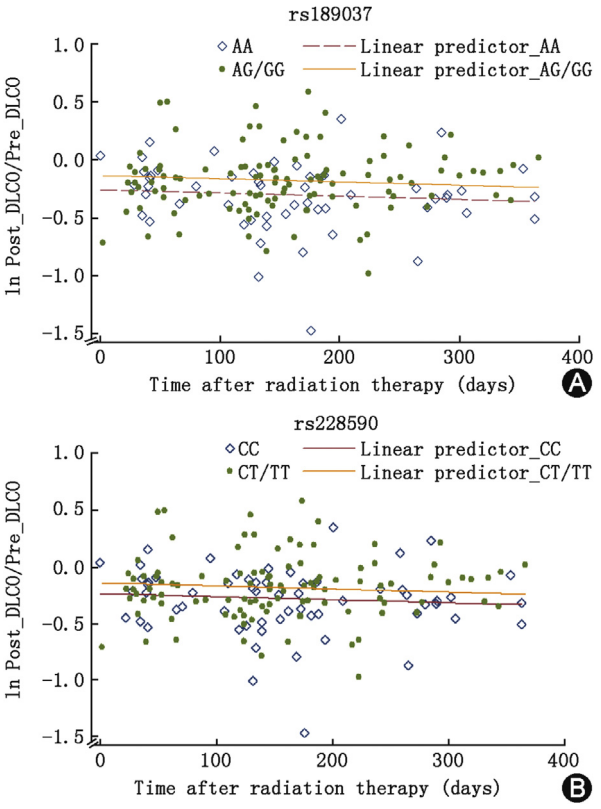

Fig. 2. Scatter plot of changes in DLCO *vs*. time after radiotherapy for patients with SNPs in (A) rs189037 and (B) rs228590 (n=167). DLCO: diffusing capacity of the lung for carbon monoxide; SNPs: single-nucleotide polymorphisms.

further identified MLD as being the most important risk factor for risk of developing severe RP. Our findings are partially consistent with these results. We noted that patients carrying the AA genotype at *ATM* rs189037 had a higher probability of decreased DLCO after radiotherapy. We also found that MLD was strongly associated with impaired DLCO. However, the present study did not support an association between SNPs at *ATM* rs228590 and change in DLCO after radiotherapy.

Previous studies[17,18] of Chinese Han populations also evaluated the potential role of *ATM* variants in RP risk. They found that the *ATM*−111G > A (rs189037) polymorphism was independently associated with increased RP risk among 253 Asian patients receiving thoracic irradiation between 2004 and 2006. The hazard ratio for the *ATM*−111GA genotype (*vs*. the −111GG genotype) in the RP group was 3.03 (95% *CI*: 1.23−7.46, $P = 0.021$). However, the −111AA genotype was not associated with increased risk of RP (adjusted hazard ratio 1.70, 95% *CI*: 0.56−5.23, $P = 0.313$), perhaps because of small numbers of patients in that analysis. The divergence between these findings and our own could also be partially explained by differences in the frequency of the variant alleles of *ATM* rs189037 between these two populations (the frequencies of alleles A *vs*. G were 41% *vs*. 59% in Asian group and 53% *vs*. 47% in our group).

Indeed, our finding that rs189037 and rs228590 SNPs were associated with a higher probability of decrease in DLCO soon (within 3−6 months) after radiotherapy suggests that early lung impairment could be predicted, before radiotherapy, by using a simple genetic assessment. If these findings are validated in prospective studies of other patient populations, this information could be used to inform clinical decisions[19] about which patients are most likely to benefit from a given treatment as well as those who are at risk of developing substantial side effects.

Other than the constraints inherent in any retrospective analysis, our study had several limitations. First, to limit the scope and thus increase the feasibility of this analysis, we selected and analyzed only two *ATM* SNPs, and we did not find a significant correlation with change in DLCO for one of them (rs228590). Development of DLCO impairment is likely to involve a complex interplay of several genetic processes that were not analyzed in this study. Second, although we were able to establish strong statistical associations between genotype, clinical factors, and DLCO outcomes, we did not explore the biological mechanism by which the selected genetic polymorphisms led to radiation-induced impairment in DLCO in patients with NSCLC; this issue is the topic of ongoing assessment at our institution. Thus we acknowledge that our findings do not fully elucidate the complex mechanism between gene expression and toxicity, but rather demonstrate a correlation that will be further illuminated as findings such as these are incorporated into future prospective investigations of genotype and adverse events in large multi-institutional trials.

In conclusion, our goal was to identify biomarkers associated with lung injury after radiotherapy for patients with NSCLC that could be used before radiotherapy is begun. Our results demonstrated that patients with NSCLC carrying the AA genotype at *ATM* rs189037 was associated with a greater risk of decreased DLCO after radiotherapy compared with patients with GG/AG genotypes. This finding strongly supported our previous finding of the association between *ATM* polymorphism and radiation induced lung injury.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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